

A Pathway from the Midbrain to the Striatum is Critical to Multiple Forms of Vocal
Learning and Modification in the Songbird

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
Neurobiology in the Graduate School
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ABSTRACT

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Abstract

Many of the skills we value most as humans, such as speech and learning to play musical instruments, are learned in the absence of external reinforcement. However, the model systems most commonly used to study motor learning employ learning paradigms in which animals perform behaviors in response to external rewards or punishments. Here I use the zebra finch, an Australian songbird that can learn its song as a juvenile in the absence of external reinforcement as well as modify its song in response to external cues as an adult, to study the circuit mechanisms underlying both internally and externally reinforced forms of learning. Using a combination of intersectional genetic and microdialysis techniques, I show that a striatonigral pathway and its downstream effectors, namely D1-type dopamine receptors, are necessary for both internally reinforced juvenile learning and externally reinforced adult learning, as well as for song modification in response to social cues or to deafening. In addition, I employ optogenetic stimulation during singing to demonstrate that this striatonigral projection is sufficient to drive learning. Interestingly, I find that neither the striatonigral pathway nor D1-type dopamine receptors are necessary for recovery of pitch after externally driven pitch learning. In all, I establish that a common mechanism underlies both internally and externally reinforced vocal learning.

Dedication

This dissertation is dedicated to my husband Matt. I couldn't have done it without you.

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1. Introduction

Some of the most remarkable forms of human learning, such as speech and language learning as well as learning to play a musical instrument, depend critically on the ability to learn through imitation in the absence of external reinforcement (Locke, 1995; Pinker, 1994). Though humans readily engage in these types of learning in everyday life, direct study of the cellular subtypes that are involved in these behaviors is not feasible. In contrast, rodent model systems have the tools needed to explore circuitry of specific cellular subtypes but the paradigms most often used to drive motor learning in rodents rely only on external reinforcement. Given the difficulty in finding an organism that displays multiple kinds of learning and can be manipulated at the cellular level, the question of whether internally and externally reinforced forms of learning rely on similar neural mechanisms is not well understood.

Birdsong provides a unique model with which to better understand both internally and externally reinforced learning. During juvenile learning, young birds memorize the song of their father, practicing this song extensively over a period of months in the absence of external rewards or punishment while relying on auditory feedback to correct mismatches to the memorized tutor song (L.A. Eales, 1985; Immelman, 1969; Marler & Peters, 1981; Price, 1979). Variability in song plays a large role in allowing the young bird to explore motor space and chose gestures that produce

sounds that best compare to the tutor song (Sohrabji, Nordeen, & Nordeen, 1990). Even after the young birds' songs crystallize into a remarkably good copy of the tutor song, birds still rely on auditory feedback as adults to maintain their songs (Konishi, 1965). In the absence of auditory feedback, the stereotyped songs of adult birds degrade first spectrally and then temporally to result in a jumble of sounds that no longer resemble a structured song (Lombardino & Nottebohm, 2000; K. W. Nordeen & Nordeen, 1992).

While juvenile learning and adult song maintenance are internally driven, songbirds are also able to change aspects of their song in response to external cues. In a pitch contingent learning paradigm in which syllables are detected and a burst of white noise is played back if the syllable falls above or below a fixed pitch threshold, birds quickly learn to adjust their pitch in order to avoid receiving a noise burst and then recover their pitch to baseline levels after white noise is turned off (Tumer & Brainard, 2007). Adult birds are also able to increase the tempo and stereotypy of their songs when presented with an external appetitive cue in the form of a female bird (Kao & Brainard, 2006; Sossinka R, 1980). Interestingly, this change in song in response to presentation of a female is accompanied by an increase in dopamine into the song-related region of the striatum, Area X, (Sasaki, Sotnikova, Gainetdinov, & Jarvis, 2006) and is blocked when D1R antagonists are infused into Area X (Leblois, Wendel, & Perkel, 2010). In other model systems, striatonigral pathways, as well as striatal dopamine receptors, are known to be key players in learning and reinforcement in classical conditioning and

other externally driven forms of learning. Given that Area X receives massive dopaminergic input from the ventral tegmental area (VTA) and the substantia nigra pars compacta (SNc) (VTA and SNc from here on referred to simply as VTA) (Gale, Person, & Perkel, 2008) and that both striatonigral pathways and the striatal dopamine receptors they act on are required for changes in associative learning in mammalian systems as well as song variability (Adamantidis et al., 2011; Gerfen & Surmeier, 2011; Steinberg et al., 2013; Tsai et al., 2009; Zweifel et al., 2009), cells in the VTA and SNc that project to Area X (referred to as VTA_x) may play a privileged role in internally guided learning that has remained unexplored.

1.1 The role of striatonigral circuits in motor learning

1.1.1 Externally reinforced forms of learning

For decades theoreticians have posited that some sort of neural signal encodes errors in reward prediction to facilitate learning (Glimcher, 2011; Sutton, 1981). The temporal difference (TD) learning model for example relies on a signal that can predict the value of future events in a changing environment. Nevertheless, the role of midbrain dopaminergic centers in reward learning was unknown until classic experiments from Schultz and colleagues were performed in the late 1990s (Schultz, Dayan, & Montague, 1997) (Figure 1). In these experiments, the activity of midbrain dopaminergic cells was recorded while a monkey performed a simple cue-based task. When the monkey was first rewarded for correctly completing the task, these neurons fired bursts upon reward delivery. However, as the monkey learned the task, the onset of this phasic firing shifted from reward delivery to presentation of the reward-predicting cue. Interestingly, when a predicted reward was omitted, firing was suppressed when reward delivery did not occur. These observations first suggested dopaminergic cells in the midbrain as a candidate for conveying a reward prediction error signal (RPE) that could effectively update expectations to improve learning by signaling the difference between an expected outcome and information to the contrary.

Due to their ability to code for RPEs, midbrain dopaminergic cells in particular have a critical role in learning. Phasic activation of dopaminergic cells, thought to be critical to encoding when an outcome of an animal's behavior occurs unexpectedly, in the VTA with optogenetics has been shown to be sufficient to induce conditioned behavior (Tsai et al., 2009) whereas disruption of phasic burst firing in DA cells via genetic inactivation of NMDARs in DA cells impairs conditioned behaviors (Zweifel et al., 2009). Phasic activation of VTA DA cells has also been shown to facilitate reward-seeking behavior in both mice (Adamantidis et al., 2011) and rats (Witten et al., 2011). Furthermore, phasic optogenetic activation of DA cells can drive learning of redundant cues in an associative blocking paradigm as well as interrupt extinction learning (Steinberg et al., 2013).

Dopaminergic midbrain areas send massive projections to the striatum and can influence learning by acting on the dopamine receptors present there. An opponent interaction between direct (D1) and indirect (D2) pathway cells can lead to action selection or suppression, respectively, as well as increases or decreases in movement kinematics, such as velocity, in the direction of reward in response to stimulation of the direct or indirect pathway. Pharmacological blockade of D1 receptors systemically in mice or locally in monkeys is able to prevent simple associative learning (Flagel et al., 2011; Puig & Miller, 2012). Notably, D1 blockade does not interfere with performance in either of the

above studies, which is a possible confound when interfering with dopaminergic signaling.

Striatal cells themselves can also encode information about actions and outcomes that can facilitate learning. While some striatal cells respond purely to reward (Tremblay, Hollerman, & Schultz, 1998), others have been reported to change their firing patterns over learning to respond preferentially to stimuli that predict reward (Hollerman, Tremblay, & Schultz, 1998; Pasupathy & Miller, 2005) as well as to encode the correctness of the animal's performance on previous trials (Histed, Pasupathy, & Miller, 2009), two functions that are critical for learning. Certain striatal cells can also encode the reward value obtained by a particular action, responding before that action is executed (Kim, Sul, Huh, Lee, & Jung, 2009; Lau & Glimcher, 2008). In a manner reminiscent of midbrain dopaminergic cells, striatal cells can also show reward prediction error-like responses, some encoding both positive and negative prediction errors with others encoding either unexpected reward or reward omission (Ito & Doya, 2009; Samejima, Ueda, Doya, & Kimura, 2005). Notably, both striatal and midbrain encoding of reward prediction errors have only been studied in the context of externally reinforced learning with paradigms that use lever pressing or joystick manipulation to produce a tangible, salient reward to the animal.

**Do dopamine neurons report an error
in the prediction of reward?**

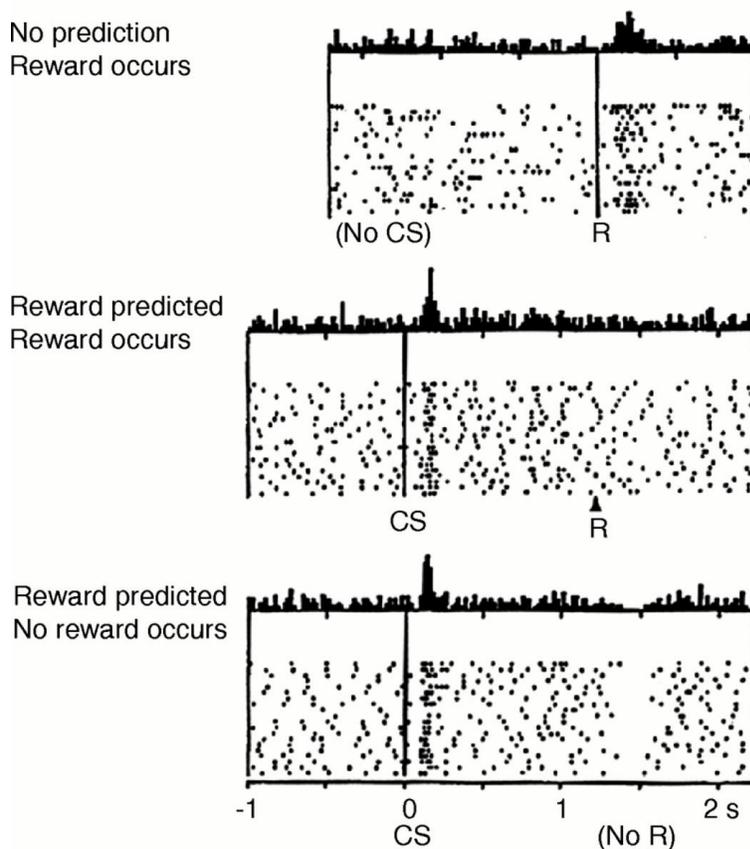


Figure 1: Dopaminergic neurons in the midbrain encode a reward prediction error

Single unit recordings in monkeys reveal an increase in firing to reward that after training turns to an increase in firing to the cue that predicts the reward. The same cells show a suppression of firing in the absence of a predicted reward. *From Schultz et al, 2007*

1.1.2 Internally reinforced forms of learning

Unlike many behaviors used to study the role of striatonigral circuitry in the laboratory, many forms of human learning occur in the absence of a tangible reinforcing stimulus such as a food reward or a foot shock. Human speech learning as well as musical instrument learning both rely on using internally held representations to shape motor output and proceed in absence of a “carrot” or a “stick” (Doupe & Kuhl, 1999; Prather, Okanoya, & Bolhuis, 2017). Though auditory cortex and primary motor areas are known to be heavily involved in both speech and music learning (Kuhl, 2010), the role of the midbrain and the striatum has not been extensively explored. Given that externally reinforced forms of learning are dependent on striatonigral circuitry and that connectivity between auditory cortex and the VTA and striatum is correlated with language proficiency in children with ASD (Abrams et al., 2013), it is likely that striatonigral circuitry plays a critical role in internally guided learning as well. Similarly to humans, and unlike many of the model animal systems commonly used in the lab, songbirds can use both internal memories of the tutor song and external stimuli to learn and modify their vocalizations.

1.2 Forms of song learning and modification in the songbird: a brief introduction

Zebra finches, a passerine songbird native to Australia, are closed ended vocal learners, meaning that while they produce learned rather than instinctive vocalizations, they are unable to learn new songs throughout their lives and are limited to singing the song of their father, or tutor (Immelman, 1969). This is in marked contrast to birds like starlings and mockingbirds that learn hundreds of songs during their lives and regularly incorporate environmental sounds into their songs (Janik & Slater, 2000). The song of the zebra finch begins with several introductory notes (indicated by an 'i'), which are not learned, followed by 2 to 6 learned notes called syllables which they "copy" from the tutor's song. The finches then sing these syllables in a stereotyped sequence called a motif (i.e. iiiABCD). Throughout the day they sing multiple song bouts, which are composed of multiple motifs sung without more than a several second gap between motifs (i.e. iiiABCD ABCD ABCD) (Figure 2). Zebra finches show no variation in transitions between the syllables of their motif (i.e. a zebra finch would never sing ACDB) but what they lack in song variability they make up for in sheer number of songs. A prolific singer can sing hundreds of song bouts per day with 5-10 motifs per bout, bringing the number of songs a day into the thousands.

Young zebra finches first begin learning their song by listening to and memorizing the song of their father (referred to as tutor song) from the time they fledge (~20 dph (days post hatch)) until approximately 40 dph (Figure 3). This phase of

listening and memorizing the tutor song is often referred to as the sensory phase of juvenile learning. Even in the absence of exposure to a live tutor, juveniles will readily key peck to hear playback of adult zebra finch song and will memorize songs that are played back to them through a speaker (Adret, 1993). Juveniles are able to memorize and copy an entire tutor song with a limited amount of exposure to the song; juveniles can copy tutor songs that they have heard as few as 40 times (Tchernichovski, Lints, Mitra, & Nottebohm, 1999), which is quite remarkable given the spectral and temporal complexity of adult zebra finch song.

After the sensory phase of learning is complete, juvenile zebra finches will produce strings of simple sounds that are likened to babbling in babies (Aronov, Andalman, & Fee, 2008). During the sensorimotor phase of learning, which begins around 40-50 dph, the juveniles begin to practice the tutor song extensively, shaping their babbling into definable sequences of syllables. Juveniles in this stage have been observed to use two different learning strategies: one in which they first perfect one syllable at a time before stringing the syllables into the proper order to form a motif and another strategy in which they begin by singing the entire motif which they gradually perfect in a more global manner (Tchernichovski, Mitra, Lints, & Nottebohm, 2001).

By the time the juvenile birds reach 80-90 dph they can sing a recognizable copy of the tutor song though the spectral structure and speed of the song has more variability than that of an adult song. At 110-120 dph birds are considered sexually

mature and sing what is called a crystallized song, which has minimal spectral and temporal variability. The now-adult birds will continue to sing this one song throughout their lives. Even though their song is now “crystallized”, adult birds still rely on auditory feedback to maintain their songs (Konishi, 1965). In the absence of auditory feedback, such as when the cochlea are bilaterally removed, song will deteriorate within a matter of days on a spectral level and within one month on a temporal level until their song lacks any recognizable features of learned zebra finch song (K. W. Nordeen & Nordeen, 1992).

Adult zebra finches are also able to learn to modify their songs, however unlike the juvenile this form of learning is externally, rather than internally, reinforced. Using real-time pitch detection software (Tumer & Brainard, 2007), we can detect and target specific syllables birds sing. While the adult zebra finch’s song is remarkably stereotyped, there is a small amount of within-syllable variability in pitch that will allow us to employ the paradigm described below (Figure 4). If the pitch of the targeted syllable falls below the experimenter-determined threshold, the bird receives a short, loud burst of white noise (WN) playback within milliseconds of detection. Over a matter of hours the bird will learn to “escape” the WN by adjusting the pitch of the targeted syllable upwards. The experimenter can then gradually increase the pitch threshold to drive the bird’s pitch further and further from his original pitch. Interestingly, the change in pitch is only seen in the targeted, but not surrounding, syllables, emphasizing

the millisecond precision with which birds can make online modifications to their song. After the bird has achieved the desired amount of learning, the experimenter can turn off WN playback and allow the bird to recover to his original pitch. This process, referred to as “recovery”, is not simply a passive process but an active form of learning that relies on auditory feedback, which presumably allows the bird to compare his current pitch to his memory of the pitch he sang before WN and gradually modify the syllable so that it returns to its original pitch (Andalman & Fee, 2009; Warren, Tumer, Charlesworth, & Brainard, 2011).

For adult male zebra finches, an accurate song is critical for reproductive success. When presented with a female, males sing a faster, more stereotyped version of their song, known as directed song. This change, while indistinguishable to the human ear and eye, is readily calculated by measuring the change in the coefficient of variation of pitch, which is reliable across birds and most often used when comparing directed to undirected song (Stepanek & Doupe, 2010). Importantly, females prefer directed song to undirected song, which has higher levels of variability, and will orient towards and spend more time in portions of their cage in which directed song is played (Woolley & Doupe, 2008).

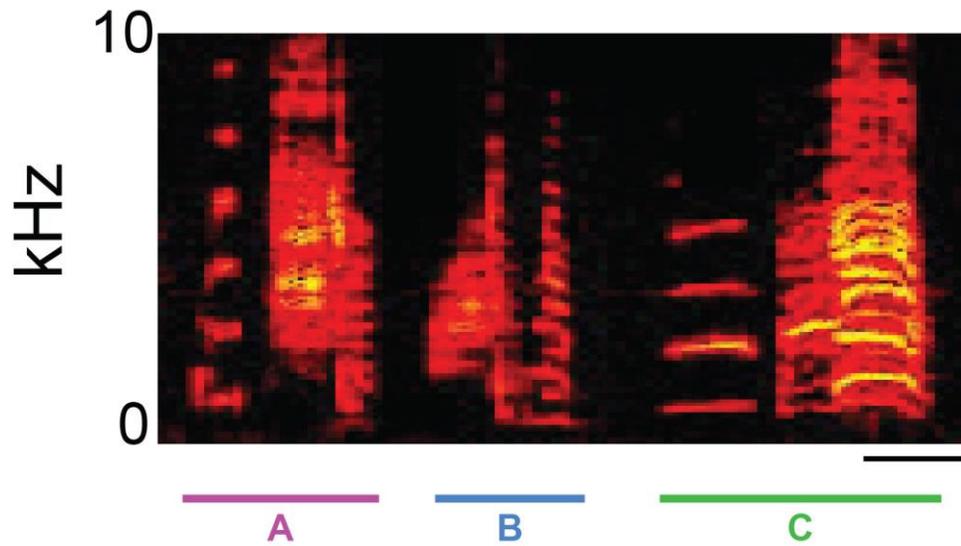


Figure 2: Sonogram of zebra finch song

This is an example of one motif of an adult male zebra finch's song. The motif is composed of three syllables, A, B and C. Y axis, frequency in kilohertz. Scale bar, 50 milliseconds. Brighter colors on sonogram indicate higher power, or loudness.

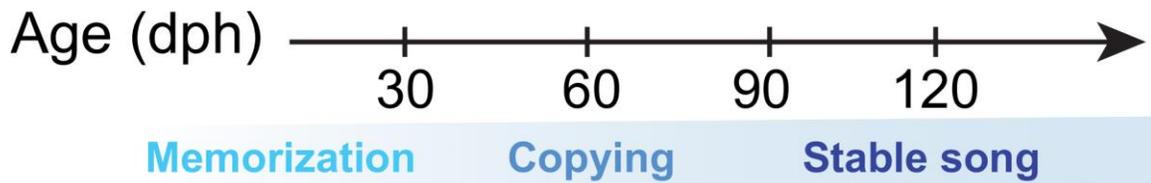


Figure 3: Timeline of song development

Song learning occurs over the first four months of the male zebra finch's life. Song learning is divided into a sensory phase, in which the pupil memorizes the tutor's song, and a sensorimotor phase, in which the pupil practices replicating the tutor's song. Song "crystallizes", or becomes highly stereotyped, at sexual maturity. Adults can sing in either the undirected state when they are alone or the directed state when they sing to a female.

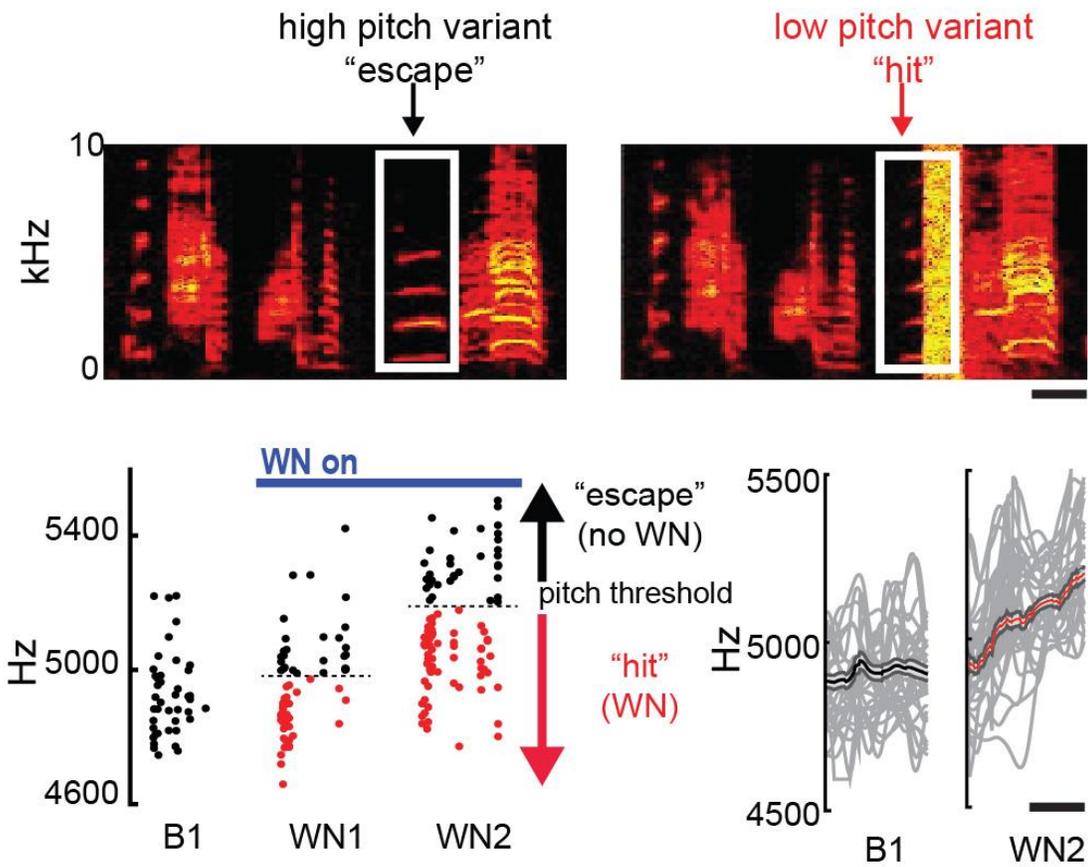


Figure 4: Adult pitch learning paradigm

Top, Example sonograms during pitch learning. Scale bar, 50 milliseconds. *Bottom left*, pitch of targeted syllable before (baseline) and during (WN1, WN2) pitch learning. Black dots, "escapes"; red dots, "hits". *Bottom right*, frequency contours and mean of target syllable before (B1) and two days after (WN2) WN. Scale bar, 20 milliseconds.

1.3 The neural circuitry dedicated to song

The circuitry underlying singing is nearly as well defined as the behavior that relies on it. Two major pathways, known as the anterior forebrain pathway (AFP) and the song motor pathway (SMP), are known to be critical to song learning and production, respectively (Figure 5). Sitting at the most dorsal position of the motor pathway, the premotor nucleus HVC sends information to both RA, the avian analogue to motor cortex, and Area X, a striatal area dedicated to singing (Nottebohm, Stokes, & Leonard, 1976). The ablation of any of these three areas in the juvenile abolishes the bird's ability to sing an accurate copy of the tutor song (Nottebohm, Kelley, & Paton, 1982; Scharff & Nottebohm, 1991). As the juvenile bird learns to sing, a cortical nucleus known as LMAN, which forms a cortico-striatal-thalamic loop with Area X and thalamic nucleus DLM, generates highly variable firing patterns that are transmitted to RA (Kao, Doupe, & Brainard, 2005; Olveczky, Andalman, & Fee, 2005). This variable firing, without which song becomes prematurely stereotyped (Bottjer, Miesner, & Arnold, 1984), is thought to aid learning by allowing the bird to explore motor space to determine the most efficient gestures to produce an accurate copy of the tutor song (Kenji Doya, 1995). As the bird learns its song, inputs from HVC to RA are preferentially strengthened over inputs from LMAN, reducing the variability in song and producing a stable motor plan (Mooney, 1992). However, how the most effective gestures for producing song are reinforced remains unknown.

1.3.1 Midbrain-basal ganglia cellular physiology in the songbird

The functions of midbrain dopamine release in the avian striatum at a cellular level are remarkably similar to that in mammals. Dopamine release in both the mammalian and avian striatum is calcium and action potential- dependent and regulated by the dopamine transporter (DAT) (Cragg & Rice, 2004; Gale & Perkel, 2005; Reiner, Laverghetta, Meade, Cuthbertson, & Bottjer, 2004). The region of the striatum dedicated to learning song, Area X, receives dense dopaminergic input from the midbrain and has higher levels of dopamine release than surrounding striatal areas (Gale & Perkel, 2005; Gale et al., 2008). In the presence of receptor-specific agonists in zebra finch brain slices, D1Rs and D2Rs respectively increase and decrease striatal medium spiny neuron (MSN) excitability with a net inhibitory effect (Ding & Perkel, 2002), as is also seen in mammalian brain slices. DA appears to directly affect excitability of zebra finch MSNs by modulating the “slow ramp” depolarization that occurs before MSN spiking in both mammals and birds (Farries & Perkel, 2002; Nisenbaum & Wilson, 1995), with D1R activation increasing, and D2R activation decreasing the slope of the slow ramp (Ding & Perkel, 2002). As in mammals, D1Rs can dampen neuronal activity through retrograde messengers, driving presynaptic depression in MSNs in a PKA-dependent manner by reducing glutamate release via retrograde messenger adenosine (Ding, Perkel, & Farries, 2003). Interestingly, D1Rs are also required for long term potentiation of glutamatergic inputs to MSNs (Ding & Perkel, 2004) (see figure 3 for

summary of dopamine actions at a cellular level in the songbird brain). Given that these inputs are from HVC and LMAN, two key cortical nuclei in song learning, and LTP can only occur in birds older than 40 days, at time point after which sensorimotor learning and more robust singing begin, dopamine is poised to play a bidirectional role in the modification of corticostriatal synapses during song learning, strengthening synapses that are active concurrently while decreasing transmission of synapses that are not simultaneously active with other synapses.

1.3.2 Midbrain-basal ganglia circuit physiology in the songbird

At a circuit level, the avian and mammalian striatum both receive dopaminergic input from anatomically and physiologically similar VTA and SNc but differ in the circuit mechanisms that drive dopamine release in the striatum. Both avian and mammalian VTA and SNc are composed of dopaminergic and nondopaminergic populations. These dopaminergic cells in both classes of animals share similar properties such as slow, broad action potentials, strong spike-frequency adaptation and autoinhibition mediated by D2Rs (Gale & Perkel, 2006). Though the physiological properties of the VTA and SNc as well as dopamine release, uptake, and receptor action in the striatum are similar in birds and mammals, the pathways through which striatal dopamine release occurs differ. Briefly, in the classical mammalian corticostriatal loop, the striatum disinhibits the thalamus by inhibiting the globus pallidus and substantia nigra pars reticulata and

allowing the thalamus to provide excitatory input to the cortex which then sends excitatory input back to the striatum (Figure 6). The GABA-ergic MSNs of the striatum also send direct inhibitory input to the VTA and SNc (Haber, Fudge, & McFarland, 2000). In contrast to the mammalian striatum, avian MSNs do not project outside the striatum and instead inhibit targets within the striatum, namely pallidal-like cells. These pallidal cells then send inhibitory axons to a thalamic area involved in song learning, DLM, as well as axon collaterals to the ventral pallidum (VP), an area implicated in reward and pleasure seeking in mammals (Smith, Tindell, Aldridge, & Berridge, 2009). VP in turn bilaterally inhibits VTA and SNc to decrease dopamine release in Area X (Gale & Perkel, 2010).

Though MSNs have been the main cell type studied in slice physiology experiments in avian striatum, the role of striatal inputs and outputs in vivo has been more thoroughly characterized in the projection-type pallidal cells. Importantly LMAN and HVC provide excitatory input to pallidal cells as well as MSNs in Area X. Excitatory input to MSNs from HVC drive inhibition of pallidal cells which allows for disinhibition of thalamic nucleus DLM which in turn drives LMAN firing (Leblois, Bodor, Person, & Perkel, 2009). Pallidal cells are active during singing and responsive to playback of the bird's own song (BOS) under anesthesia and have been found to drive BOS responses in dopaminergic VTA and SNc cells through disinhibition of VP (Gale & Perkel, 2010). In the presence of dopamine however, BOS responsiveness and firing variability in pallidal cells

decrease though spontaneous firing rates increase. This decrease in firing variability reduces firing in DLM, which depends on the irregular firing of pallidal cells, and decreases excitatory drive to LMAN (Leblois et al., 2009). One could imagine that the presence of dopamine could induce the increased stereotypy observed in directed song by modulating the activity of both variability-generating LMAN and midbrain dopaminergic areas, decreasing LMAN-induced song variability and strengthening and stabilizing cortical inputs to MSNs that produce favorable song gestures.

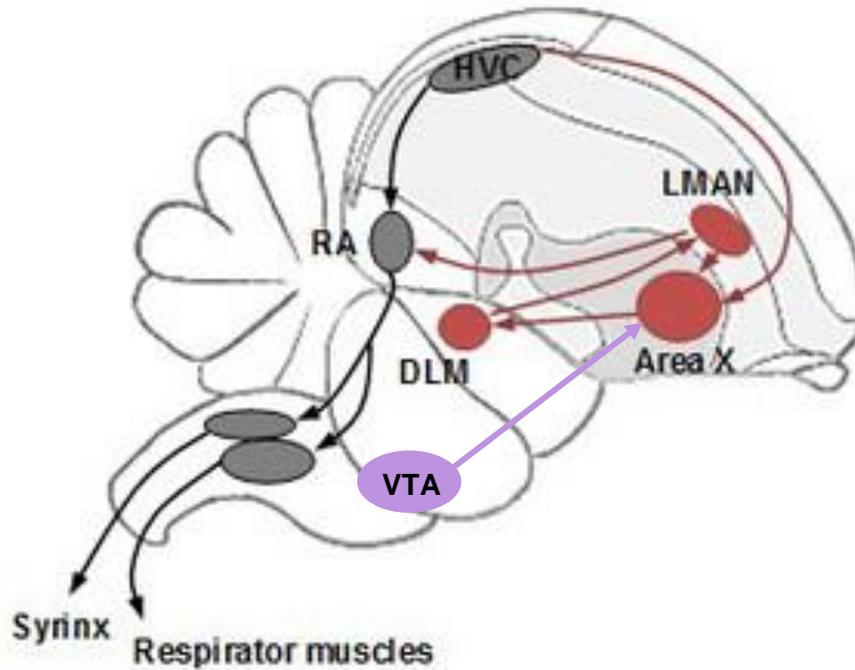
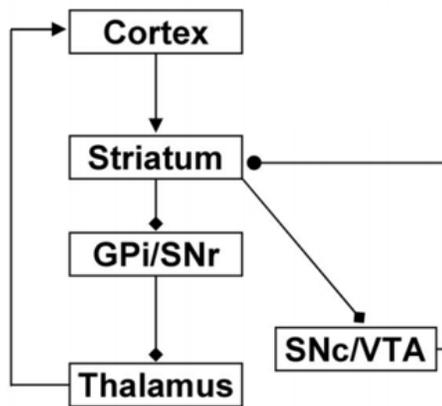


Figure 5: A simplified schematic of the song system

A sagittal view of the zebra finch brain. Black indicates the song motor pathway, red indicates the song learning pathway. HVC used as a proper name; RA, robust nucleus of the arcopallium; Area X, striatopallidal component of the song system; LMAN, lateral magnocellular nucleus of the anterior nidopallium; DLM, medial nucleus of the dorsolateral thalamus; Area X receives dense dopaminergic input from the ventral tegmental area (VTA); VTA (purple). This schematic does not show all nuclei and connections for simplicity. *From Wada 2012.*

mammalian



avian

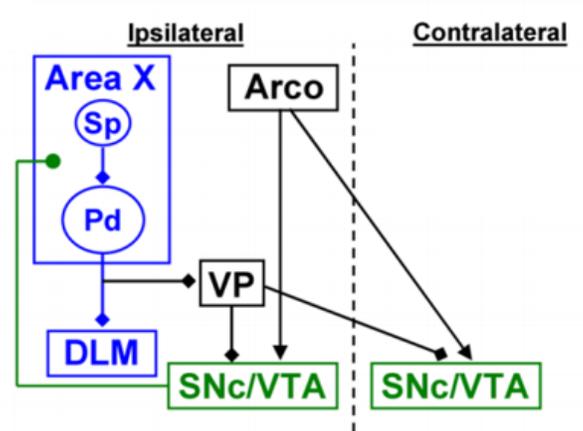


Figure 6: Comparison of avian to mammalian striatum

Left, schematic of canonical corticostriatal pathway in mammals. Right, schematic of analogous corticostriatal pathway in zebra finches. Note that medium spiny neurons (Sp) do not project out of the song striatum, Area X, and that pallidal cells (Pd) are a type of cell in Area X rather than a specific structure as seen in mammals. GPi, globus pallidus; SNr, substantia nigra pars reticulata; SNc, substantia nigra pars compacta; VTA, ventral tegmental area; Arco, arcopallium; VP, ventral pallidum; DLM, medial nucleus of the dorsolateral thalamus. *From Gale et al, 2008.*

1.4 The role of midbrain-basal ganglia circuitry in songbirds

As described briefly above, the anterior forebrain pathway (AFP), composed of LMAN, Area X, and DLM is critical for both juvenile and adult vocal learning. Here I will describe the role of the AFP, in particular the role of Area X, the area of the striatum dedicated to song, and dopamine, presumably released by the midbrain into the striatum, in various forms of song learning and modification in the zebra finch.

1.4.1 Juvenile learning

Juvenile learning is composed of an early sensory phase, in which the juvenile pupil memorizes his tutor's song, and a later sensorimotor phase, in which the pupil practices the tutor song extensively.

In sensory learning, higher secondary auditory areas and premotor area HVC are known to be necessary for encoding of the tutor memory (London & Clayton, 2008; Roberts, Gobes, Murugan, Olveczky, & Mooney, 2012) while the roles of the striatum and the dopaminergic midbrain in sensory learning are less well understood. Young birds infused with NMDA receptor antagonists in LMAN, which sends a large projection to Area X, during tutoring copy less of their tutor's song than control siblings, though their ability to discriminate between zebra finch and canary song remained intact (Basham, Nordeen, & Nordeen, 1996). Exposure to a familiar tutor induces Fos+ expression in both dopaminergic and non-dopaminergic cells in the VTA and SNc (E. J. Nordeen, Holtzman, & Nordeen, 2009); however these experiments are unable to test the

necessity of midbrain populations to song copying. Preliminary experiments in our laboratory suggest that infusion of D1 and D2-type receptors antagonists into HVC during sensory learning has a subtle effect on song memorization (Tanaka and Mooney, unpublished), though the role of dopamine receptors in the striatum during tutoring has not yet been tested. Together these experiments may hint at involvement of the striatum and midbrain in sensory learning but more substantial evidence as to the role of these areas in sensory learning remains to be found.

In contrast to sensory learning, the role of the AFP in sensorimotor learning has been more extensively examined. LMAN is required for babbling during the subsong phase of sensorimotor learning (Aronov et al., 2008) and inactivation of LMAN reduces trial-to-trial variability of song during later phases of learning (Oliveczky et al., 2005). Both LMAN and Area X have necessary and apparently opposing functions during sensorimotor learning. LMAN ablation causes young birds' songs to crystallize prematurely (Bottjer et al., 1984) while Area X lesions prevent stabilization of song sequence and spectral structure (Scharff & Nottebohm, 1991; Sohrabji et al., 1990). Knockdown of the FOXP2 gene in Area X, which is thought to mediate its effects through D1-type receptors, reduces both spectral and temporal accuracy in copying of the tutor song as well as syllable omission or repetition (Haesler et al., 2007). Though the studies described above establish that Area X and dopamine signaling are necessary for

song learning, they hint at but do not explore the necessity or sufficiency of dopaminergic projections from the midbrain to sensorimotor learning.

1.4.2 Pitch learning and recovery in adults

The role of the AFP in pitch learning has been extensively tested. Given the role of LMAN in injecting variability into song, many were curious as to its role in pitch learning, namely if adult birds could still learn to shift their pitch in the absence of LMAN. If variability is important for exploring motor space and facilitating changes in learned behaviors, inactivation or ablation of LMAN would render birds unable to learn in response to WN. Indeed, inactivating and lesioning LMAN or blocking the NMDA receptors in RA that LMAN acts through greatly impairs birds' ability to learn in response to WN playback as well as their ability to return to their original pitch after pitch learning (Ali et al., 2013; Andalman & Fee, 2009; Charlesworth, Tumer, Warren, & Brainard, 2011; Warren et al., 2011). Lesions of Area X also abolish pitch learning though in contrast to LMAN do not impair recovery from pitch learning (Ali et al., 2013). Interestingly, the effects of chemically lesioning tyrosine hydroxylase positive (TH+) terminals in Area X with 6-OHDA mirror those of Area X lesions (Hoffmann, Saravanan, Wood, He, & Sober, 2016), suggesting that dopaminergic innervation of Area X may be critical to pitch learning but not recovery. However one cannot specifically attribute this effect to the death of dopaminergic terminals given that 6-OHDA kills noradrenergic as well as dopaminergic terminals. Though the lesioning studies

described above suggest Area X as a locus for pitch learning that may act through dopaminergic signaling, the type of receptor in Area X as well as the type of cell that gives rise to TH+ terminals in Area X remains unknown.

1.4.3 Deafening-induced song degradation

As adults, zebra finches actively maintain their songs using auditory feedback. In the absence of auditory feedback, song begins to degrade spectrally a few days after the removal of feedback, becoming completely unrecognizable six to ten weeks after feedback removal (K. W. Nordeen & Nordeen, 1992). Temporal degradation appears on a slightly slower timescale, usually not becoming apparent until at least a month after feedback removal. Concurrent deafening and lesioning of LMAN prevent both spectral and temporal degradation indefinitely (Brainard & Doupe, 2000b). In birds with songs with variable syllable transitions, such as Bengalese finches, pairing deafening and LMAN lesioning results in song degradation that occurs on an even faster time scale than in zebra finches (Okanoya & Yamaguchi, 1997). Deafening combined with Area X lesions also prevents song degradation for up to two months after deafening (Kojima, Kao, & Doupe, 2013). The combination of AFP lesions and removal of auditory feedback strongly suggest a role for the AFP in song maintenance though there are no studies to date that examine the role of dopamine or the midbrain in deafening-induced song degradation.

1.4.4 Social context-dependent changes in song

Directed singing is dependent on cortical nucleus LMAN, which is also necessary for song learning (Kao & Brainard, 2006). During directed song, the firing rate and bursting of cells in LMAN decreases dramatically and becomes more stereotyped (Kao et al., 2005), likely allowing its output, motor cortex analog RA, to fire in a more regular fashion and drive a more stereotyped song.

Dopamine plays a critical role in the transition from undirected to directed song. During directed song, levels of dopamine in striatal Area X increase relative to baseline and undirected song whereas decreased levels of dopamine in the presence of DAT blockers prevents the increased stereotypy associated with directed song (Sasaki et al., 2006). The infusion of D1R antagonists into Area X can also prevent decreased variability during directed song (Leblois et al., 2010), providing further evidence that dopamine is necessary for directed song. The VTA and SNc provide substantial input to Area X and likely provide the majority of the dopamine required for directed song. Lesion studies of the VTA and SNc in zebra finches have been inconclusive as to the necessity of these areas to directed song (Hara, Kubikova, Hessler, & Jarvis, 2007), likely due to the massive area covered by the VTA/SNc complex and the inability of traditional lesioning methods to efficiently and safely ablate large regions in the midbrain.

A more recent study (Murugan, Harward, Scharff, & Mooney, 2013) has shown that corticostriatal transmission is strongly influenced by dopamine. In normal adult

birds, propagation of activity from HVC to LMAN via Area X is slowed in the presence of D1R agonists, suggesting that dopamine modulates propagation of corticostriatal activity. However, adult birds with knockdown of the FoxP2 gene in the striatum showed insensitivity to both D1R agonists and antagonists. Western blots revealed that both D1Rs and DARPP32 levels were decreased in these knockdown birds. Given that these same knockdown birds were unable to transition from undirected to directed song, one can conclude that dopamine is indeed necessary for normal corticostriatal signal propagation required in context-dependent singing. In all, studies of context-dependent singing have demonstrated a critical role for striatal dopamine for changes in motor output.

1.5 How do midbrain-basal ganglia circuits drive learning in the songbird?

The increases in stereotypy seen in directed song that results from an influx of dopamine and D1R activation strongly suggest that dopamine may act to reinforce favorable gestures during song learning. In the striatum, glutamatergic synapses from HVC and LMAN synapse on the same MSN. It has been posited that concurrent activation of HVC and LMAN synapses on the same MSN establishes an eligibility trace that “tags” the HVC synapse (Fiete, Fee, & Seung, 2007). This synapse can then be later “identified” and strengthened by an influx of dopamine from the VTA if its activation results in a favorable motor outcome (Figure 7) (Fee & Goldberg, 2011). While interesting, this model does not describe how modulation of VTA, and thus dopamine release into Area X, by its inputs might occur. Two of the largest inputs to the VTA, the ventral pallidum (VP) and the ventral arcopallium (Aiv), may act in a push-pull reinforcing-punishing manner similar to that seen in mammalian systems (Lammel et al., 2012). VP is a major center for reward and pleasure seeking in the mammalian brain, as seen in classic studies in which rats implanted with stimulating electrodes over VP will adopt behaviors that drive self-stimulation (Smith et al., 2009). Aiv, on the other hand, receives auditory inputs from areas across the forebrain including *Avalanche*, a higher order auditory structure that receives input from HVC and may convey an error signal to its outputs (Mandelblat-Cerf, Las, Denisenko, & Fee, 2014). Depending on whether auditory feedback indicates that current song is a good or poor match to the tutor song, VP or Aiv, respectively, may increase or

suppress dopaminergic firing in VTA/SNc. This may occur through direct contact with dopaminergic cells or through indirect inhibition by local interneurons; further research is needed to understand the connectivity between VTA/SNc and its inputs. It seems reasonable to hypothesize that if the song does happen to be a good match to the tutor song, dopaminergic firing in the VTA/SNc will increase and dopamine levels in Area X will rise, increasing the regularity of firing of Area X pallidal cells and suppressing excitatory drive from DLM to LMAN (Leblois et al., 2009). This will then dampen the amount of variable input from LMAN into motor cortex analog RA and result in increased stability of the version of song that best matches the tutor song. However, though dopamine and midbrain-striatal circuits have long been thought to play a critical role in models of vocal learning, neither the role of a particular midbrain cell type or dopamine signaling has been explored in juvenile or adult forms of learning

Here I use an intersectional genetic strategy to show that VTA_x specifically are necessary in multiple forms of song learning and modification across different ages. By utilizing an approach that affects only our projection of interest, I avoided potential confounds, such as a reduction in motivation or motor control, associated with eliminating activity across all VTA cells. I find that VTA_x cells, which project almost exclusively to Area X, are required for both imitative learning of tutor song in the juvenile and for pitch contingent learning in the adult, as well as for context-dependent and deafening-induced song modification. I also show that striatal D1-type dopamine

receptors are necessary for both internally driven juvenile learning and externally reinforced adult learning. Furthermore, pitch-contingent optogenetic stimulation of VTA terminals in Area X revealed that the projection from VTA to Area X is sufficient to drive vocal learning. Surprisingly, I find that neither VTA_x cells nor striatal D1-type receptors are required for recovery from pitch-contingent learning in the adult. My findings show that VTA_x and striatal D1-type receptors are indeed required for forms of learning outside the realm of association and suggest that both internally and externally driven learning, as well as imitative and non-imitative learning, rely on similar cellular substrates.

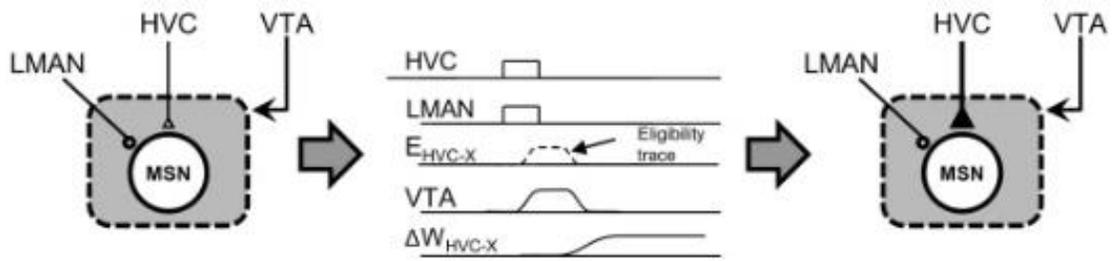


Figure 7: Model of how the VTA reinforces favorable song gestures

Cortical glutamatergic synapses from HVC and LMAN synapse on the same MSN in Area X. Concurrent activation of HVC and LMAN synapses on the same MSN “tags” the HVC synapse with an eligibility trace. This synapse can then be later “identified” and strengthened (change in W) by an influx of dopamine from the VTA if its activation results in a favorable motor outcome. *From Fee and Goldberg 2011.*

2. VTA_x cells and D1-type receptors are necessary for juvenile learning

2.1 Introduction

Though striatonigral pathways and the striatal dopamine receptors they act on are known to be necessary to externally reinforced forms of learning such as operant learning and classical conditioning (Adamantidis et al., 2011; Tsai et al., 2009; Witten et al., 2011), their roles in forms of learning that occur in the absence of external cues or reinforcement are unknown. In contrast to mice or monkeys that learn tasks to receive food or liquid rewards or to avoid punishment, juvenile zebra finches memorizes, practices and eventually precisely copies its father's song all in the absence of external cues or guidance (L.A. Eales, 1985; Immelman, 1969; Price, 1979). Zebra finches are also valuable model system in which to study this question because the zebra finch brain contains a striatal region dedicated to purely to song (Nottebohm et al., 1976). This characteristic of zebra finch brain anatomy allows the experimenter to avoid conflating effects of striatonigral manipulations on movement or motivation with effects on singing, potentially allowing one to tease apart the mechanisms that underlie learning from those that are critical for movement or motivation. In this chapter I will describe the results of experiments in which I tested the necessity of both VTA_x cells and D1-type receptors to juvenile song learning using intersectional cell ablation and *in vivo* microdialysis, respectively.

2.2 Results

2.2.1 VTA_x cells are necessary for copying tutor song at the motif level

Research into the role of midbrain structures in the songbird has been limited due in large part to a lack of techniques with which particular subsets of cells can be manipulated. As the VTA both sends and receives a massive amount of outputs and inputs (Watabe-Uchida, Zhu, Ogawa, Vamanrao, & Uchida, 2012) I wanted to use a technique that could target a specific projection type without interfering with behaviors not related to song. I used a combination of a virally encoded Cre-dependent caspase3 (Yang et al., 2013), a molecule involved in apoptosis, and a virally encoded retrogradely transported Cre to selectively drive apoptosis in VTA_x cells. To confirm that this technique effectively ablated VTA_x cells I injected a subset of birds with AAV2/9 CMV-Cre and AAV2/1-FLEX-Caspase3 into Area X and VTA, respectively, and gathered another subset of control birds that I either did not inject or injected with only Cre or only caspase. After injecting both groups of birds with retrograde tracer into Area X and then sacrificing them five days later, I confirmed that the intersectional ablation did indeed significantly reduce the number of VTA_x cells in experimental birds compared to controls (unpaired two-tailed t-test: control: 4423 ± 143 cells, $n = 10$ birds; experimental: 3669 ± 156 cells, $n = 10$ birds; $p = 0.003$) (Figure 8).

In order to examine the role of VTA_x cells in sensorimotor learning in juvenile birds I employed the intersectional ablation technique described above to selectively ablate only the VTA cells that project to Area X. I injected 25-30 day old birds bilaterally with AAV2/9 CMV-Cre and AAV2/1-FLEX-Caspase3 into Area X and VTA, respectively, in order for viral

expression to coincide with sensorimotor learning at ~60 days. After viral injection, juveniles were raised with their tutor until 60 days post hatch (abbreviated as 'dph'), after which they were housed with other birds of similar age and recorded monthly until they reached adulthood (120 dph). They were then injected bilaterally with dextran in Area X and sacrificed 5 days later to provide an anatomical readout as to whether we had effectively ablated VTA_x cells (Figure 9).

After obtaining cell counts of VTA_x cells, we found that birds injected with both caspase and Cre had significantly lower similarity to the tutor than age-matched controls that were either injected with Cre or caspase alone or not injected (two-tailed t-test: experimental: n = 12, 56.25 +/- 6.61%; control: n = 5, 83.00 +/- 4.87%; p = 0.027). Notably, given that our injections failed to ablate VTA_x in some of the experimental birds, the number of VTA_x cells was strongly correlated with percent similarity to tutor song ($R^2 = 0.689$, p = 0.0008) (Figure 10). In the most extreme cases, six of experimental birds were unable to develop complex syllables much less accurately copy their tutor's song. These six birds were able to sing a string of introductory and call-like syllables with a tempo similar to a typical zebra finch song but were only able to copy roughly 1 of 6 tutor syllables on average (n = 6 out of 12 birds, 4/24 tutor syllables copied across 6 birds) (Figure 11).

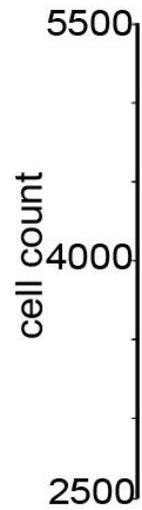
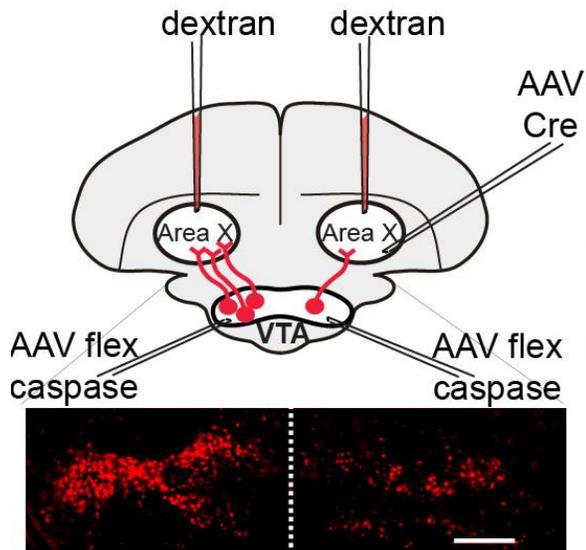


Figure 8: Intersectional ablation of VTAX cells

Left, Unilateral VTAX ablation on right hemisphere with VTA histology. *Right*, VTAX cell counts for control (grey, n = 10) and VTAX ablation (red, n = 10) birds. **P < 0.01, two-tailed t-test. Scale bar, 500 μ m.

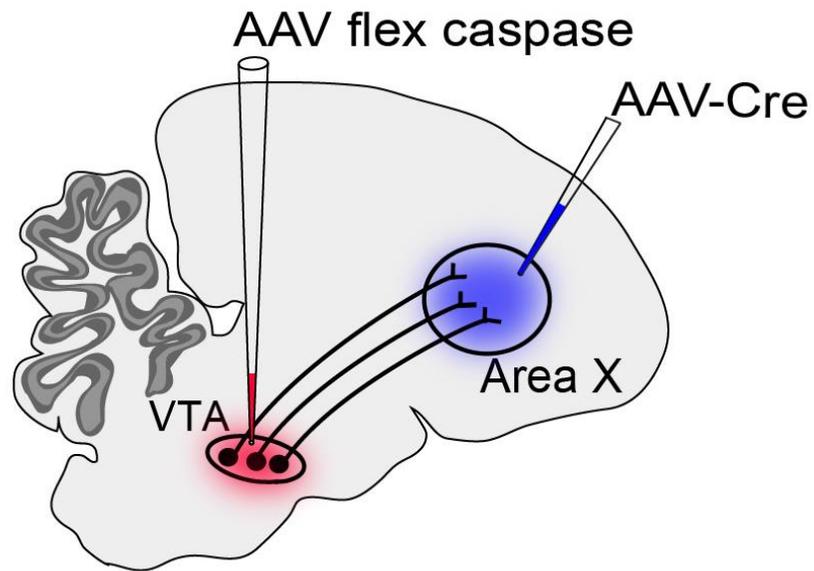
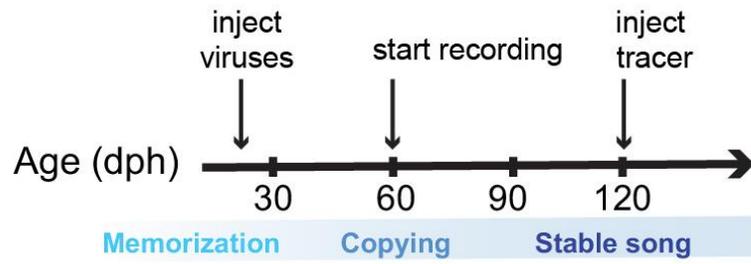


Figure 9: Experimental design of juvenile ablation experiments

Top, timeline of experiment. *Bottom*, sagittal section of zebra finch brain with locations of viral injections.

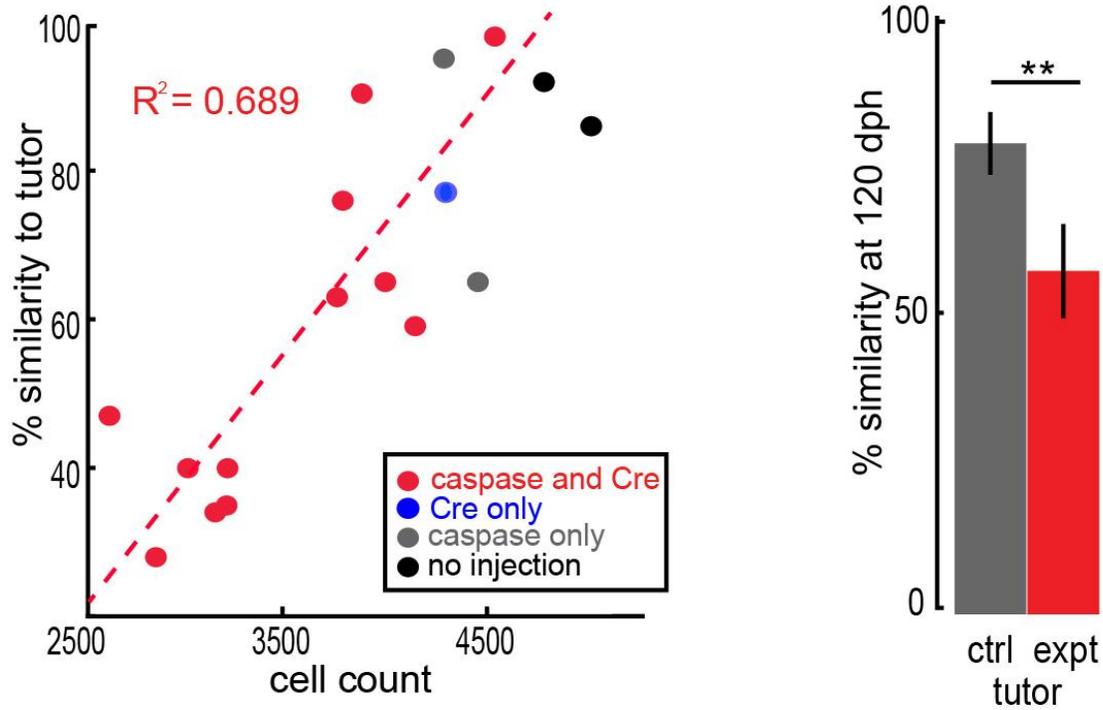


Figure 10: The number of VTAX cells strongly correlates with the pupil's ability to copy the tutor song

Left, percent similarity to tutor as a function of remaining VTAX neuron number ($R^2 = 0.689$, $p = 0.0008$ for $n = 12$ birds (Cre and caspase injected birds)). *Right*, percent similarity to tutor. *Red*, experimental birds ($n = 12$). *Grey*, control birds ($n = 5$). * $P < 0.05$, paired two-tailed t-test.

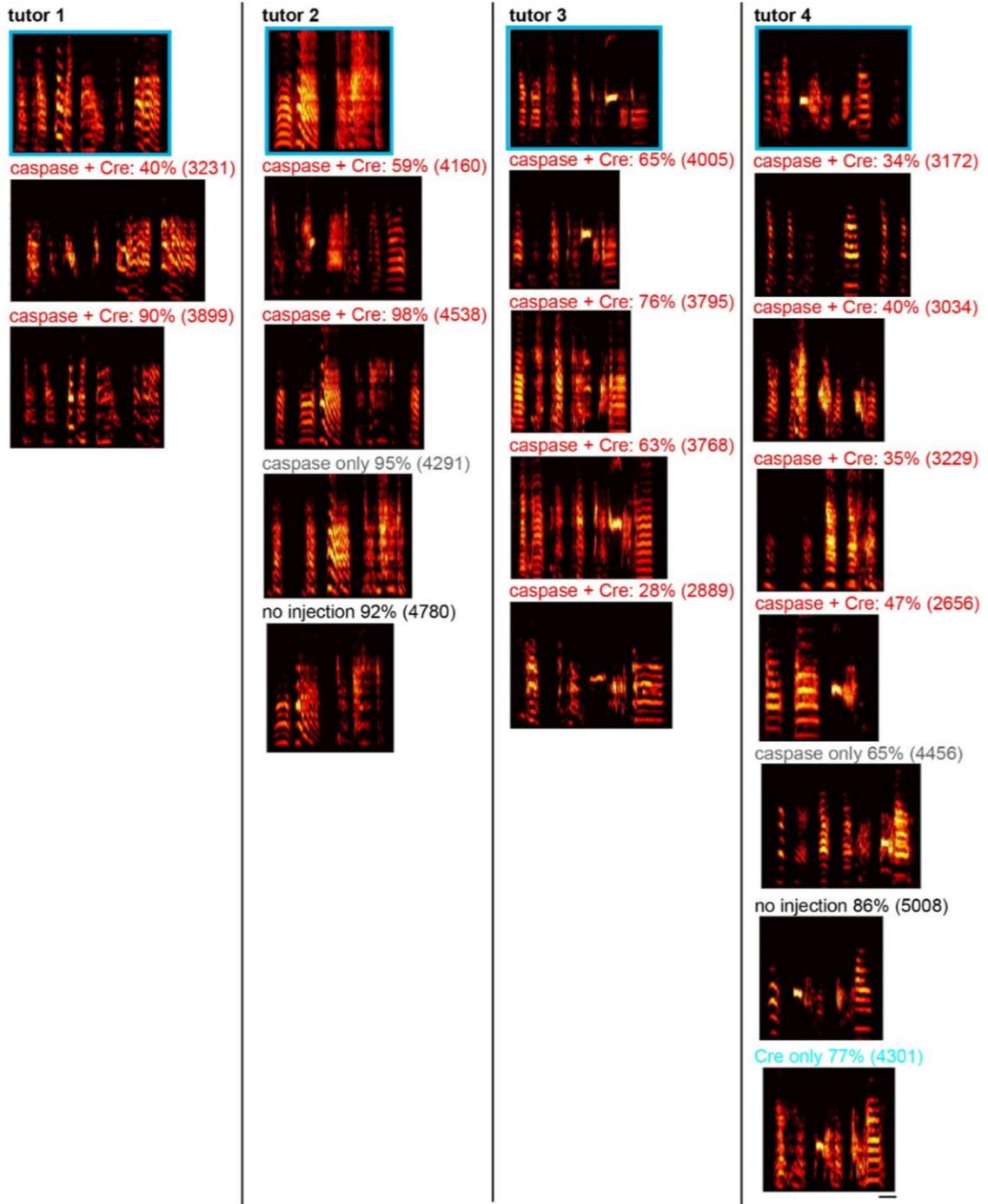


Figure 11: Sonograms of each pupil's song and their tutor songs

Each column includes sonograms from a tutor (outlined in blue) and his pupils. The percent similarity to the tutor is listed above each pupil's sonogram as well as the number of VTA_x cells in parentheses. Red font indicates birds injected with both Cre and caspase, grey indicates birds injected with only caspase, blue indicates birds injected with only Cre, black indicates birds that were not injected. Scale bar, lower right 50 milliseconds. All sonograms span 0-10 kHz on the y-axis.

2.2.2 Ablation of VTA_x cells does not affect motif stereotypy, individual syllable structure, or amount of singing

2.2.2.1 Motif stereotypy

In order to examine whether the experimental juveniles could sing stereotyped motifs we compared song motifs at different developmental time points to the bird's own song motif as an adult to generate a measure of self-similarity. Though similarity to tutor was markedly reduced in experimental birds, the self-similarity scores at 120 dph were not significantly different between control and experimental groups (two-tailed t-test: experimental: $n = 12$, $90.98 \pm 2.77\%$; control: $n = 5$, $95.07 \pm 1.82\%$; $p = 0.34$). Across development, even the six birds with the most severe deficits in tutor copying paralleled the increase in self-similarity seen in control birds (Figure 12).

2.2.2.2 Syllable structure

In the subset of birds with the most severe deficits in tutor copying, I also examined the spectral features of individual syllables at 60 and 90 dph to adult syllables at 120 dph to determine whether syllable features changed dramatically over time, as is seen in normal song development, or whether song remained "frozen" in place from the time of viral expression (~60 dph) onward. Though the experimental birds sang extremely poor copies of tutor song, neither the entropy nor the goodness of pitch of individual syllables significantly differed from controls ($n = 6$ syllables from 6 experimental birds, 4 syllables from 4 control birds; 2 factor ANOVA with Repeated Measures on One Factor; entropy AxB $p = 0.676$; goodness of pitch AxB $p = 0.104$) (Figure 13). These birds' normal developmental trajectory coupled with an inability to copy tutor song is reminiscent of birds with decreased dopamine signaling in Area X due to

FoxP2 knockdown (Haesler et al., 2007; Murugan et al., 2013), though unlike the VTAx lesioned birds, the FoxP2 birds were able to copy a number of their tutor's syllables.

2.2.2.3 Amount of singing

In order to account for the possibility that by disrupting dopaminergic signaling in this pathway we may have inadvertently impaired the bird's motivation to sing, I also compared number of songs per day and motif length of experimental birds to control birds with good copies of their tutor's song. Experimental birds showed no significant differences in number of songs per morning at different times during development compared to control birds (number of songs over development at 60, 90, and 120 dph: 2-factor ANOVA with Repeated Measures on One Factor: AxB: $p = 0.599$) (Figure 14). As adults, experimental birds also adapted normal courtship posturing and readily sang when presented with a female. The ability of the experimental birds to sing as much as control birds throughout development as well as show motivation in courtship allay concerns that the deficits in tutor copying might have been due to a motivational impairment and a resultant lack of practice needed to produce a good imitation of the tutor's song.

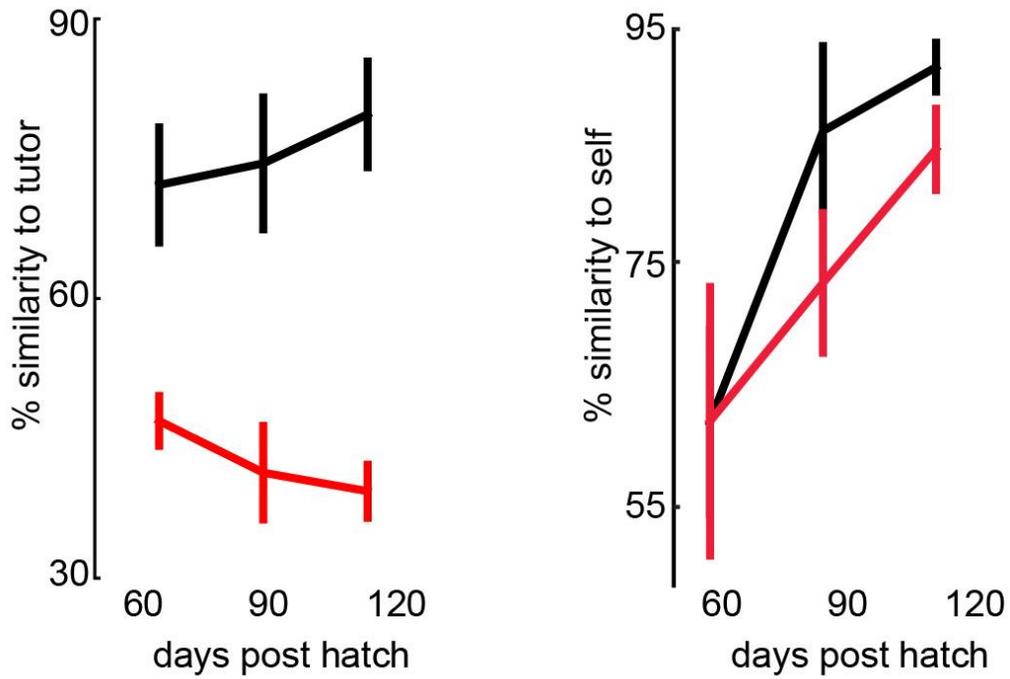


Figure 12: Percent similarity to tutor and to self over development

Left, percent similarity to tutor over the course of sensorimotor learning. *Right*, percent similarity to adult self over the course of sensorimotor learning. Red indicates six experimental birds with the most severe deficits in tutor copying, black indicates four control birds.

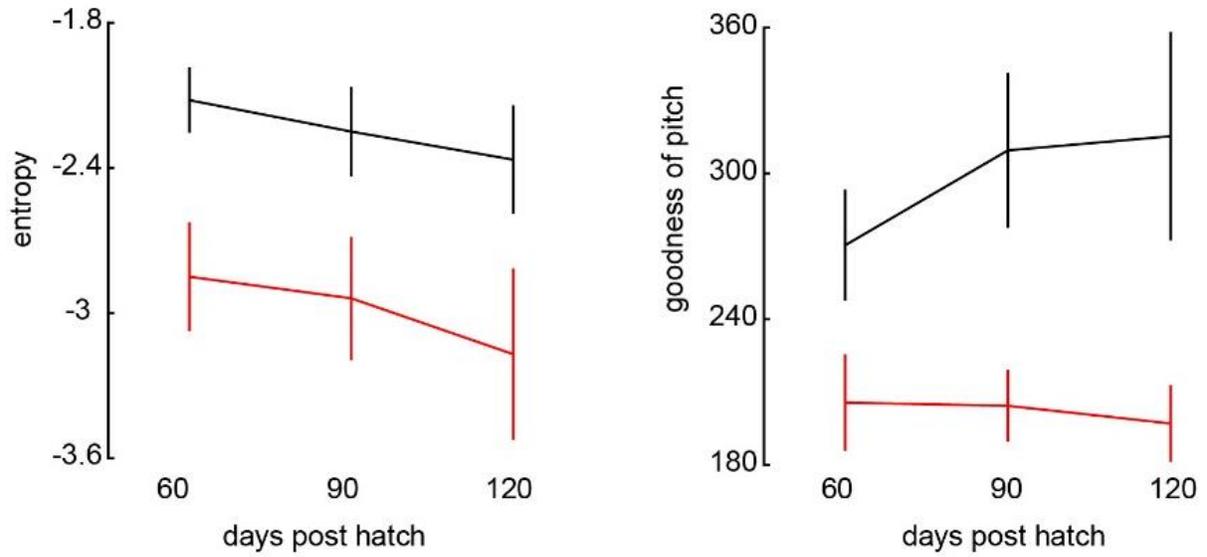


Figure 13: Spectral features of pupils' song over development

Left, entropy of single syllables over development. *Right*, goodness of pitch of single syllables over development. Red, experimental birds, $n = 7$; black, control birds, $n = 5$.

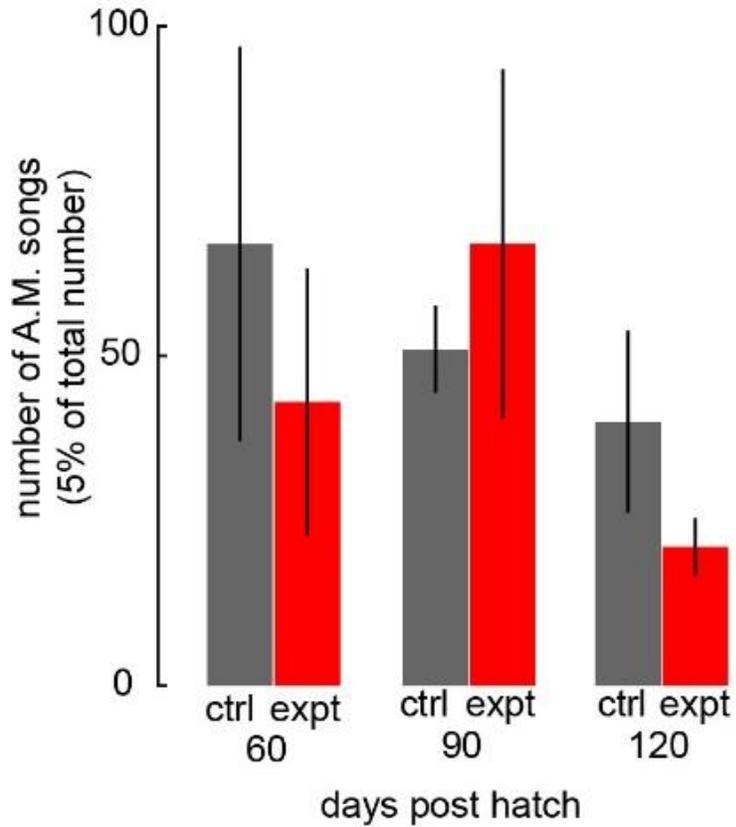


Figure 14: The number of experimental birds' songs over development is no different than the number of control birds' songs over development.

Number of songs over development. Five percent of songs between 8 and 11 a.m. were randomly sampled and counted for all birds. Counts listed represent a percentage of songs sung in the morning. Red, experimental birds, n = 6; grey, control birds, n = 4.

2.2.3 VTA_x cells project almost entirely to Area X

Earlier work in the song system has shown that a number of song nuclei other than Area X, namely HVC, LMAN, and Nif, are innervated with TH+ fibers and express higher levels of dopamine receptors than surrounding tissue (Kubikova, Wada, & Jarvis, 2010; Soha, Shimizu, & Doupe, 1996). Given the dramatic effects to song production seen from lesions to any of these four nuclei alone, I wanted to know if the deleterious effects of VTA_x ablation to juvenile song learning could be due to ablating collaterals critical to the function of other song nuclei. To determine if VTA_x cells send axon collaterals to other song nuclei that receive dopaminergic input, I performed dual tracer injections of dextran 594 into Area X and dextran 488 into either HVC, LMAN, or Nif, of 60 day old birds, the approximate age at which viral constructs should express in the juvenile experiments described above.

Though I was able to confirm that my injections were correctly targeted by examining the tissue for appropriate retrograde and anterograde label, I was only able to find small populations of cells co-labeled for both HVC and X (HVC_{VTA}: 23/1323 or 1.7%, 3 hemispheres, 2 birds; LMAN_{VTA}: 74/1549 or 4.8%, 3 hemispheres, 2 birds) in VTA, evidence which suggests that VTA_x do not send axon collaterals to HVC or LMAN (Figure 15). Retrograde label from LMAN and HVC was more dense in A11 than VTA, a dopaminergic area required for adult pitch contingency learning (Hamaguchi & Mooney, 2012), suggesting LMAN and HVC likely receive TH+ innervation from A11 rather than VTA (Figure 16A,B). Unlike HVC or LMAN injections, which resulted in sparse label in VTA, injections into Nif resulted in a larger amount of label in VTA that nevertheless shared minimal overlap with cells labeled by Area X injection (Nif_{VTA}: 95/1696 or 5.6%, 3 hemispheres, 2 birds). Cells retrogradely labeled from Nif and X injections

separated into two homogenous regions within VTA, with cells projecting to X located more rostrally and cells projecting to Nif more caudally. The small number of cells that were co-labeled was found in the area between these regions (Figure 16C).

To determine if these cells might send collaterals to HVC, LMAN or Nif once birds reach maturity, I performed the same set of tracer injections in 120 day old adult birds. The number of cells co-labeled from X and HVC or LMAN or Nif injections was small and comparable to the number of co-labeled cells found in juveniles (HVC_{VTA}: 14/1033 or 1.36%; 3 hemispheres, 2 birds; LMAN_{VTA}: 50/979 or 5.12%, 3 hemispheres, 3 birds; Nif_{VTA}: 95/1696 or 5.6%; 54/1054 or 5.10%; 3 hemispheres, 2 birds, paired two-tailed t-test between juvenile and adult percentages, $p = 0.56$). Given that the vast majority of VTA_X do not send axon collaterals to three of the critical players in song learning, I believe that the behavioral deficits produced by the intersectional cell ablation described above can be attributed specifically to VTA_X.

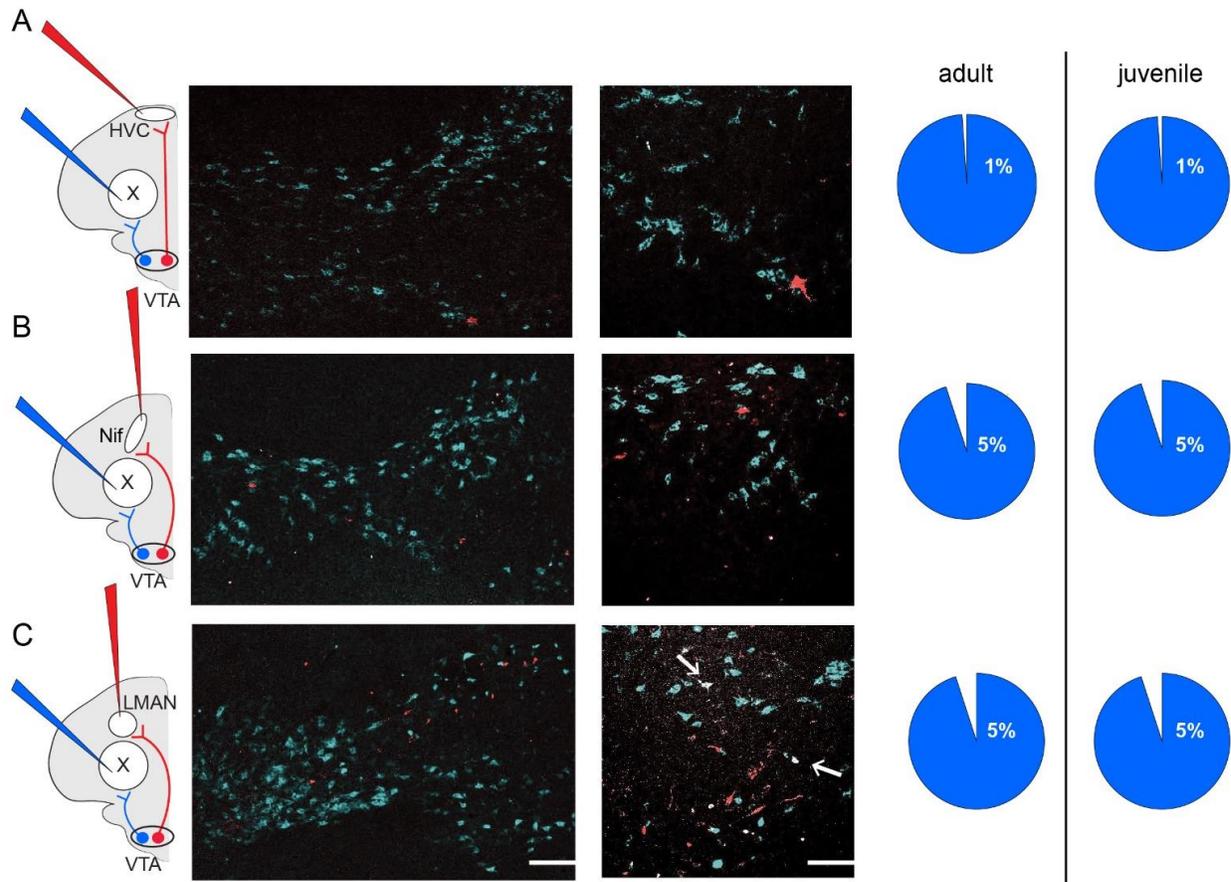


Figure 15: VTA_x cells project almost exclusively to Area X

From left to right: A) schematic of tracer injections into HVC and X; image of VTA with blue (X) and red (HVC) retrograde label; magnified image of VTA with cyan (X) and red (HVC) retrograde label; pie chart demonstrating percentage of cells co-labeled (white) out of total VTA_x cells (blue) in adult (left) and juvenile (right) birds. B) As in A) but with injections into Nif and X. C) As in A) but with injections in LMAN and X. Arrows in C) point to double-labeled cells. Scale bars, 100 μ m.

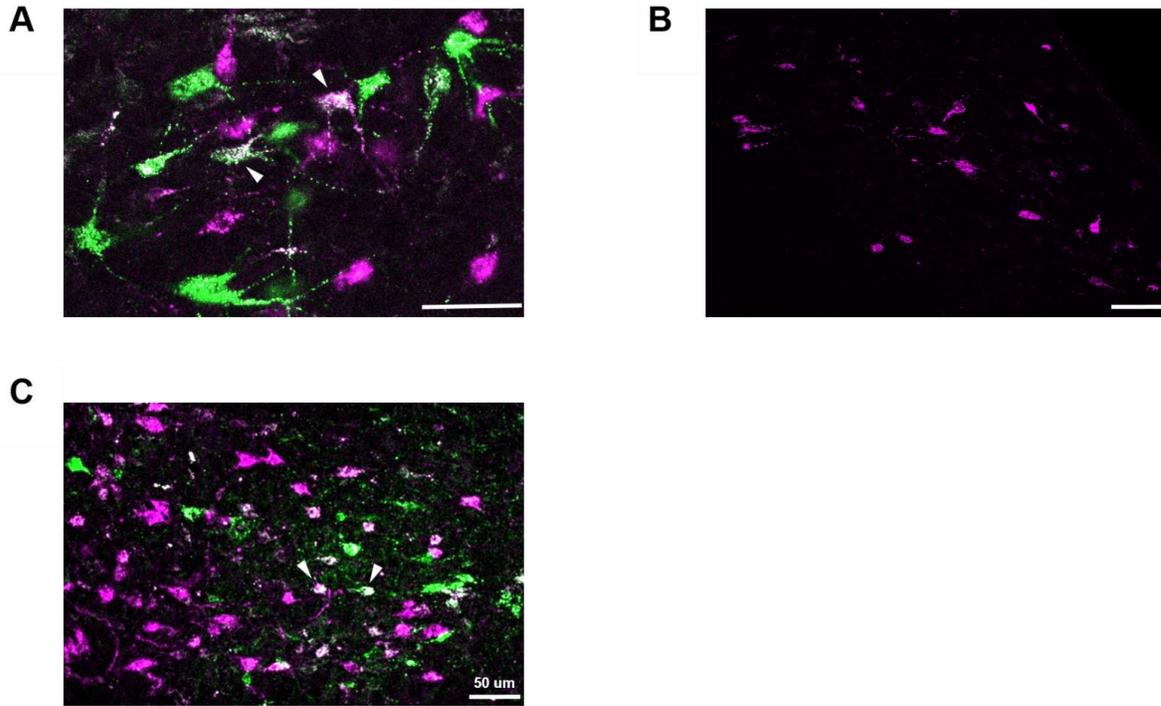


Figure 16: Retrograde labeling in A11 and posterior VTA from injections in song nuclei

A) Retrograde labeling from HVC (green) and X (magenta) in A11. B) Retrograde label from LMAN (magenta) in A11. C) Retrograde label Nif (green) and X (magenta) with X label located more rostrally and Nif label located more caudally. White arrowheads point to double labeled cells. All scale bars 50 µm.

2.2.4 D1-type receptors in Area X are necessary for improving song during sensorimotor learning

In order to examine whether dopamine receptors in Area X that presumably receive input from VTA_x cells are critical for juvenile song learning, I implanted a cohort of 45-50 day old juvenile siblings with microdialysis probes over Area X and infused the D1 antagonist SCH23390 to determine the dependence of juvenile learning on dopamine receptors (Figure 17). Over a period of 10 days I recorded the juveniles' songs and compared them to the song of their tutor. Though control juveniles, either implanted with microdialysis probes and infused with saline or not implanted, showed a steady upward trend in the similarity of their song to that of their tutor, juveniles infused with a D1 antagonist showed little to no improvement in similarity to tutor over the infusion period (2-factor repeated measures ANOVA: A x B: $p = 0.002$; paired two-tailed t-test: control birds: saline day -1: $49.04 \pm 3.79\%$; saline day 10: $60.75 \pm 2.46\%$, $n = 6$, $p = 0.001$; paired two-tailed t-test: experimental birds: drug day -1: $49.29 \pm 1.72\%$; drug day 10: $45.78 \pm 1.03\%$, $n = 5$, $p = 0.33$) (Figure 18). Once infusion of SCH23390 ended and the birds matured in the absence of drug infusion however, the experimental birds were able to increase the similarity of their song to the tutor's to a level that was statistically indistinguishable from that of control birds (unpaired two-tailed t-test: control birds: 40+ days after beginning of treatment: $67.08 \pm 2.30\%$, $n = 5$; experimental birds: 40+ days after beginning of treatment: $54.19 \pm 6.40\%$, $n = 4$, $p = 0.17$) (Figure 19).

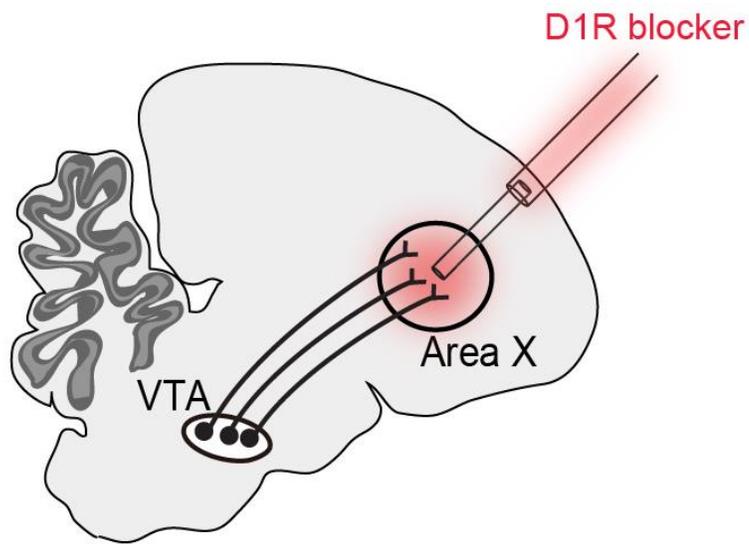
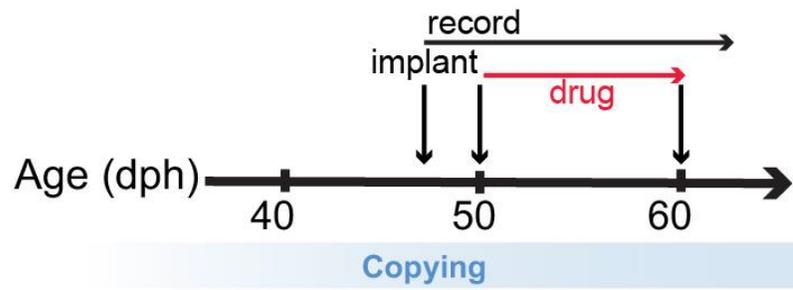


Figure 17: Experimental design of juvenile microdialysis experiments

Top, timeline of experiment. *Bottom*, sagittal section of zebra finch brain with location of microdialysis probe.

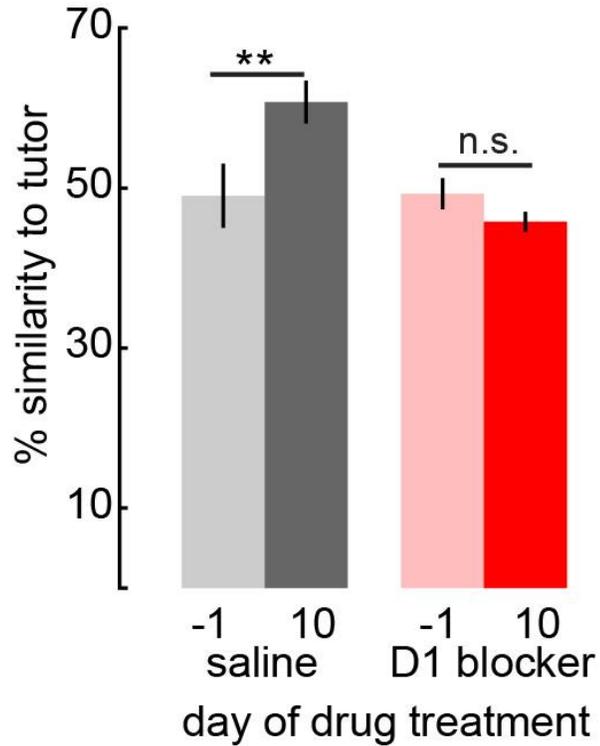


Figure 18: D1-type receptor antagonist infusion in Area X halts juvenile sensorimotor learning

Percent similarity to tutor one day before and 10 days after beginning of daily microdialysis for control (grey) (n = 3 implanted birds infused with saline and n = 3 intact birds) and SCH23390 birds (red) (n = 5 birds). ***P<0.001, 2-factor repeated measures ANOVA, paired two-tailed t-test.

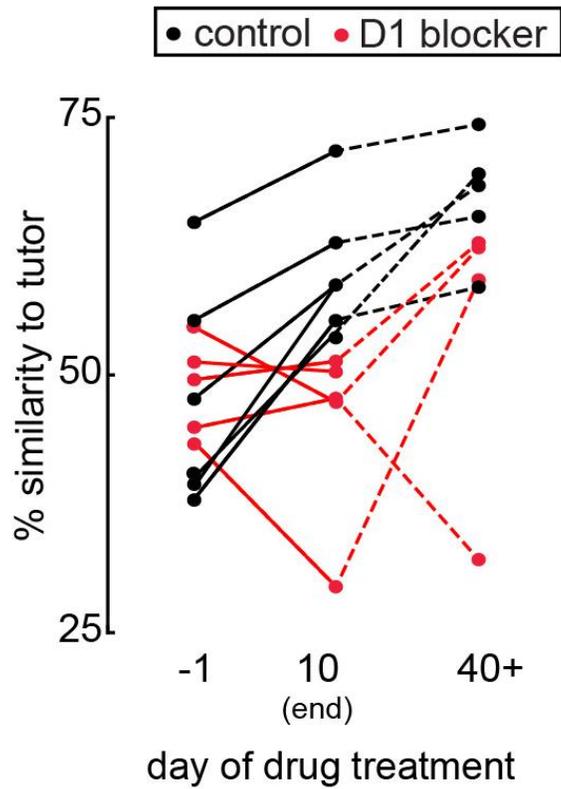


Figure 19: D1-type receptor antagonist infusion in Area X does not permanently prevent an increase in tutor song similarity over development
 Percent similarity to tutor one day before, 10days after, and 40+ days after beginning of microdialysis for SCH23390 (red, n = 5 birds) and control birds (black, n = 4 birds).

2.3 Discussion

In this chapter I show that both VTA_x cells and striatal D1-type receptors are necessary for juvenile song learning. Interestingly, though VTA_x ablation impairs the ability of juvenile birds to copy their tutor's song in a manner that is proportional to the number of cells ablated, neither the motif stereotypy, syllable structure, nor the motivation to sing and to court females is affected in birds with deficits in tutor song copying. Using a dual tracer strategy I show that the effects of VTA_x cell ablation cannot be attributed to inadvertent damage of collaterals to other nuclei involved in song learning. Inactivation of the receptors that VTA_x cells presumably act on, D1-type receptors in Area X, also halts improvements to tutor song copying during sensorimotor learning. These results demonstrate a role for this pathway and its downstream effectors in sensorimotor learning that is independent of pathways involved in motivation or motor control.

2.4 Methods

Juvenile (18–49 dph) male zebra finches were obtained from the Mooney lab breeding colony within the Duke University Medical Center animal facility.

Experimental procedures were conducted in accordance with the National Institutes of Health guidelines and were reviewed by the Duke University Medical Center Animal Care and Use Committee. Viral vectors were acquired from University of Pennsylvania Vector Core and University of North Carolina, Chapel Hill Vector Core.

2.4.1 Intersectional ablation of VTA_x cells

Male zebra finches (20-30 days post hatch (dph)) were food deprived for 30 minutes and then anesthetized with 2% isofluorane gas before being placed on top of a small heating pad in a custom stereotaxic apparatus. Rate of breathing and stability of surgical plane were monitored throughout surgery. The feathers over the skull were trimmed and topical anesthetic (0.25% bupivacaine) was applied before an incision was made in the skin from anterior to posterior with a scalpel. After pushing skin from the center of the skull with a cotton swab doused in 70% ethanol, craniotomies were made with a smaller scalpel at a predetermined distance from the bifurcation of the midsagittal sinus (the 'y-sinus'; coordinates measured from y-sinus: VTA: head angle 37 degrees, 1.65mm anterior, 0.5 and 1.8 mm lateral, 6.2 mm ventral; Area X: head angle 43 degrees, 5.0 mm anterior, 1.6 mm lateral, 3.2, 2.9 and 2.7mm ventral). To selectively ablate VTA_x cells, a pressure injection system (Drummond Nanoject II) was used to

make bilateral injections of a retrogradely transported Cre construct (AAV2/9.CMV.HI.GFP-Cre.SV40; Penn Vector, a total of 15 injections of 32.2 nl of Cre per hemisphere) into Area X at 3 different depths. A locally expressed Cre-dependent caspase construct was then injected into the VTA at 2 different locations along the medial-lateral axis (AAV2/1.Ef1 α .FLEXCasp3-2A-TEV; construct courtesy of Nirao Shah (Yang et al., 2013), UCSF, 15 injections of 32.2 nl of Casp3 per site per hemisphere i.e. a total of 4 caspase injection sites per bird). After these viral injections, the craniotomies were sealed with bone wax, the incision site was closed with tissue adhesive, and the bird was allowed to recover from anesthesia under a heat lamp. At the endpoint of each experiment and 5 days prior to perfusion, birds were injected with AlexaFluor 594 in Area X to retrogradely label VTA_x neurons. Five days after these tracer injections, birds were deeply anesthetized with an intraperitoneal injection of pentobarbital solution (Euthasol) and then perfused through the heart with 0.025 M phosphate-buffered saline followed by 4% paraformaldehyde. The brain was then removed from the skull and placed in a cryoprotective formalin sucrose solution (30% sucrose in 4% paraformaldehyde) overnight. The next day consecutive sagittal sections of the cryoprotected brain were cut on a freezing microtome and alternate sections were mounted on glass slides. Sections containing VTA were visualized and imaged under a confocal microscope (Zeiss Axioskop 2). The images were then examined in an image-

processing program and the number of fluorescent retrogradely labeled cells in VTA was counted in a semi-automated manner (ImageJ, CellCounter plug-in).

2.4.2 Microdialysis in Area X

Young (40-49 dph) juvenile male zebra finches that had recognizable syllables but had not yet developed a stereotyped motif were implanted bilaterally with microdialysis probes in Area X. Probes were constructed in house from plastic tubing which served as a drug reservoir fitted at the end with a 0.7-1.0 mm-long semipermeable membrane which allowed drug to slowly diffuse throughout the day (see Hamaguchi and Mooney, 2012 for probe design). Using surgical procedures and stereotaxic coordinates described above, craniotomies were made over Area X and neural recordings were made to confirm its depth (Differential A-C Amplifier 1700, A-M Systems). We approached Area X rostrally as to avoid LMAN (anterior Area X coordinates: initial head angle 35 degrees, 5.3 mm anterior marked with scalpel on skull, then adjusted head angle to 72 degrees, 1.2 mm anterior from scalpel mark, 1.7 mm lateral, 2.9-3.2 mm ventral). Probes were then implanted with the tip of the semipermeable membrane placed at the most ventral part of Area X so that the membrane extended through the dorsal-ventral extent of Area X. The surgical site was covered with melted bone wax, and the probes were secured in place first using MetaBond and then a coating of VetBond. Birds were then removed from the apparatus and recovered under a heat lamp. After they recovered from surgery they were infused

with saline until they began singing again. After recording at least one day of singing with saline infusion, the birds were infused in the morning ~10-20 minutes before 'lights on' with either SCH23390 (experimental birds) or saline (3 of 6 of the control birds were implanted and infused with saline only, the 3 other birds were not implanted and were recorded continuously for 12 days) each morning for the next 10 days. After 10 days of drug or saline treatment, juveniles were infused with saline and recorded for 2-3 more days. They were then placed back in the colony until they reached early adulthood (90-110 dph), when they were again isolated and recorded for 1-2 days. Following perfusion, as described above, the fixed tissue was examined for correct placement of microdialysis probes in Area X. Because the probes clogged 2-3 weeks after implantation, we were not able to infuse tracers to estimate drug diffusion in these birds.

2.4.3 Dual retrograde tracer injections

Male zebra finches (60 days post hatch (dph) for juvenile experiments, 100-110 dph for adult experiments) were food deprived for 30 minutes and then anesthetized with 2% isoflurane gas before being placed on top of a small heating pad in a custom stereotaxic apparatus. Rate of breathing and stability of surgical plane were regularly examined throughout surgery. The feathers over the skull were trimmed and topical anesthetic (0.25% bupivacaine) was applied before an incision was made in the skin from anterior to posterior with a scalpel. After pushing skin from the center of the skull with a cotton swab doused in 70% ethanol, craniotomies were made with a smaller

scalpel at a predetermined distance from the bifurcation of a major blood vessel ('y-sinus'; coordinates measured from y-sinus: Area X: head angle 37 degrees, 5.1mm anterior, 1.6mm lateral, 3.0mm ventral, Nif: head angle 40 degrees, 2.2 mm anterior, 1.7 mm lateral, 2.0 mm ventral; LMAN: head angle 43 degrees, 4.9 mm anterior, 1.85 mm lateral, 2.2 mm ventral; HVC: head angle 35 degrees, 0.0 mm anterior, 2.4 mm lateral, 0.4 mm ventral). A glass pipette connected to a pressure injection system (Drummond Nanoject II) was filled with either AlexaFluor 488 or AlexaFluor 594 (10,000 MW; Life Technologies) and lowered into the brain to the desired depth. Small amounts of AlexaFluor (30-60 nl) were then injected into the brain. After waiting 5 minutes to allow the tissue to absorb the dextran, the pipette was retracted from the brain. The craniotomies were then sealed with melted bone wax and the margins of the skin were sealed together using a tissue adhesive (VetBond). Birds were then placed under a heat lamp for ~30 minutes until they recovered from anesthesia. Birds were then monitored every few hours throughout the day to ensure they recovered fully (i.e. eating, drinking, and perching normally).

After 5 days, birds were deeply anesthetized with an intraperitoneal injection of pentobarbital solution (Euthasol). Once feathers were removed from the chest, the chest cavity was opened with surgical scissors and a needle connected to a peristaltic pump was inserted into the heart to perfuse the system with 0.025 M phosphate-buffered saline followed by 4% paraformaldehyde. The brain was then removed from the skull and placed in a cryoprotective formalin sucrose solution (30% sucrose in 4%

paraformaldehyde) overnight. The next day a freezing sliding microtome was used to cut 50 μm -thick coronal sections from the cryoprotected brain. After mounting sections containing VTA and the injection sites on glass slides, the slides were coverslipped with mounting medium (Fluoromount) and examined and imaged under a confocal microscope (Zeiss LSM 510).

2.4.4 Song recordings and analysis

For VTA_x cell ablation, juvenile male zebra finches (20-25 dph) were injected with viruses as previously described to ablate VTA_x cells, then isolated with their siblings and father until 60 dph. At 60 dph juveniles were housed with other virally injected birds and isolated temporarily for recording at 60, 90, and 120 dph using a custom song recording program (SOUND ANALYSIS PRO 2011 (SAP)). We relied on percent similarity, a measure that combines measures of pitch, amplitude modulation, frequency modulation, Weiner entropy and goodness of pitch, to gauge the similarity of song elements between two sets of songs (i.e., the pupil's song and that of his tutor). SAP was also used to measure spectral features of single syllables, such as entropy and entropy variance, over development in a subset of birds. Once birds reached 120 dph, they were injected with dextran in Area X and the number of VTA_x cells was quantified as previously described.

For juvenile microdialysis, juvenile birds were recorded with SAP for at least one day before surgery and for several days after surgery to determine when they recovered

pre-surgery levels of singing. Birds were continuously recorded throughout the period they were infused with either saline or drug as well as for several days after infusion. Birds were again recorded for several days approximately 40-60 days after the end of the infusion period.

3. VTA_x cells and D1-type receptors are necessary for pitch learning in the adult

3.1 Introduction

The birdsong system provides an excellent system in which to study both internally guided learning, as seen in juveniles, as well as externally driven learning via aversive associative learning in adults. Being able to examine two different forms of learning in the same organism allows us to determine if the same mechanisms required for one form of learning are also necessary for the other. Though externally driven associative learning in mammals is known to rely on midbrain to striatal circuits as well as signaling through striatal dopamine receptors (Adamantidis et al., 2011; Gerfen & Surmeier, 2011; Schultz et al., 1997; Steinberg et al., 2013; Surmeier, Plotkin, & Shen, 2009; Tsai et al., 2009; Witten et al., 2011; Yttri & Dudman, 2016; Zweifel et al., 2009), the circuit mechanisms underlying externally reinforced learning in songbirds are unknown. Lesions of dopaminergic and noradrenergic terminals with 6-OHDA in Area X hint that projections from the dopaminergic midbrain may be required for externally reinforced pitch learning (Hoffmann et al., 2016). However, these experiments cannot determine the cell type that is necessary for pitch learning or confirm if the deficits in pitch learning are purely due to disruption of dopaminergic signaling. Here I use a combination of intersectional ablation and microdialysis techniques to demonstrate that both VTA_x cells and dopaminergic signaling, in particular signaling through D1-type receptors in Area X, are required for externally driven adult pitch learning.

3.2 Results

3.2.1 VTA_x cells are necessary for pitch learning in the adult

Though adult zebra finches sing a remarkably stereotyped song across days, there are small amounts of variability in the pitch of individual syllables over a given day. We can capitalize on this variability to drive shifts in the pitch of syllables by pairing an aversive burst of white noise (WN) with pitch variants that fall below an experimenter-determined pitch threshold. To do this we use a software program (EvTAF, Brainard lab, UCSF) (Tumer & Brainard, 2007) that allows us to identify a particular syllable in real time, detect its pitch and then in a matter of milliseconds play back WN to the bird if the pitch falls below our predetermined threshold. As the bird learns to associate the aversive WN burst with the range of pitches that fall below the pitch threshold, over the day he will shift his pitch upwards to avoid the white noise; the syllables that have high enough pitches to avoid the WN we refer to as “escapes” while the variants that fall below the pitch threshold are called “hits”. We can then gradually raise the pitch threshold in order to drive the bird’s pitch higher and higher until we reach the upper bounds of pitch for that particular syllable and the bird’s pitch asymptotes (see Figure 4). By setting the pitch threshold so that a larger percentage of syllables will receive WN, we can drive learning more rapidly (i.e. a pitch contingency in which pitches below the seventieth percentile receive WN will drive learning more rapidly than a contingency set to the fiftieth percentile). Notably, this paradigm can also

be used to shift the bird's pitch downwards by targeting higher, rather than lower, pitch variants with white noise.

In order to examine the role of VTA_x cells in the pitch learning described above I again relied on the intersectional ablation strategy I used previously in juveniles (Figure 20). I selected young adult birds (n = 6, 95 +/- 5 dph) with clear tonal elements of song and injected them with a virally encoded Cre-dependent caspase (AAV2/1.EF1α.FLEX-Casp3-2A-TEV) in the VTA and injected a retrogradely traveling virally encoded Cre in Area X (AAV2/9.CMV.HI.GFP-Cre.SV40). After the birds recovered from surgery and began singing readily (5 +/- 1 days post surgery) I recorded two days of baseline song then drove pitch learning with WN for four days. After four days of WN I turned off WN and recorded the bird's song for three more days. At the end of this experiment, which occurs well before the window in which I see viral expression, I returned the bird to its home cage. After the viruses I injected had adequate time to express, generally four to six weeks, I repeated the same experiment, recording two days of song then driving learning with four days of WN playback then recording the bird's song for three days after discontinuing WN playback. At the end of the experiment I injected a retrogradely transported tracer into Area X in order to quantify the proportion of VTA_x cells ablated.

In contrast to the rapid learning exhibited by all birds in the time window before viral expression, after ablation of VTA_x cells five of the six birds tested learned significantly less than they had previously (paired two tailed t-test: early: $6.11 \pm 0.84\%$; late: $4.18 \pm 0.96\%$, n = 6, p = 0.042) (Figure 21). The effect I see on learning appears to not

be mediated by a loss of ability to regulate pitch variability as the pitch variability of the targeted syllable measured before and after viral expression was not significantly different (paired two tailed t-test: early: 0.022 ± 0.003 ; late: 0.016 ± 0.004 ; $n = 6$, $p = 0.099$) (Figure 22). I also was unsure as to whether the effect I saw could be recapitulated by ablating cells in the VTA that projected to the striatum more generally. However, this effect was restricted to ablation of VTA_x cells as two birds in which I ablated VTA cells projecting to the medial striatum showed no changes in learning before and after viral expression (Figure 23).

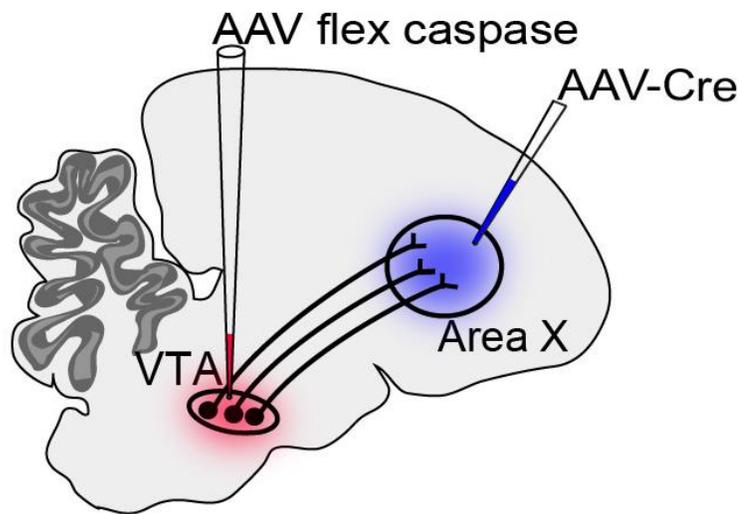
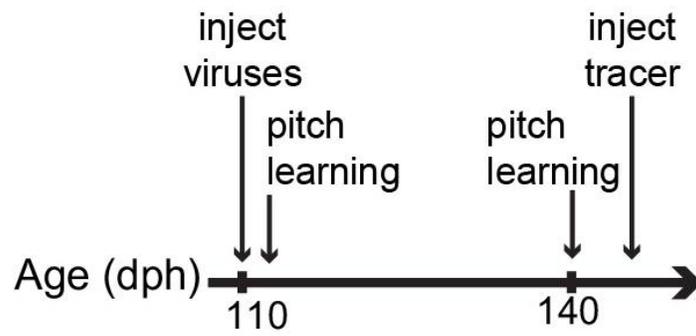


Figure 20: Experimental design for adult pitch learning experiments
Top, timeline of experiment. *Bottom*, sagittal section of zebra finch brain with locations of viral injections.

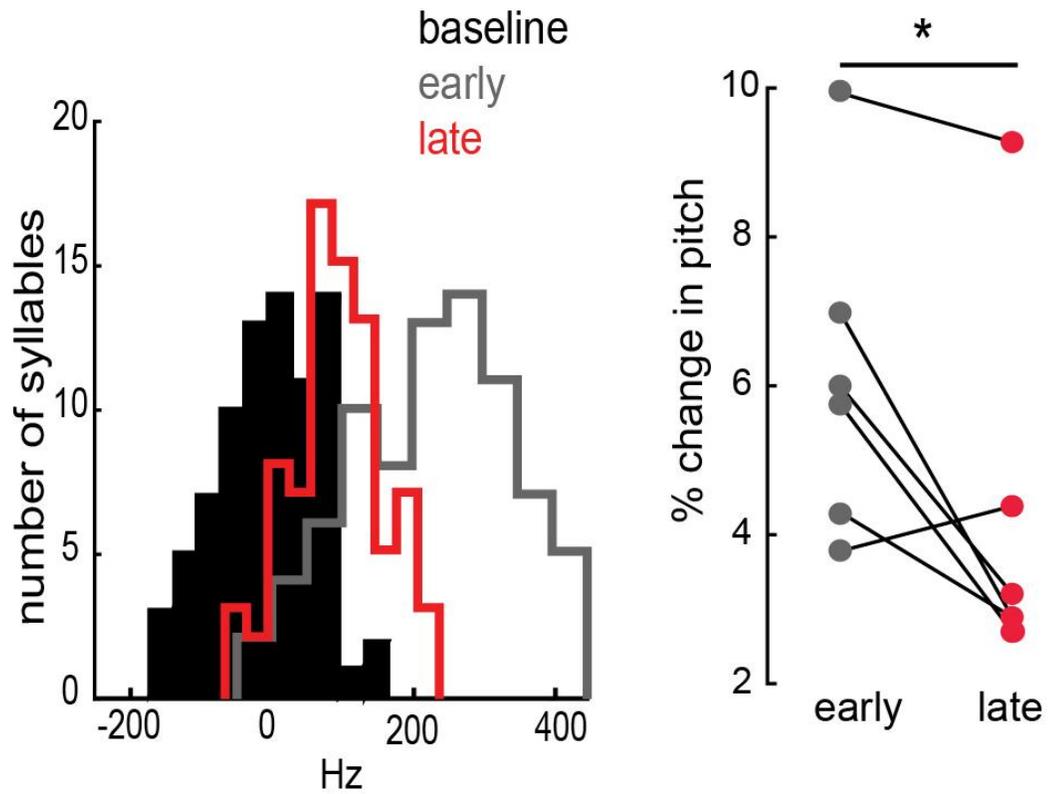


Figure 21: VTA_x cell ablation reduces adult pitch learning

Left, distribution of pitch of the targeted syllable for one bird at baseline (black) and after learning early (grey) and late (red) in the time window for viral expression *Right*, Percent change in pitch of target syllables early and late in the viral expression window (n = 6 birds). *P<0.05, paired two tailed t-test.

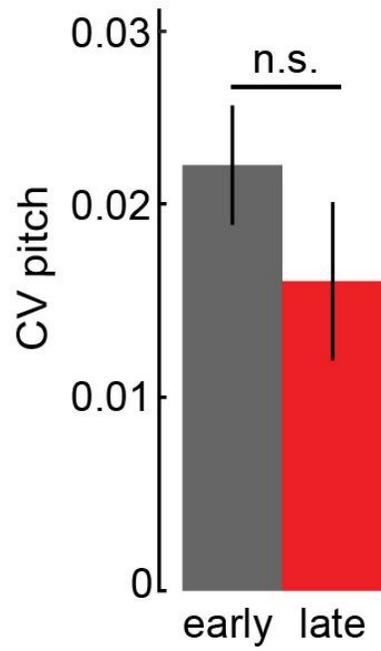


Figure 22: VTA_x cell ablation does not affect adult pitch variability
Coefficient of variation of target syllable pitch early (grey) and late (red) in the viral expression window.

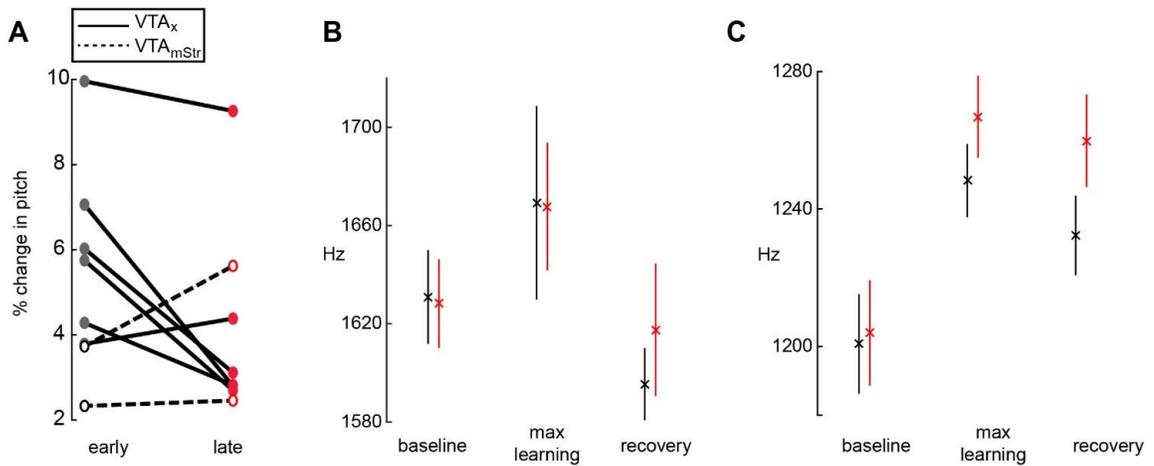


Figure 23: VTA_{mStr} cell ablation does not affect adult pitch learning

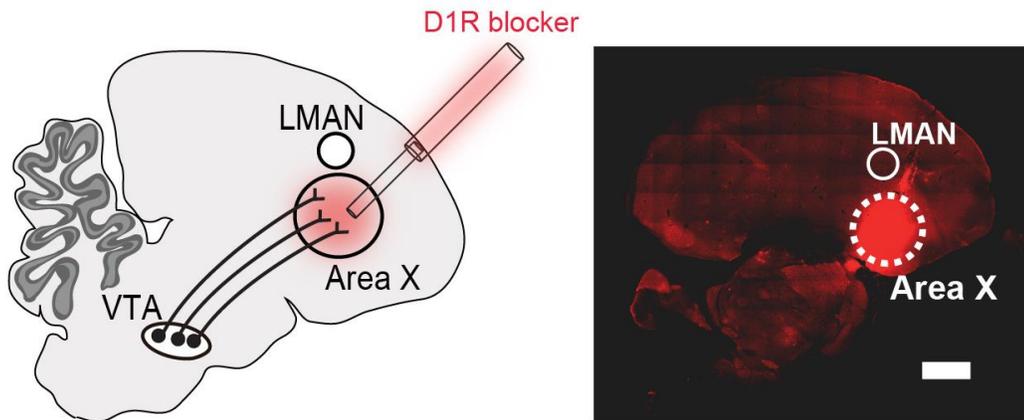
A) Percent change in pitch during exposure to pitch-contingent noise early (grey) and late (red) in viral expression window in VTA_x ablation birds (as in Figure 21) with 2 VTA_{mStr} ablation birds included early (white with black outline) and late (white with red outline) in viral expression window (solid black line connects VTA_x ablation birds, dashed black line connects VTA_{mStr} ablation birds). B) Mean and S.E.M. frequency of targeted syllable on baseline day 2, day of maximal learning, and recovery day 3 early (black) and late (red) in expression window for bird 1 with VTA_{mStr} ablation. C) As in B) but for bird 2 with VTA_{mStr} ablation.

3.2.2 D1-type receptor blockade in Area X abolishes pitch learning in the adult

Though I determined that juvenile learning requires both VTA_x cells and D1 type receptors in Area X I was curious as to whether externally reinforced pitch learning in the adult also required D1-type receptors in Area X. To test this I implanted young adult birds (n = 6, 99 +/- 9 dph) with clear tonal elements with microdialysis probes over Area X bilaterally. After the birds recovered from surgery I recorded their songs for an hour in the morning. I used this one-hour period of morning song to confirm that my syllable targeting with our syllable detection software was accurate. I then infused the birds with saline and drove pitch learning with WN for eight hours. At the end of the eight hours I discontinued white noise and flushed the probes with saline. After waiting for one day for pitch to recover back to baseline I again recorded song for one hour in the morning but instead infused the birds with a D1-type receptor antagonist, SCH23390, before again driving pitch learning with WN for eight hours (Figure 24). For both the saline and SCH23390 experiments I compared pitch from the first third of songs in the WN learning period to the last third of songs in the WN learning period. At the end of the experiment, birds were infused with muscimol-BODIPY to visualize the spread of drug. Only birds with sufficient drug spread and probe placement, confirmed through *post hoc* histological methods, were included in my analysis (Figure 24).

In comparison to learning during saline infusion, learning in the presence of the D1-type receptor antagonist SCH23390 was significantly reduced in all birds both in

terms of the mean change in pitch (percent change in target syllable pitch during exposure to pitch-contingent noise in the presence of saline or SCH23390: paired two tailed t-test for learning: $n = 6$, for saline learning vs. SCH23390 learning (day 1 vs. day 3): $p = 0.006$) (Figure 25, 26) and in the change in the distribution of pitch from the first third to the last third of songs as measured by the change under the receiver operator characteristic (Change in auROC values of target syllable pitch in the presence of pitch-contingent noise while either saline or SCH23390 was infused in Area X: paired two tailed t-test: saline learning: 0.663 ± 0.034 ; SCH23390 learning: 0.512 ± 0.027 ; $n = 6$, $p = 0.004$) (Figure 26). As there were no significant changes in either syllable variability (coefficient of variation of pitch during saline or drug infusion: paired two tailed t-test: saline: 0.025 ± 0.004 ; SCH23390: 0.0268 ± 0.004 ; $n = 6$, $p = 0.735$) (Figure 27) or amount of singing during SCH23390 infusion compared to saline infusion (number of catch songs per day: paired two tailed t-test: saline: 75 ± 14 ; SCH23390: 69 ± 19 ; $n = 6$, $p = 0.764$) I believe that the deficit in learning can be attributed to an effect of blocking D1-type receptors on learning rather than an inadvertent interference with motor control or motivation, respectively.



day	1	2	3	4	5
noise	+	-	+	-	+
saline	+	+	-	+	+
drug	-	-	+	-	-

Figure 24: Experimental design of adult microdialysis experiments

Top left, sagittal section of zebra finch brain with location of microdialysis probe. *Top right*, infusion of muscimol-BODIPY through microdialysis probes to visualize drug spread. Scale bar, 1 mm. *Bottom*, experimental schedule.

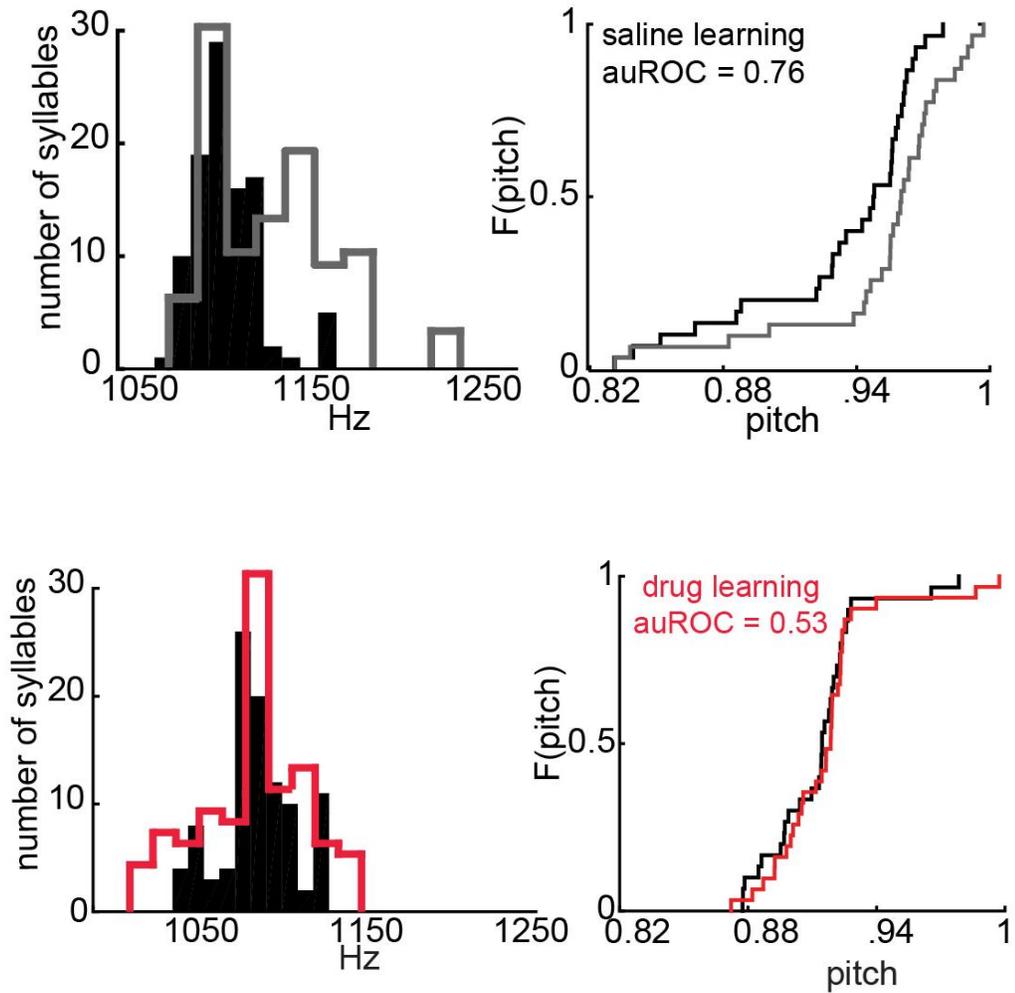


Figure 25: Example of pitch learning during saline infusion and D1-type receptor antagonist infusion

Top left, target syllable pitch distribution before (black) and after WN with saline infusion (grey) from one bird on day 1. *Top right*, cumulative distribution of normalized target syllable pitch during first (black) and last third (grey) of day 1. *Bottom panel*, as above panel but with infusion of SCH23390 (red) instead of saline.

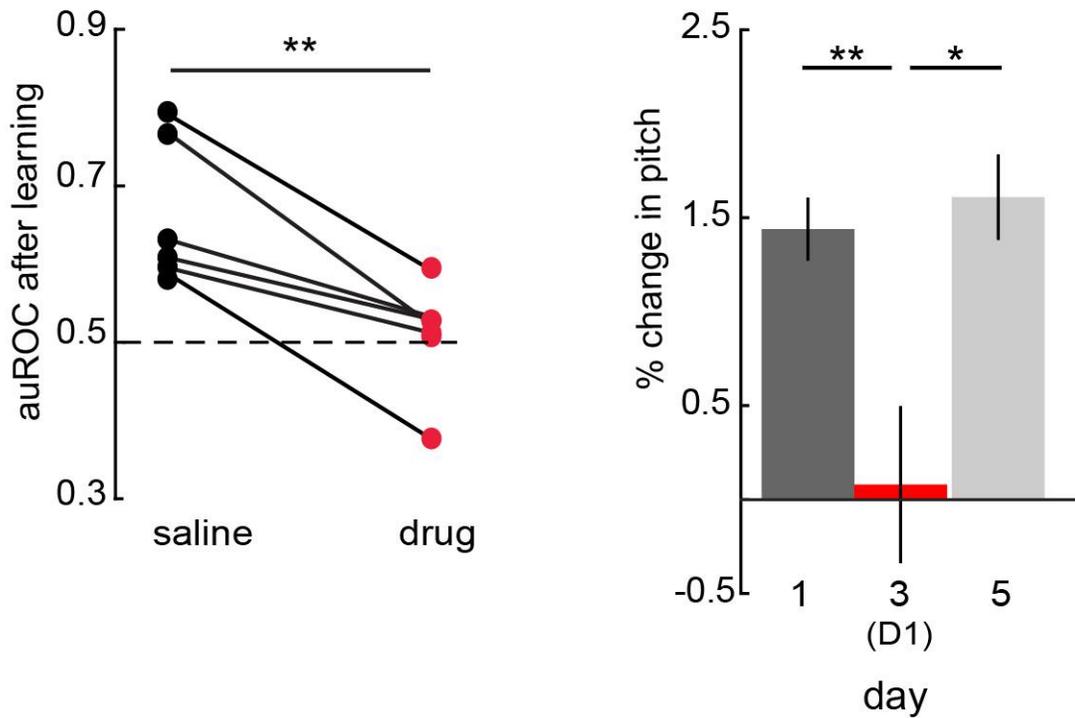


Figure 26: D1-type receptor activation is necessary for changes in mean pitch and pitch distribution during learning

Left, change in auROC of target syllable pitch after WN during saline (black) or SCH23390 (red) infusion. ** $P < 0.01$, paired two tailed t-test, $n = 6$ birds. *Right*, Percent change in target syllable pitch after WN during saline (dark and light grey) or SCH23390 (red) infusion. * $P < 0.05$, ** $P < 0.01$, paired two tailed t-test, $n = 6$ birds.

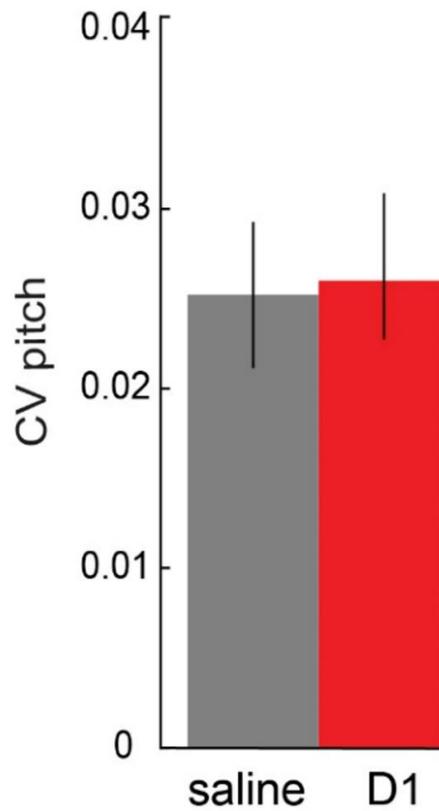


Figure 27: D1-type receptor blockade in Area X does not affect pitch variability

Coefficient of variation of pitch during saline (grey, n = 6 syllables from 6 birds) or SCH23390 (red, n = 6 syllables from 6 birds) infusion.

3.2.3 D2-type receptor blockade in Area X reduces the amount of singing in adult birds

After observing the effect of D1-type receptor blockade on learning I was curious if this effect was specific to a particular dopamine receptor type or if it was due to a disruption in dopamine signaling more generally. To this end I repeated the experiments described above in five birds but with sulpiride, a D2-type receptor antagonist, rather than SCH23390 infusion.

Interestingly, the most consistent effect I saw in birds infused with sulpiride was a dramatic decrease in singing compared to singing in the presence of saline. The percent change in the amount of singing from saline to sulpiride infusion days was also significantly reduced in comparison to the percent change in the amount of singing from saline to SCH23390 infusion days (percent change from saline baseline in number of songs sung during D1 antagonist SCH23390 infusion or D2 antagonist sulpiride infusion: unpaired two-tailed t-test: percent change in number of songs: D1 antagonist: $-7.810 \pm 23.175\%$, $n = 6$ birds; D2 antagonist: $-66.12 \pm 17.38\%$, $n = 6$ birds; $p = 0.05$) (Figure 28). Infusion of sulpiride appeared to have variable effects on learning in the four birds that sang enough to support pitch learning experiments though learning during sulpiride infusion was not significantly different from learning during saline infusion (percent change in target syllable pitch during exposure to pitch-contingent noise in the presence of saline or the D2 antagonist sulpiride: paired two tailed t-test for learning: saline learning (day 1): $1.282 \pm 0.266\%$; sulpiride learning (day 3 (D2)): $1.159 \pm 0.725\%$; n

= 5, $p = 0.91$) (Figure 29). However, when I corrected for the reduced amount of singing by calculating the change in pitch per syllable rendition I found that the rate of pitch learning was either enhanced or unchanged in three of the four birds tested and once again revealed no significant difference from saline infusion (percent change in target syllable pitch per rendition during exposure to pitch-contingent noise in the presence of saline or D2 antagonist sulpiride: paired two tailed t-test for learning per rendition: saline learning (day 1): $0.00058 \pm 0.00008\%$; sulpiride learning (day 3 (D2)): $0.00246 \pm 0.001834\%$; $n = 5$, $p = 0.42$) (Figure 29).

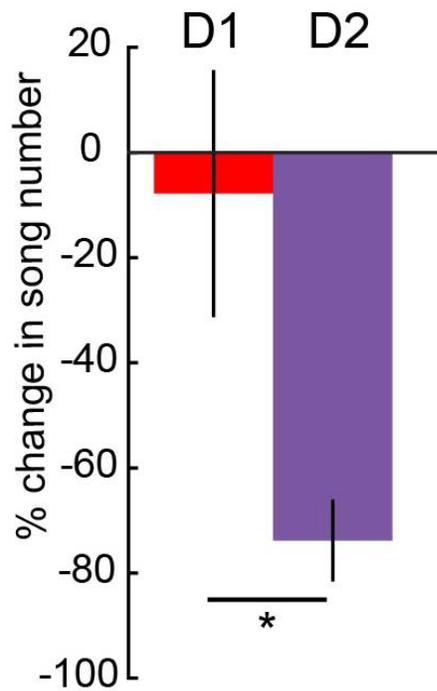


Figure 28: D2-type receptor blockade in Area X reduces the amount of singing

Percent change from baseline number of songs sung during D1 antagonist SCH23390 (red, n = 6 birds) or D2 antagonist sulpiride infusion (purple, n = 6 birds). *P<0.05, two tailed t-test.

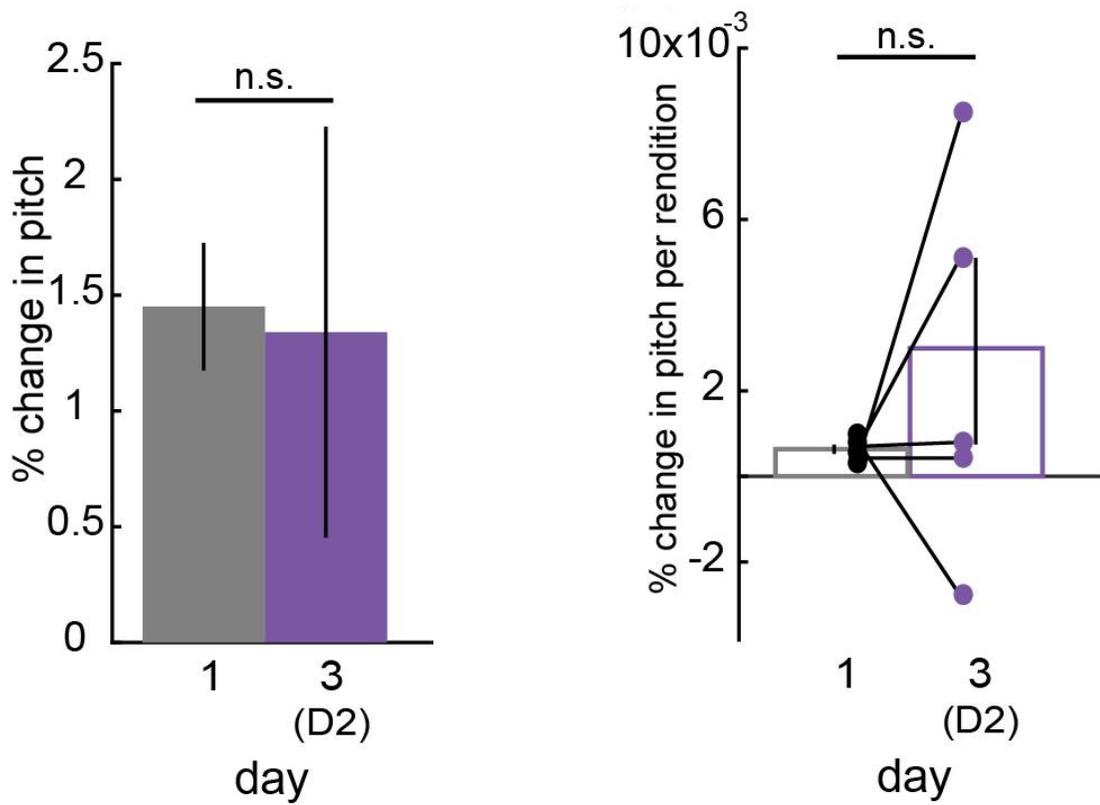


Figure 29: D2-type receptor blockade has variable effects on learning

Left, percent change in target syllable pitch after WN during saline (grey) or sulpiride (purple, $n = 5$ birds). *Right*, percent change in target syllable pitch per rendition after WN with saline (grey) or sulpiride (purple).

3.3 Discussion

Overall, I found that similarly to juvenile learning, adult pitch learning depends on both VTA_x cells and D1-type receptors in Area X. These results strongly argue that a common mechanism underlies both forms of learning and may more generally mean that a common mechanism underlies both internally and externally reinforced learning. Both ablation of VTA_x cells and blockade of D1-type receptors significantly decreases the amount of learning during WN driven pitch learning. Blockade of D2-type receptors, in contrast, affects amount of singing rather than learning, which confirms that the effect on learning is specific to a particular dopamine receptor type. This effect is curious, given that D2-type receptors are thought to act in action termination and blockade of them would presumably increase, rather than decrease, the amount of singing according to their role in action selection. It may be that I am interfering with motivation rather than action termination.

3.4 Methods

Adult (82-436 dph) male zebra finches were obtained from the Mooney lab breeding colony within the Duke University Medical Center animal facility.

Experimental procedures were conducted in accordance with the National Institutes of Health guidelines and were reviewed by the Duke University Medical Center Animal Care and Use Committee. Viral vectors were acquired from University of Pennsylvania Vector Core and University of North Carolina, Chapel Hill Vector Core.

3.4.1 Intersectional ablation of VTA_x cells

Male zebra finches (~100-110 days post hatch (dph)) were food deprived for 30 minutes and then anesthetized with 2% isoflurane gas before being placed on top of a small heating pad in a custom stereotaxic apparatus. Rate of breathing and stability of surgical plane were monitored throughout surgery. The feathers over the skull were trimmed and topical anesthetic (0.25% bupivacaine) was applied before an incision was made in the skin from anterior to posterior with a scalpel. After pushing skin from the center of the skull with a cotton swab doused in 70% ethanol, craniotomies were made with a smaller scalpel at a predetermined distance from the bifurcation of the midsagittal sinus (the 'y-sinus'; coordinates measured from y-sinus: VTA: head angle 37 degrees, 1.65mm anterior, 0.5 and 1.8 mm lateral, 6.2 mm ventral; Area X: head angle 43 degrees, 5.0 mm anterior, 1.6 mm lateral, 3.2, 2.9 and 2.7mm ventral). To selectively ablate VTA_x cells, a pressure injection system (Drummond Nanoject II) was used to

make bilateral injections of a retrogradely transported Cre construct (AAV2/9.CMV.HI.GFP-Cre.SV40; Penn Vector, a total of 15 injections of 32.2 nl of Cre per hemisphere) into Area X at 3 different depths. A locally expressed Cre-dependent caspase construct was then injected into the VTA at 2 different locations along the medial-lateral axis (AAV2/1.Ef1 α .FLEXCasp3-2A-TEV; construct courtesy of Nirao Shah, UCSF, 15 injections of 32.2 nl of Casp3 per site per hemisphere i.e. a total of 4 caspase injection sites per bird) (Yang et al., 2013). After these viral injections, the craniotomies were sealed with bone wax, the incision site was closed with tissue adhesive, and the bird was allowed to recover from anesthesia under a heat lamp. At the endpoint of each experiment and 5 days prior to perfusion, birds were injected with AlexaFluor 594 in Area X to retrogradely label VTA_x neurons. Five days after these tracer injections, birds were deeply anesthetized with an intraperitoneal injection of pentobarbital solution (Euthasol) and then perfused through the heart with 0.025 M phosphate-buffered saline followed by 4% paraformaldehyde. The brain was then removed from the skull and placed in a cryoprotective formalin sucrose solution (30% sucrose in 4% paraformaldehyde) overnight. The next day consecutive sagittal sections of the cryoprotected brain were cut on a freezing microtome and alternate sections were mounted on glass slides. Sections containing VTA were visualized and imaged under a confocal microscope (Zeiss Axioskop 2). The images were then examined in an image-

processing program and the number of fluorescent retrogradely labeled cells in VTA was counted in a semi-automated manner (ImageJ, CellCounter plug-in).

3.4.2 Microdialysis in Area X

Young adult birds (> 80 dph) with clear tonal elements in their song were chosen for implantation of microdialysis probes. Probes were constructed in house from plastic tubing which served as a drug reservoir fitted at the end with a 0.7-1.0 mm-long semipermeable membrane which allowed drug to slowly diffuse throughout the day (see Hamaguchi and Mooney, 2012 for probe design). Using surgical procedures and stereotaxic coordinates described above, craniotomies were made over Area X and neural recordings were made to confirm its depth (Differential A-C Amplifier 1700, A-M Systems). We approached Area X rostrally as to avoid LMAN (anterior Area X coordinates: initial head angle 35 degrees, 5.3 mm anterior marked with scalpel on skull, then adjusted head angle to 72 degrees, 1.2 mm anterior from scalpel mark, 1.7 mm lateral, 2.9-3.2 mm ventral). Probes were then implanted with the tip of the semipermeable membrane placed at the most ventral part of Area X so that the membrane extended through the dorsal-ventral extent of Area X. The surgical site was covered with melted bone wax, and the probes were secured in place first using MetaBond and then a coating of VetBond. Birds were then removed from the apparatus and recovered under a heat lamp. After recovery birds were placed in a sound isolation box and their first full day of song was recorded and used to make an EvTAF template

to target a tonal syllable as described above. Birds were recorded in the absence of white noise for two hours the morning after their first full day of song then infused with saline and recorded in the presence of pitch-contingent white noise for the next 8 hours, after which they were again infused with saline and white noise was turned off (“learning day”, day 1 on figure 2A).

This protocol was repeated the following day with the white noise remaining off (“recovery day”, day 2) The next day the protocol from the saline “learning day” (day 3) was repeated but either 5 mM SCH23390 or 0.5 µg/ml sulpiride were infused after two hours of recording instead of saline and washed out with saline after 8 hours of recording. After this drug “learning day,” the bird underwent a “recovery day” (day 4) after which followed another “learning day” (day 5) with saline only. Before perfusion, birds were infused with fluorescent muscimol-BODIPY for 2 hours to allow for post-hoc visualization of drug diffusion through the semi-permeable membrane into Area X. Birds were then perfused and histology was performed as described above to assess correct placement of the dialysis probes in Area X and the extent of drug diffusion.

3.4.3 Pitch-contingent learning paradigm

Young adult male birds (100-110 dph) were screened for syllables with clear tonal components and for the amount of song produced. Birds that fit both these criteria were then bilaterally injected with viruses to ablate VTA_x cells. After the birds recovered

from surgery and began singing readily, their songs were recorded and a template to detect the fundamental frequency (i.e., pitch) of a tonal syllable was made in a custom software program (EvTAF, Tumer and Brainard, 2007). The template was designed to detect no less than 75% of the renditions of the targeted syllable with no more than a 5 millisecond jitter in detection onset. After collecting two days of “baseline” song, a threshold at the upper 70th percentile of the target syllable’s pitch distribution was set and a 50 millisecond white noise burst (~70 dB) was played through a nearby speaker to the bird whenever the program template detected that the pitch of the targeted syllable was below this threshold; over hours and days, this manipulation results in an adaptive shift in the pitch of the target syllable. The bird’s pitch for the targeted syllable was measured in the late morning and early evening for the next four days and the threshold was adjusted each morning and early evening to the upper 70th percentile of the bird’s pitch distribution to promote more rapid learning. After four days of pitch-contingent white noise experience, the white noise was discontinued and the bird’s song was recorded for the next three to four days as the pitch of the targeted syllable recovered toward its baseline value.

This entire process was repeated one month after viral injections, when VTAx neurons had been ablated by the intersectional viral treatment. At the end of the recovery period from this second pitch learning experiment, birds were injected with

dextran in Area X in order to allow for the number of VTA_x cells remaining to be quantified, as described in Section 3.4.1.

4. VTA_x cells are necessary for two forms of song modification in the adult

4.1 Introduction

Though adult zebra finches sing highly stereotyped songs with little variability in pitch from rendition to rendition, they can modify their songs in response to the presence or absence of environmental cues (Konishi, 1965; Woolley & Doupe, 2008). When presented with a female, adult males will sing a faster version of song that is more stereotyped in terms of pitch. While it is known that dopamine levels rise when male zebra finches sing to females (Sasaki et al., 2006) and that D1-type receptor blockade in Area X prevents changes in variability in song directed towards females (Leblois et al., 2010), the cell type that mediates the decrease in variability during directed song is unknown.

In the absence of auditory feedback, adult birds' songs begin to degrade within weeks after deafening. This degradation is indicative of the fact that while adults sing very stereotyped song, they do constantly modify and adjust their songs using auditory feedback. Ablation of cortical nuclei such as LMAN and specific cortical cell types such as HVC_{Av} cells are known to prevent deafening-induced song degradation (Brainard & Doupe, 2000b), whether the midbrain plays any role in song maintenance is unknown. Here I test the necessity of VTA_x cells to both processes using the intersectional genetic ablation technique described in previous chapters.

4.2 Results

4.2.1 VTA_x cells are necessary for the “switch” from undirected to directed song

Before VTA_x ablation, I found that birds showed a normal decrease in variability during directed song compared to undirected song, as measured by the comparison of coefficient of variance of pitch for a given syllable in undirected and directed states (Figure 30). Though this decrease in variability from undirected to directed song was significant across birds before viral injection, 1 month after injection the same birds were unable to significantly modulate variability when singing to a female (n = 12; repeated measures ANOVA: AxB: p < 0.01; paired two-tailed t-test: pre-injection undirected CV = 0.0202 ± 0.004, directed CV = 0.0141 ± 0.003, p = 0.005; paired two-tailed t-test: post-injection undirected CV = 0.0135 ± 0.003, directed CV = 0.0145 ± 0.003, p = 0.65) (Figure 31). In some cases birds actually sang a more variable directed than undirected song (6 of 12 birds). Overall, birds showed a reduction in the magnitude of change in variability between undirected and directed song after injection. This reduction in magnitude was also weakly correlated with number of VTA_x cells remaining (R² = 0.278, p = 0.078) (Figure 32), supporting the idea that VTA_x are necessary for context-dependent changes to song.

Interestingly enough, when I compared CV of pitch from undirected song before ablation to undirected song after ablation I found that variability in undirected song did not significantly increase after ablation (paired two-tailed t-test: pre-injection undirected

CV = 0.0202 \pm 0.004, post-injection undirected CV = 0.0135 \pm 0.003, $p = 0.073$; pre-injection directed CV = 0.0141 \pm 0.003, post-injection directed CV = 0.0145 \pm 0.003, $p = 0.901$; pre-injection directed CV = 0.0141 \pm 0.003, post-injection undirected CV = 0.0135 \pm 0.003, $p = 0.873$), lending further evidence to the notion that the inability to modulate context-dependent song was not due to inadvertent disruption of song production. The reason why neither undirected nor directed CV before injection were significantly different from undirected or directed CV after injection is likely due to the fact that roughly half of the birds sang more variable directed than undirected song after injection while the other half of birds still sang a directed song that was less variable than undirected song but to a much lesser extent than before injection.

To confirm that the effect I saw was not simply due to a deficit in song production, I compared the number of elements per bout as well as bout duration of lesioned birds before and after viral expression and found no significant changes in song production after VTA \times production in terms of the number of song elements sung or the duration of singing bouts (elements per bout: pre = 12.529 \pm 0.949, post = 12.836 \pm 0.719, $p = 0.829$; bout duration: pre = 2.032 \pm 0.155 seconds, post = 1.969 \pm 0.108 seconds, $p = 0.779$).

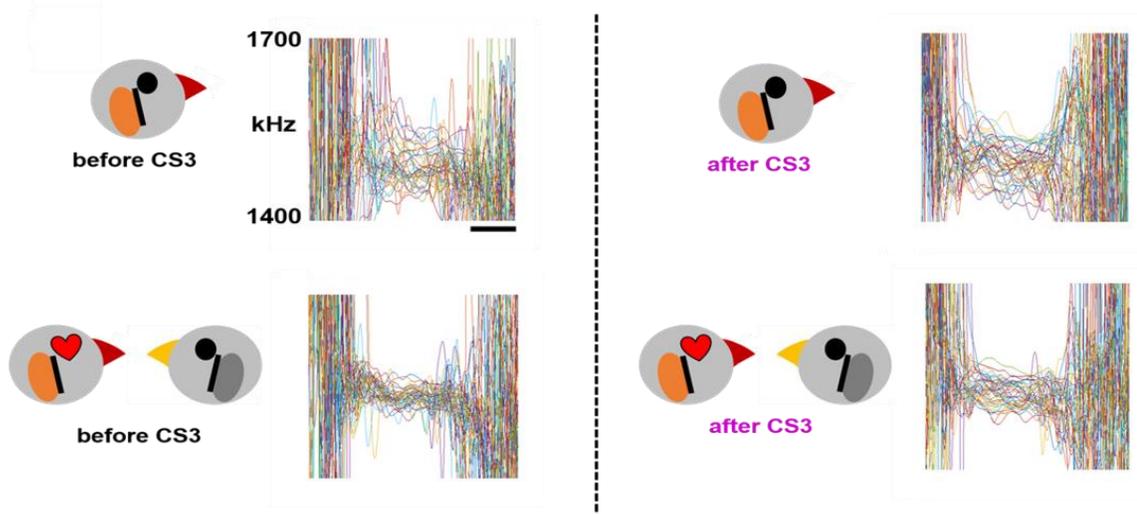


Figure 30: Undirected and directed song before and after VTA_x cell ablation

Frequency of 40 renditions of one syllable during undirected (*top left*) and directed song (*bottom left*) before viral expression and during undirected (*top right*) and directed song (*bottom right*) after viral expression. Scale bar, 20 milliseconds.

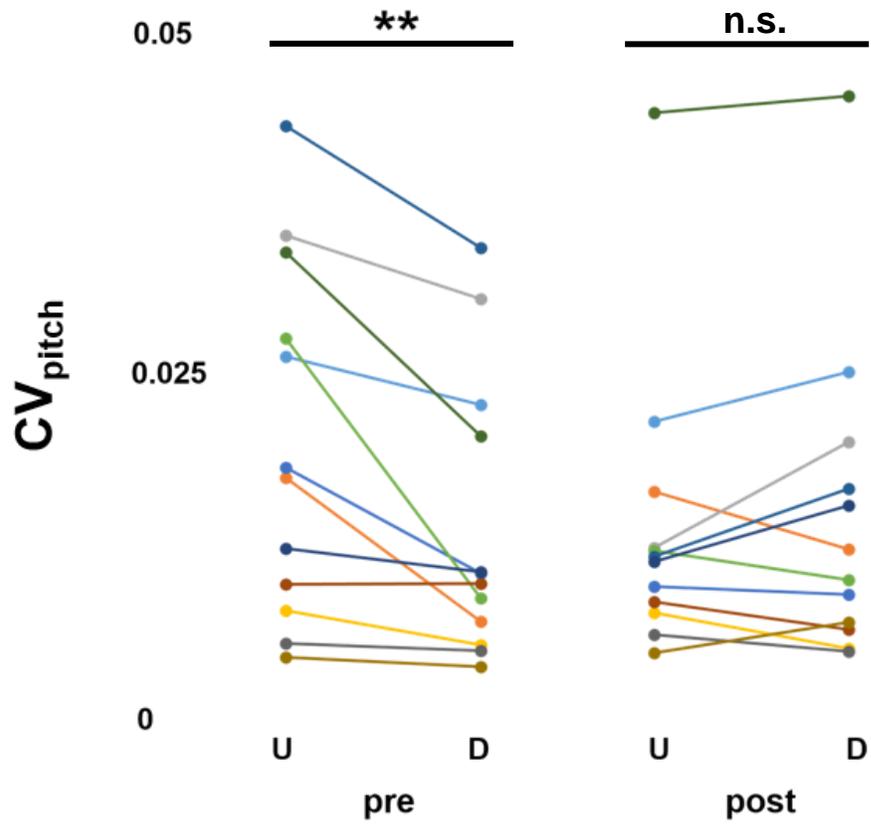


Figure 31: Ablation of VTA_x in adult birds abolishes social context dependent changes in song

Coefficient of variation of pitch for undirected (U) and directed (D) song before (left) and after (right) viral ablation (n = 12 birds).

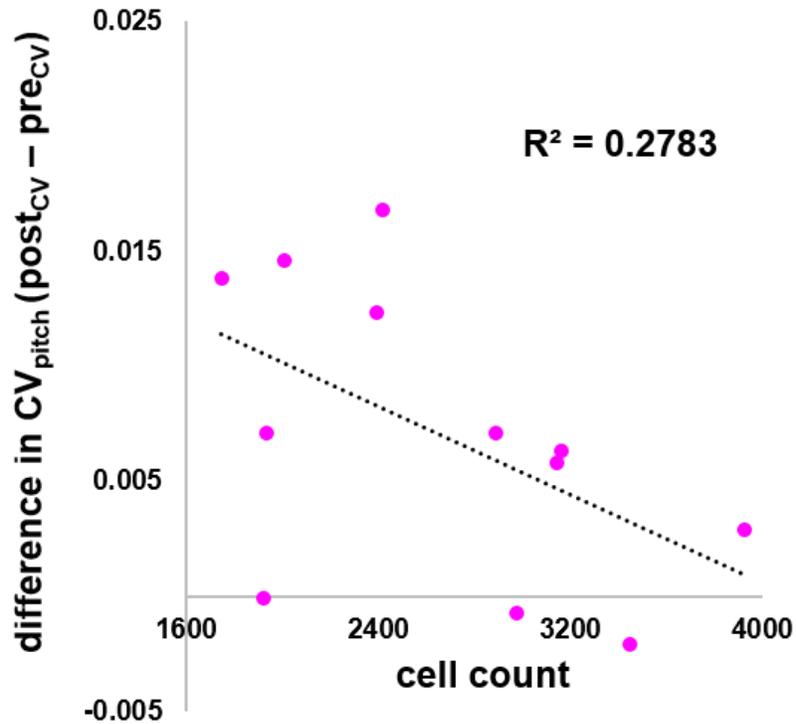


Figure 32: The reduction in the magnitude of change in variability between undirected and directed song after injection is weakly correlated with number of VTA_x cells remaining

A plot of the difference in CV before and after lesioning (post CV – pre CV) as a function of VTA_x cell count.

4.2.2 Ablation of VTA_x cells slows deafening-induced song degradation in adult birds

Though I already determined the necessity of VTA_x cells to juvenile and adult learning, I was curious what, if any, role these cells might play in auditory-feedback dependent changes to song in the adult bird. As an initial step to determining if these cells were at all necessary for changes to adult song, I performed the same intersectional viral injection strategy as described above in adult birds which we then deafened by bilateral cochlear removal one month after injection (Figure 33).

Lesioned birds showed delayed deafening-induced song degradation in comparison to unlesioned birds 1 month after deafening (similarity scores comparing motifs before and 1 month after deafening for each group, two-tailed paired t-test: experimental: $n = 6$, $83.678 \pm 3.82\%$, control: $n = 4$, $50.645 \pm 6.47\%$, $p = 0.007$) (Figure 34, 35). Lesioned birds' similarity scores 1 month after deafening were high but still significantly different from their similarity scores before deafening (similarity scores comparing motifs before and 1 month after deafening for the same birds, paired two-tailed t-test: self-similarity for experimental birds before deafening: $n = 6$, $95.07 \pm 1.21\%$; experimental birds 1 month after deafening: $n = 6$, $83.678 \pm 3.82\%$, $p = 0.015$) (Figure 35). Though song did eventually degrade in the 3 birds we tracked out to 2 months after deafening to an amount comparable to control birds 1 month after deafening (paired two-tailed t-test: motif similarity score before deafening: $95.070 \pm 1.205\%$ versus 1) experimental (two months after deafening: $n = 3$, $63.76 \pm 5.50\%$, $p = 0.065$; or 2) control (one month after deafening): $n = 4$, $50.645 \pm 6.47\%$, $p = 0.007$) (Figure 35), the delayed degradation of song

suggested a role for VTA_x cells in song maintenance in the absence of auditory feedback along as well as its role in learning.

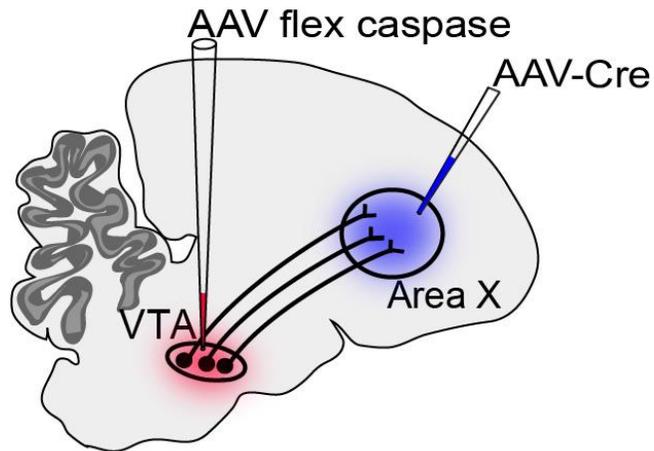
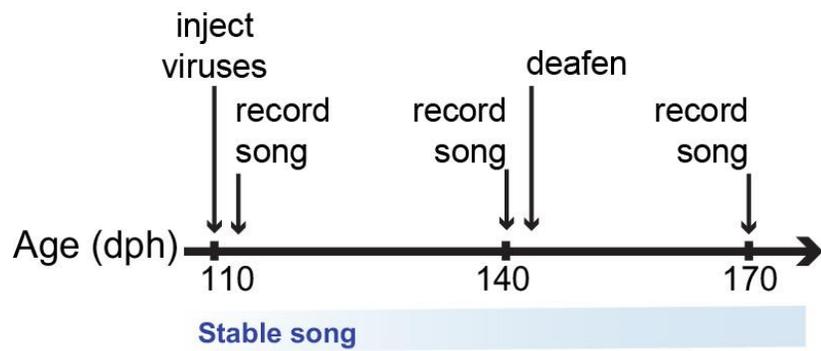


Figure 33: Experimental design of adult deafening experiments

Top, experimental timeline. *Bottom*, sagittal section of the zebra finch brain showing location of viral injections.

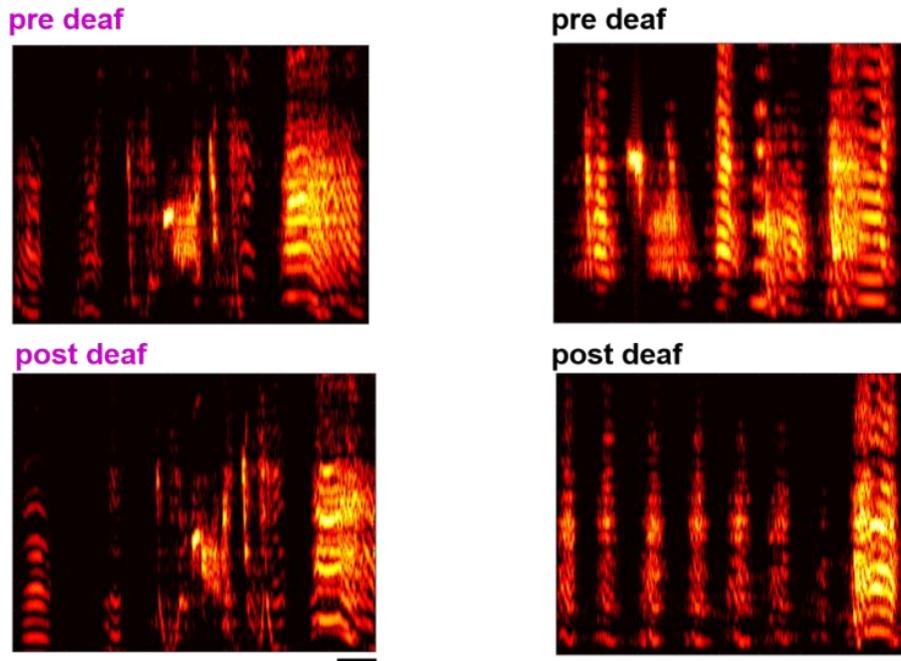


Figure 34: Examples of song before and after deafening in control birds and VTAX ablation birds

Representative sonograms from VTAX ablation birds (*left*) and control birds (*right*) before and one month after deafening. Scale bar, 50 milliseconds.

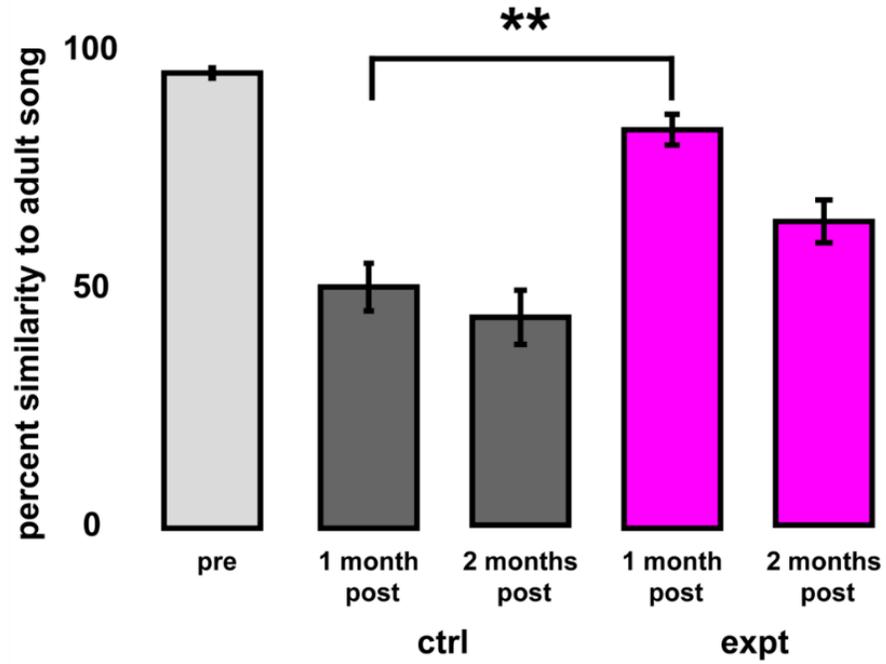


Figure 35: VTAx ablation delays deafening-induced song degradation

Average percentage similarity to song before deafening to bird's song before deafening (light grey, from experimental birds before deafening), control birds one and two months after deafening (dark grey) and experimental birds one and two months after deafening (magenta).

4.3 Discussion

In this chapter I demonstrate the necessity of VTA_x cells to two forms of adult song modification. VTA_x ablation greatly decreases the “distance” in pitch variability between undirected and directed song, rendering the difference between CV of pitch for directed versus undirected song insignificant. Song degradation after deafening is also slowed after VTA_x ablation when compared to control birds. However, song degradation was not completely blocked by VTA_x ablation as the birds we recorded song from two months after deafening showed song degradation comparable to deafened control birds. This may be due to the fact that the ablation strategy I use only kills on average only forty to fifty percent of the VTA_x population. The lesioning of other song nuclei like LMAN is known to completely block song degradation after deafening so it could also be that other brain regions play a more critical role in deafening-induced song degradation than the VTA_x population.

4.4 Methods

4.4.1 Intersectional ablation of VTA_x cells

Using identical procedures to those described in Chapter 3, male zebra finches (~100-110 dph for adult experiments) were anesthetized and placed in a stereotaxic apparatus. After making an incision in the skin, craniotomies were made with a smaller scalpel at a predetermined distance from the bifurcation of the midsagittal sinus using the stereotaxic coordinates described previously for VTA and Area X. Bilateral injections of a retrogradely transported Cre construct (AAV2/9.CMV.HI.GFP-Cre.SV40; Penn Vector, a total of 15 injections of 32.2 nl of Cre per hemisphere) were made into Area X at 3 different depths. A locally expressed Cre-dependent caspase construct was then injected into the VTA at 2 different locations along the medial-lateral axis (AAV2/1.Ef1 α .FLEXCasp3-2A-TEV; construct courtesy of Nirao Shah, UCSF, 15 injections of 32.2 nl of Casp3 per site per hemisphere i.e. a total of 4 caspase injection sites per bird). After these viral injections, the craniotomies and the incision site were sealed and the bird was allowed to recover from anesthesia under a heat lamp.

At the endpoint of each experiment and 5 days prior to perfusion, birds were injected with AlexaFluor 594 in Area X to retrogradely label VTA_x neurons. Five days after these tracer injections, birds were perfused and tissue was processed as described above. Sections containing VTA were visualized and imaged under a confocal microscope (Zeiss Axioskop 2). The images were then examined in an image-processing program and the number of fluorescent retrogradely labeled cells in VTA was counted in a semi-automated manner (ImageJ, CellCounter plug-in).

4.4.2 Bilateral removal of cochlea

Young adult male birds (100-120 dph) were anesthetized with an intramuscular injection of a pentobarbital and chloral hydrate solution (Equithesin) and secured to a custom-made metal platform. The tympanic membrane and inner ear bones occluding the oval window were removed with forceps and the cochlea were removed with a small wire hook. The cochlea were then examined under a dissecting microscope to ensure complete removal and stored in 4% paraformaldehyde. The incision site in the skin over the ears was closed with tissue adhesive and birds were placed under a heat lamp during recovered from anesthesia. Birds were then closely monitored over the next week as they recovered.

4.4.3 Song recordings and analysis

Social context-dependent changes in adults: Within a few days of bilateral viral injections to ablate VTA_x cells, birds were recorded in isolation to obtain renditions of undirected song. Female birds were then placed inside the chamber for no more than 3 minutes to elicit directed song from the male bird. The male's behavior was monitored with a video camera inside the recording booth to ensure he directed his song towards the female and displayed typical courtship behavior (beak wiping, plumage ruffling, hopping towards and/or attempting to mount female). One month after injection, the same process was repeated to collect both undirected and directed song. The stability of the bird's pitch for a given syllable was measured in a custom Matlab program (EvTAF song analysis, Brainard lab, UCSF). Syllables chosen to

measure pitch stability had clear tonal components and no fewer than 30 renditions of <5 millisecond syllable segments were used to calculate the coefficient of variation of pitch for directed and undirected song before and after viral expression. After song was collected, the male birds were injected with dextran in Area X in order to allow for the number of VTA_x cells remaining to be quantified. Birds were perfused 5 days after dextran injection and brain tissue was processed as described above.

Recordings before and after deafening: Within a few days of bilateral viral injections to ablate VTA_x cells, birds were recorded in isolation to obtain renditions of undirected song. At this early time point expression of caspase would be minimal. After obtaining at least a full day of “pre-deafening” song, I removed the cochlea bilaterally using the procedure described above. After the bird recovered from surgery and began singing readily, I recorded his song using SAP at 10-day intervals until at least 1 month after deafening. Motif similarity scores were calculated in SAP, comparing no fewer than 30 renditions of “post-deafening” song to “pre-deafening” song.

5. Activation of VTA terminals in Area X is sufficient to drive vocal learning

5.1 Introduction

The ablation and microdialysis experiments described above demonstrated that VTA_x cells and D1-type receptors in Area X are necessary for juvenile and adult learning; however, these experiments cannot give insight into how exactly these cells and receptors drive learning. I hypothesized that VTA_x cells, which receive information about errors in song from upstream areas, would become active when young birds sing song variants that best match the tutor's song. In adult birds we know from *in vivo* single unit recordings that VTA_x cells appear to signal a reward prediction error during pitch learning, increasing their firing when white noise is avoided and suppressing their firing when white noise does occur (Vikram Gadagkar, 2016). It thus seems likely that VTA_x cell activity paired with a range of pitches could increase singing of those paired pitches.

Here I use optogenetic terminal field stimulation of VTA terminals in Area X to determine if VTA_x terminal activity is sufficient to drive pitch learning. By substituting brief pulses of laser light for white noise, I used the pitch learning paradigm described previously to drive changes in pitch. In contrast to adult pitch learning with white noise, I find that optogenetic stimulation of VTA terminals drives learning towards, rather than away from, pitches paired with stimulation. I find that the effects of optical stimulation are specific to the targeted syllable and do not affect the pitch of surrounding syllables. Optical stimulation also fails to acutely change the mean pitch, pitch distribution, or pitch variability of the targeted syllable. Multiple controls show that the effects seen with stimulation of Chr2-expressing

terminals is not due to tissue heating, inadvertent fluorophore excitation, or non-contingent stimulation. Stimulation does appear to reinforce singing more generally, increasing the amount of singing over days of stimulation.

5.2 Results

5.2.1 VTA terminal activation in Area X drives pitch shifts in the direction of stimulation

In order to determine if VTA terminals in Area X are sufficient to drive learning, I combined song-triggered optogenetic stimulation with the learning paradigm I used previously to drive pitch learning in adults (Figure 36). Briefly, young birds were injected with AAV2/9.CAGChR2.mCherry ($n = 2$) or AAV2/9.CAG-Neurexin-ChR2.YFP ($n = 6$) bilaterally in the VTA, allowed to recover and then were returned to the colony. After allowing enough time for robust expression of ChR2 in the VTA terminals in Area X, I simultaneously recorded multiunit activity in Area X and stimulated with pulses of blue laser light to test for optogenetic responses in Area X in six of the eight birds I implanted (Figure 37). I implanted bilateral cannulas over Area X using similar surgical procedures to those described earlier and allowed the bird to fully recover before coupling the bird to fiberoptic cables for stimulation ($n = 8$ animals; mean interval between viral injection in the VTA and fiber implantation in Area X: 116 ± 16 days; mean age at implantation: 250 ± 33 dph).

Using the same custom software to target syllables that I used to drive adult pitch learning, after recording song for two days I targeted a syllable for a fifty millisecond pulse of laser light stimulation if the pitch of the syllable fell above (or below) a particular pitch threshold. Over the next several days (5 ± 1 days, range: 4-10 days), I gradually increased the threshold as the bird learned to shift his pitch upwards in order to receive more light stimulation. I then turned off the laser and recorded several days of song. As with the pitch learning experiments, syllables could be driven either up or down depending on whether

stimulation was applied above or below the pitch threshold determined by the experimenter. At the end of every experiment, birds were perfused and, following histological procedures described in the methods, tissue in the VTA and Area X was examined for channelrhodopsin labeled cell bodies and terminals, respectively. In a subset of birds the VTA was also co-stained with an antibody against tyrosine hydroxylase, which overlapped with cells labeled with ChR2 (Figure 38).

In response to light stimulation, the eight birds tested gradually shifted the pitch of the targeted syllable in the direction that received light stimulation. Seven out of ten syllables from six of the eight birds followed a gradual linear trajectory, steadily increasing their pitch each day (Figure 39, 40). Two out of the ten syllables from two birds showed the largest amount of learning on the second day of stimulation while one syllable from one bird showed the largest change in pitch three days after light stimulation began. Across birds, the absolute change in pitch (Figure 41) as well as the shift in distribution of pitch, measured by the absolute change in the area under the receiver operator characteristic (auROC) (Figure 42), were significantly larger when the last day of stimulation was compared to the second day of baseline than when baseline days were compared to one another (paired two-tailed t-test: absolute change in auROC from baseline day 2 versus light day 4: $n = 10$ syllables from 8 birds, $p = 0.00005$) (paired two-tailed t-test: absolute percent change in pitch from baseline day 2: versus light day 4: $n = 10$ syllables from 8 birds; $p = 0.008$). I also found no significant differences in the absolute change in pitch or auROC between syllables shifted up or syllables shifted down, indicating that light stimulation did not simply bias the bird's ability to sing higher or lower pitches (unpaired two-

tailed t-test: absolute change in pitch for “up” syllables versus “down” syllables: n = 5 syllables from 5 birds for “up” syllables, n = 5 syllables from 5 birds for “down” syllables, p = 0.789)
(unpaired two-tailed t-test: absolute change in auROC for “up” syllables versus “down” syllables: n = 5 syllables from 5 birds for “up” syllables, n = 5 syllables from 5 birds for “down” syllables, p = 0.397).

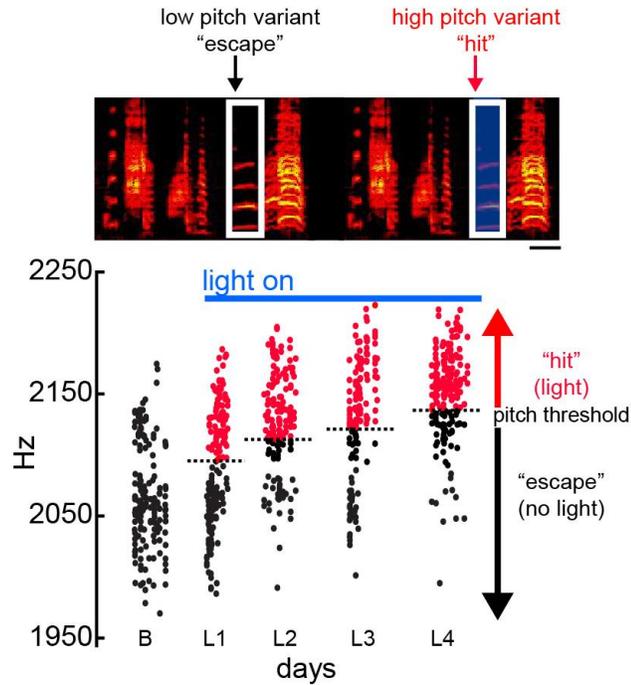


Figure 36: Experimental design for pitch-contingent optogenetic stimulation experiments

Top, Pitch learning paradigm described previously but white noise is replaced with optical stimulation of VTA_x terminals. *Bottom*, Sagittal section of the zebra finch brain indicating the injection site of virally encoded channelrhodopsin and the placement of fiberoptic cannula.

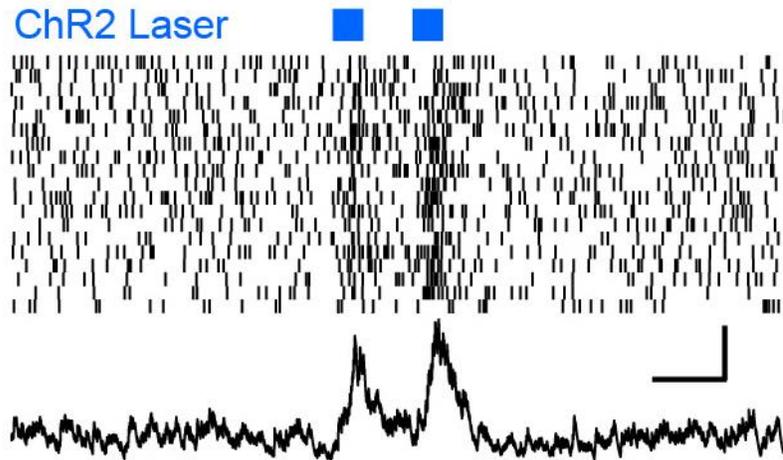


Figure 37: Optical stimulation of ChR2-expressing VTA terminals in Area X evokes multiunit activity

Multunit activity in Area X during optogenetic stimulation of VTA terminals. Scale bars, 500 milliseconds, 50 spikes/sec, n = 20 trials.

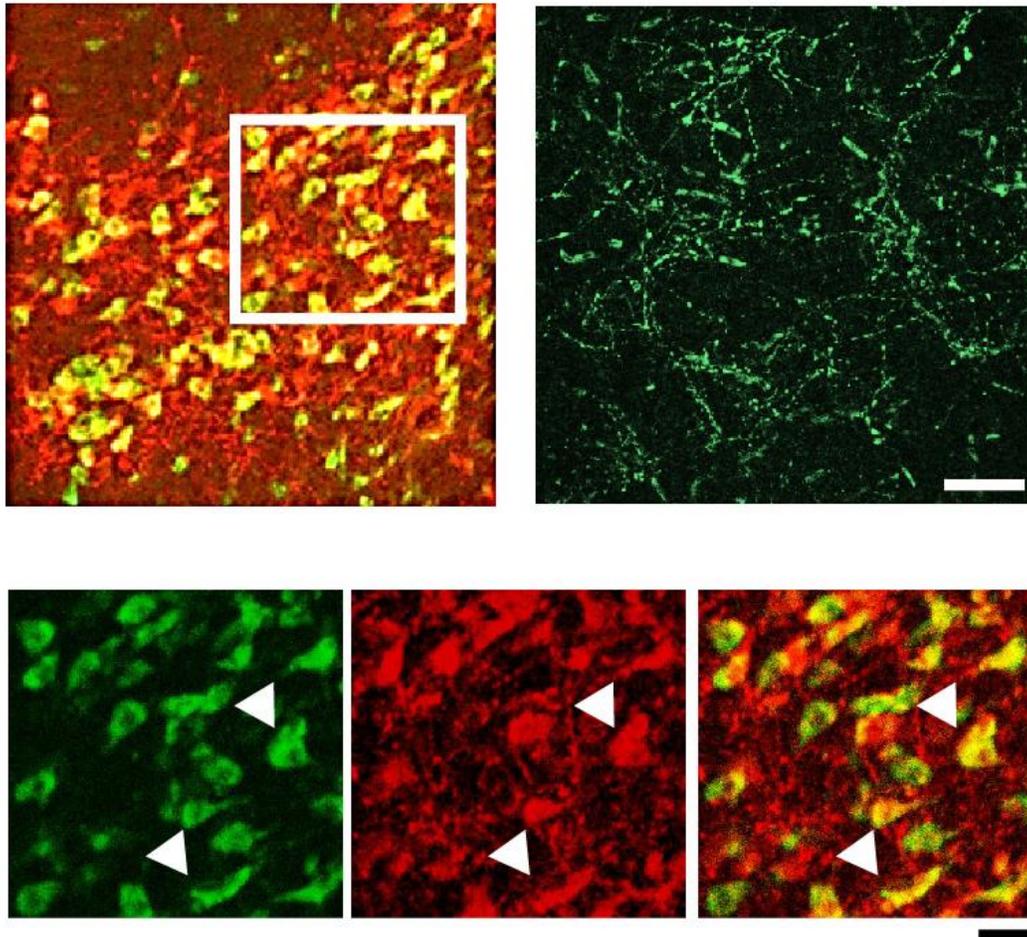


Figure 38: Channelrhodopsin expresses robustly in both VTA cell bodies and VTA terminals in Area X

Top left, merged image of TH+ VTA cells (red) with ChR2 (green) expression. Top right, ChR2 terminals in Area X. Scale bar, 100 μm . Bottom row, inset from top left. Scale bars, 50 μm .

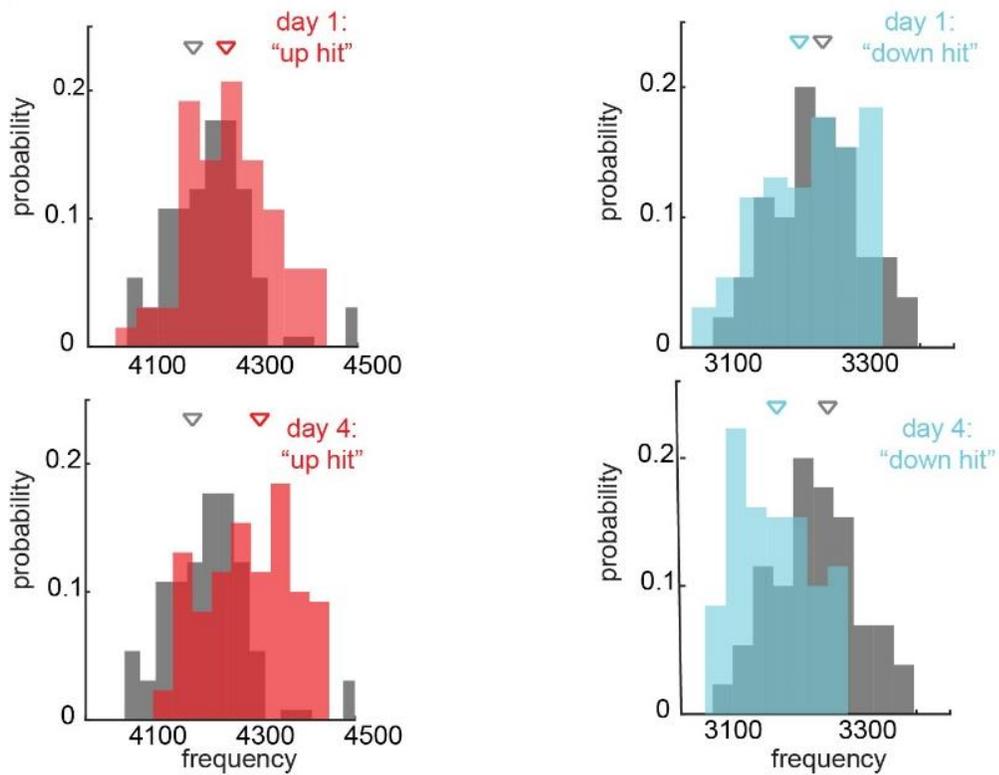


Figure 39: Change in pitch over consecutive days of optogenetic stimulation of VTA terminals in Area X for two example syllables

Left, distribution of pitch for an “up” syllable on the first (top) and fourth (bottom) day of pitch-contingent optogenetic stimulation of VTA_x terminals. Grey histogram indicates baseline pitch distribution; red histogram indicates pitch distribution on day of stimulation. *Right*, distribution of pitch for a “down” syllable on the first (top) and fourth (bottom) day of light stimulation. Grey histogram indicates baseline pitch distribution, blue histogram indicates pitch distribution on day of stimulation. Arrowheads indicate the mean pitch for the day.

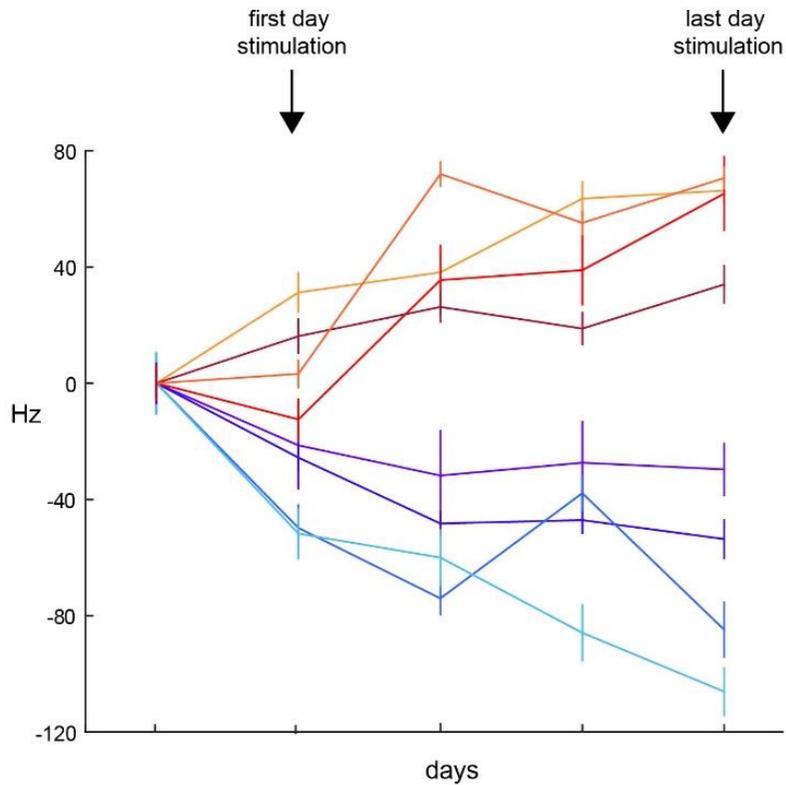


Figure 40: Change in pitch over consecutive days of optogenetic stimulation of VTA terminals in Area X across birds

Absolute change in hertz for one baseline day and four days of stimulation ($n = 8$ syllables from 6 birds). Each color indicates the trajectory of a single syllable. Warm colors correspond to “up” syllables, cool colors correspond to “down” syllables. This plot only shows birds that were stimulated at seventy percent contingency and does not include the two birds that were stimulated at seventy, as well as at one hundred percent contingency.

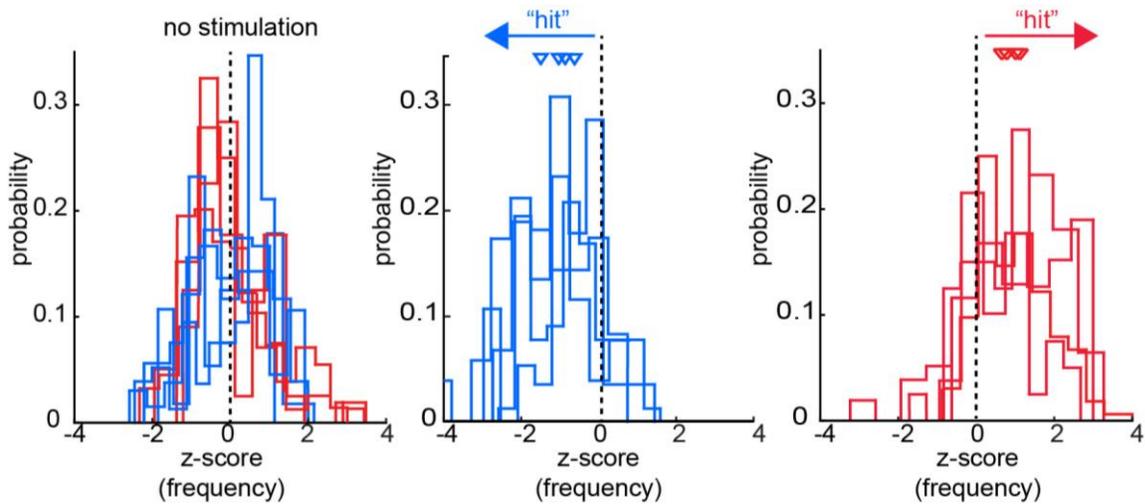


Figure 41: Pitch-contingent stimulation of VTA_x terminals drives appetitive shifts in syllable pitch

Left, z-scored frequency of all syllables before stimulation (n = 8 syllables, 6 birds). *Middle*, z-scored frequency of “down” syllables (n = 4 syllables) after stimulation. *Right*, Z-scored frequency of “up” (n = 4 syllables) syllables after stimulation. This plot only shows birds that were stimulated at seventy percent contingency and does not include the two birds that were stimulated at seventy, as well as at one hundred percent contingency.

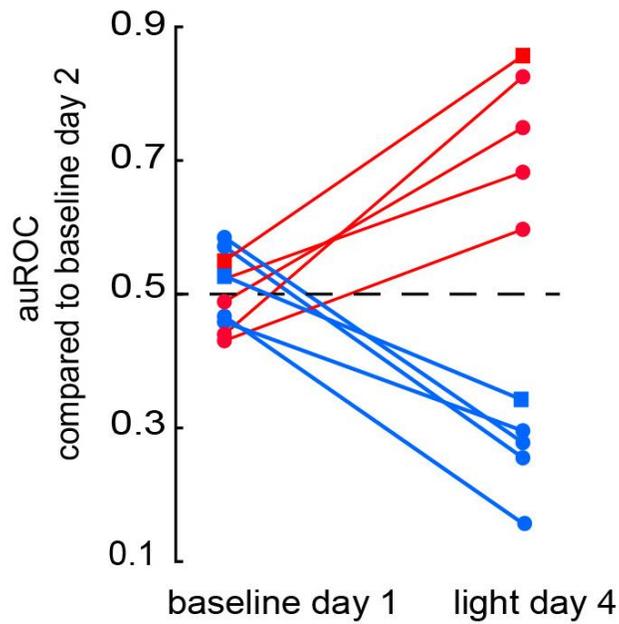


Figure 42: Pitch-contingent stimulation of VTA_x terminals drives appetitive shifts in the distribution of syllable pitch

auROC for “up” (red) and “down” (blue) syllables (n = 10 syllables, 8 birds). Square markers correspond to 100% contingency birds that were also run at 70% contingency.

5.2.2 VTA terminal activation does not affect nontargeted syllables and does not acutely affect targeted syllables

After determining that optical stimulation of VTA terminals in Area X was able to drive learning in the targeted syllable, I was curious about the temporal specificity of this stimulation and whether it was acutely changing the structure of syllables. To determine how temporally specific the effect of optical stimulation was, I compared the mean pitch and the auROC of syllables that occurred either before or after the targeted syllable on the last day of baseline to the last day of stimulation. Dopamine, which is presumably being released by VTA terminals in Area X to facilitate learning, is thought to act on a scale of hundreds of milliseconds rather than on the tens of milliseconds scale on which zebra finches produce song. I was surprised to find that the nontargeted syllables showed only minimal changes in pitch and auROC (two-tailed t-test: percent change in pitch of non-targeted syllable for baseline day two versus the last day of light stimulation: $0.399 \pm 0.090\%$, $n = 7$ non-targeted syllables from 4 birds, $p = 0.710$) (two-tailed t-test: absolute change in auROC of non-targeted syllable for baseline day two versus the last day of light stimulation: 0.068 ± 0.019 , $n = 7$ non-targeted syllables from 4 birds; $p = 0.498$) (Figure 43). These changes also did not scale with the proximity of the nontargeted syllable to the targeted syllable, meaning that the effect I saw on targeted syllables did not simply “spill over” to later syllables in song and was instead localized to the targeted syllable (Figure 44).

After establishing that stimulation only drove changes in the targeted syllable, I wanted to see if optical stimulation could acutely change the structure of the targeted syllable. I compared randomly sampled “catch” and “non-catch” syllables on the last day of stimulation and found that the difference in pitch as well as auROC between catch and non-catch syllables

from the same day was smaller than the change in catch syllables from baseline day one to baseline day 2. I also compared the variability of the syllable with and without optical stimulation and found that the coefficient of variation of pitch was not significantly different between catch and non-catch syllables. The lack of significant differences in measures of pitch (Figure 45) (two-tailed t-test: absolute percent change in pitch: baseline day 1 versus baseline day 2: $0.461 \pm 0.121\%$, $n = 7$ syllables from 5 birds; catch vs. not catch: $0.120 \pm 0.026\%$, $n = 6$ syllables from 6 birds; $p = 0.037$) and pitch variability (Figure 46) (two-tailed t-test: absolute change in auROC: baseline day 1 versus baseline day 2: 0.052 ± 0.010 , $n = 7$ syllables from 5 birds; catch vs. not catch: 0.032 ± 0.007 , $n = 6$ syllables from 6 birds; $p = 0.211$) between optically stimulated non-catch syllables and catch syllables, which did not receive optical stimulation, suggests that the effect of VTA terminal stimulation is not simply due to acute changes in motor execution but is rather due to a gradual learning of the association between optical stimulation and producing a syllable pitch within a particular range.

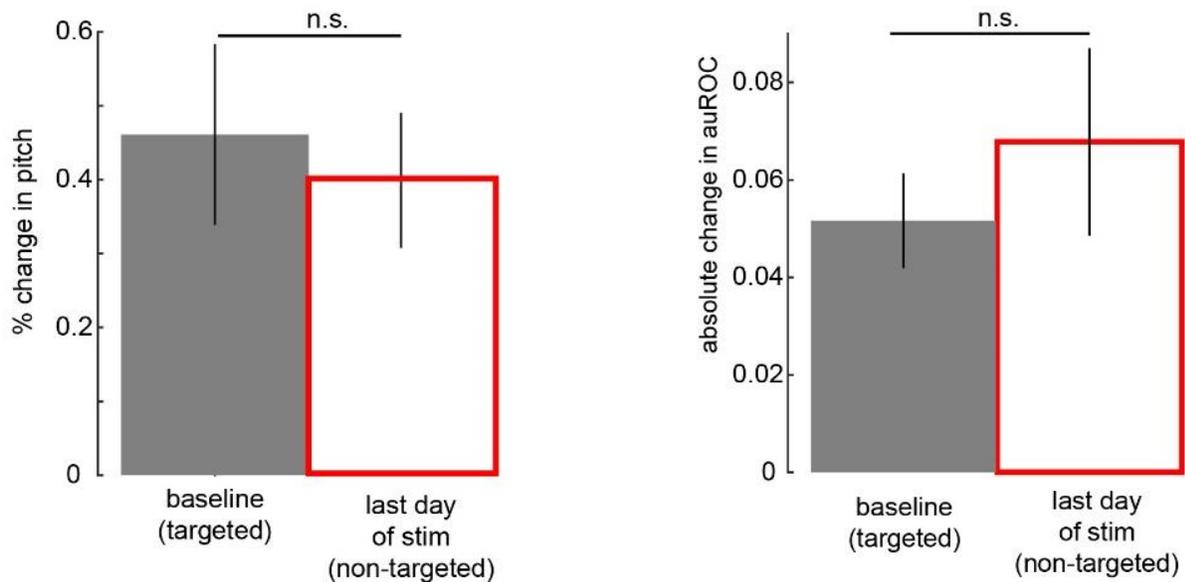


Figure 43: Syllable-triggered optogenetic stimulation of VTA terminals in Area X does not affect the pitch of non-targeted syllables.

Left, percent change in pitch for syllables that were targeted for stimulation on baseline day 2 versus baseline day 1 (grey, $n = 7$ targeted syllables from 5 birds) and syllables that were not targeted for stimulation on baseline day two versus the last day of light stimulation (white with red outline, $n = 7$ non-targeted syllables from 4 birds). *Right*, absolute change in auROC for syllables that were targeted for stimulation on baseline day 2 versus baseline day 1 (grey, $n = 7$ targeted syllables from 5 birds) and syllables that were not targeted for stimulation on baseline day two versus the last day of light stimulation (white with red outline, $n = 7$ non-targeted syllables from 4 birds).

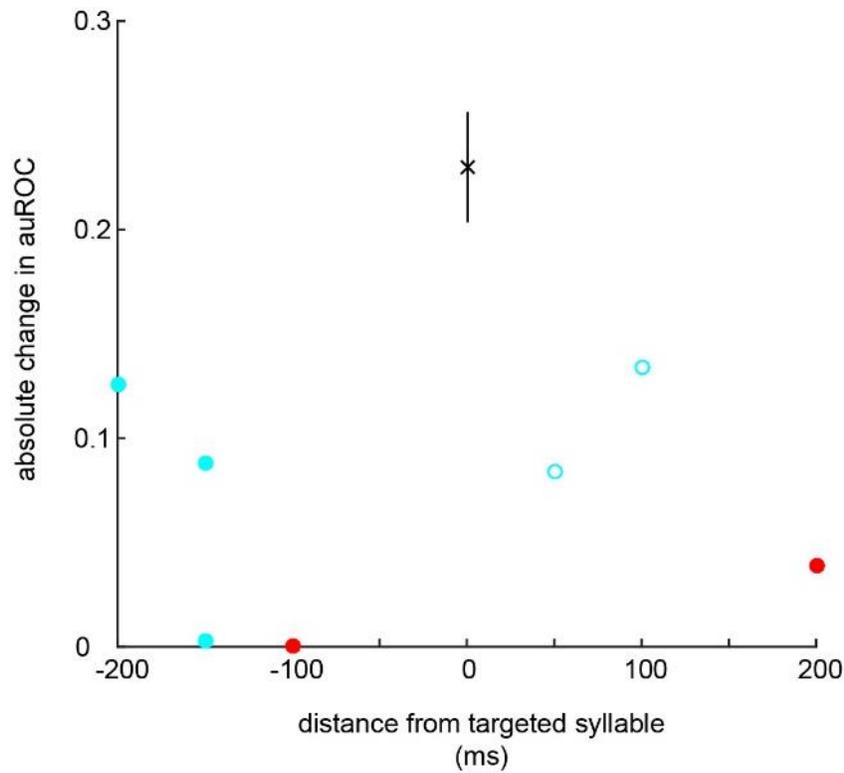


Figure 44: The proximity in song to the targeted syllable does not correlate with changes in pitch distribution in nontargeted syllables

Absolute change in auROC of non-targeted syllables as a function of milliseconds in song from the targeted syllable ($n = 7$ non-targeted syllables from 4 birds). Negative numbers indicate that the syllable preceded the targeted syllable. Red circles, targeted syllable was an “up” syllable; blue circles, targeted syllable was a “down” syllable. Open circles, later, but non-overlapping, part of the syllable that was targeted with light. ‘X’ indicates the mean and S.E.M. of the absolute change in auROC of all targeted syllables.

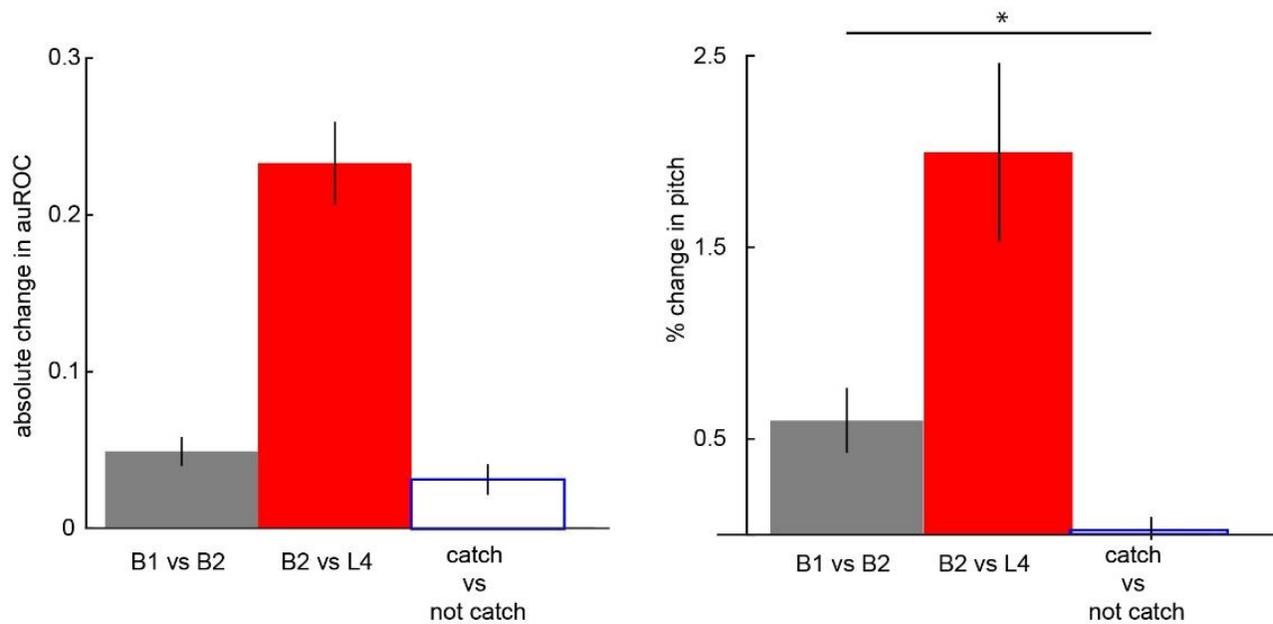


Figure 45: Optogenetic stimulation of VTA terminals does not acutely affect targeted syllable pitch

Left, absolute change in auROC for syllables on baseline day 2 versus baseline day 1 (grey, n = 7 syllables, 5 birds) and catch versus non-catch syllables on the last day of light stimulation (blue, 6 syllables, 6 birds). *Right*, percent change in pitch for syllables on baseline day 2 versus baseline day 1 (grey, n = 7 syllables, 5 birds) and catch versus non-catch syllables on the last day of light stimulation (blue, 6 syllables, 6 birds); *P<0.05, two tailed t-test.

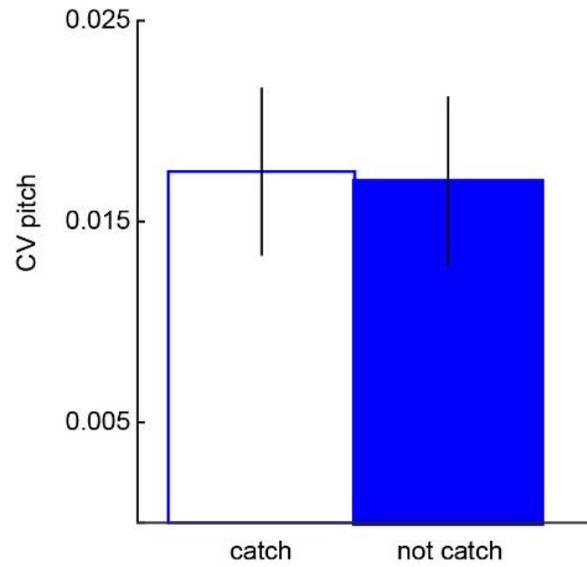


Figure 46: Optogenetic stimulation of VTA terminals does not acutely affect targeted syllable variability

Coefficient of variation of pitch for catch (white) versus non-catch (blue) syllables (n = 6 syllables from 6 birds).

5.2.3 Control stimulation does not drive changes in pitch

To control for effects of possible tissue damage or excitation of unlabeled tissue by optical stimulation I implanted a cohort of birds that had either been injected with GFP in VTA or were not injected with any virus with fiberoptic cannulas bilaterally over Area X. Neither the GFP birds (n = 3 syllables from 2 birds) nor the non-injected birds (n = 2 syllables from 2 birds) showed a significant change in either their mean pitch or pitch distribution after four days of light stimulation (unpaired t-test: absolute percent change in pitch from baseline day 2 versus light day 4 for control birds: n = 7 syllables from 6 birds; p = 0.198) (unpaired two-tailed t-test: absolute change in auROC from baseline day 2 versus light day 4 for control birds: n = 7 syllables from 6 birds, p = 0.639) (Figure 47). These controls demonstrate that the effects seen in ChR2 injected birds cannot be attributed to fluorophore excitation or tissue heating.

To demonstrate that learning was dependent on the information, or contingency, provided by the optical stimulation, I used two birds for experiments in which the birds were first stimulated every time they sang the targeted syllable regardless of its pitch (i.e. one hundred percent contingency) for four days and then were stimulated only when their pitch fell on one side of a pitch threshold (i.e. seventy percent contingency where I stimulated either syllables below the seventieth percentile or above the thirtieth percentile). Though the two birds were able to make changes to their mean pitch and pitch distribution from baseline when stimulated at a seventy percent contingency that were comparable to the other six birds stimulated at only at a seventy percent contingency, both birds only showed negligible changes in pitch from baseline when stimulated at a one hundred percent contingency (Figure 47). These

experiments confirm that stimulation of VTA terminals in Area X alone cannot drive changes in pitch but can drive rapid learning when paired with a range of pitches at a particular contingency.

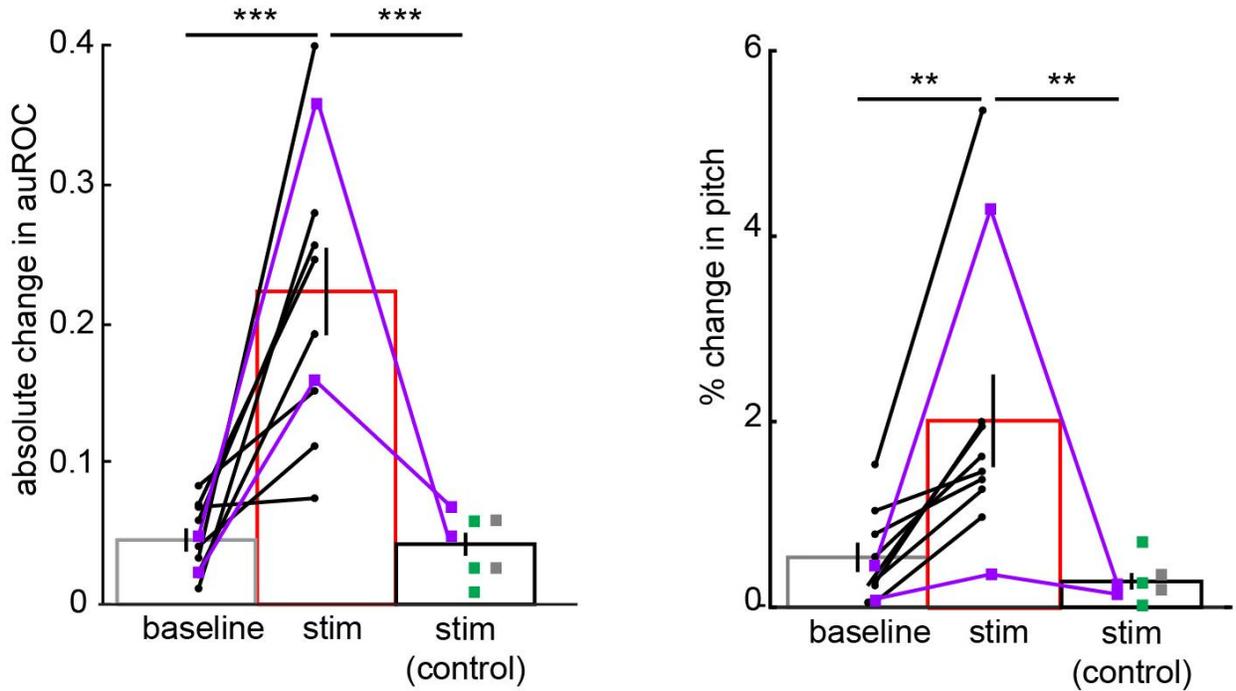


Figure 47: Control stimulation does not drive changes in pitch of the targeted syllable

Left, mean absolute change in auROC. *** $P < 0.001$, two-tailed t-test. Controls: $n = 7$ syllables, 6 birds; green, GFP; grey, no injection; purple, 100% contingency. *Right*, absolute percent change in pitch. ** $P < 0.01$, two-tailed t-test.

5.2.4 VTA terminal activation increases amount of singing

Given that mammals will self-stimulate when implanted with stimulating electrodes in dopamine-releasing areas like the VTA and the ventral pallidum (Smith et al., 2009), it seemed likely that stimulation of VTA terminals in Area X would also be positively reinforcing and would increase amounts of singing. Six of the eight birds tested dramatically increased their rate of singing as stimulation went on and four of the eight birds sharply decreased their amount of singing when stimulation was turned off. Across all eight birds there was a significant increase in amount of song between the last day of baseline and the last day of stimulation (paired two-tailed t-test: number of catch songs: baseline day 2: 94 ± 21 songs from 8 birds; light day 4: 140 ± 21 songs from 8 birds, $p = 0.038$) as well as a significant increase in the amount of song between the first day of stimulation and the last day of stimulation (paired two-tailed t-test: number of catch songs: light day 1: 101 ± 20 songs from 8 birds; light day 4: 140 ± 21 songs from 8 birds, $p = 0.033$) (Figure 48). This effect does not appear to simply be due to acclimation to the cage and fiberoptic cables as GFP and non-injected birds failed to show a similar increase in the amount of singing at the onset of stimulation which increased throughout stimulation (paired two-tailed t-test: number of catch songs: baseline day 2: 104 ± 22 songs from 4 birds; light day 4: 175 ± 47 songs from 4 birds, $p = 0.391$) (paired two-tailed t-test: number of catch songs: light day 1: 105 ± 11 songs from 4 birds; light day 4: 175 ± 47 songs from 4 birds, $p = 0.254$).

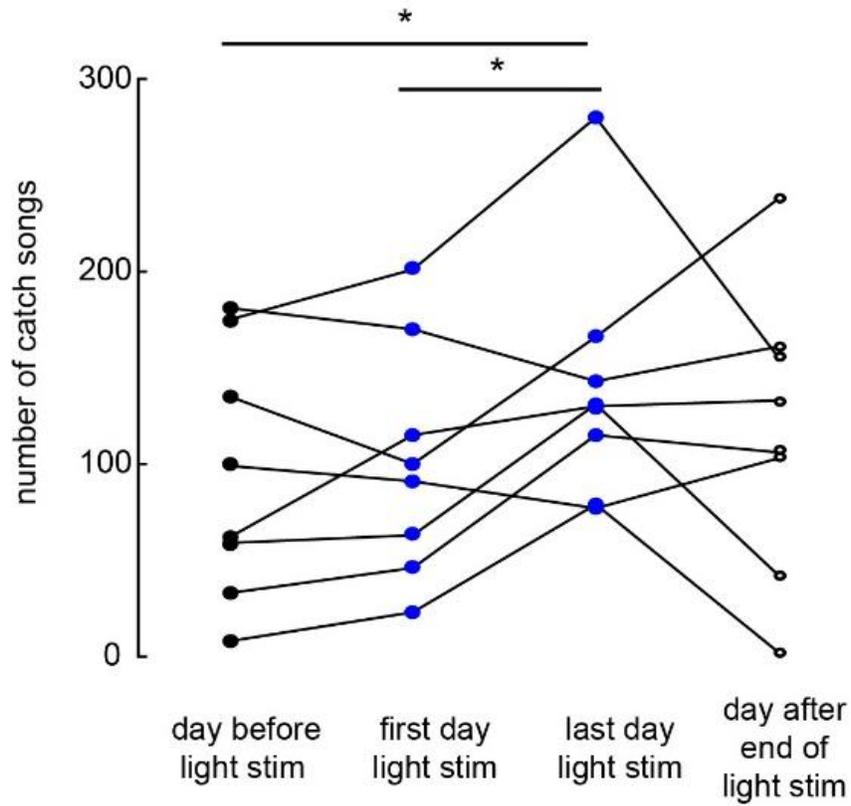


Figure 48: Optogenetic stimulation of VTA terminals increases the amount of singing

Number of catch songs per day for each bird (n = 8 birds) the day before pitch contingent optogenetic stimulation of VTA_x terminals (black circles), the first day of stimulation (blue circles), the last day of stimulation (blue circles), and the day after the last day of light stimulation (black open circles) *P<0.05, paired two-tailed t-test.

5.3 Discussion

Here I show that optogenetic stimulation of ChR2-expressing VTA terminals in Area X is sufficient to drive appetitive shifts in pitch and also reinforces singing more generally. The effects of stimulation are restricted to the targeted syllable and appear to convey a learning signal rather than a purely motor signal, as syllable pitch and variability are unaffected by acute stimulation. The appetitive effects I see are not due to tissue heating or fluorophore excitation as birds injected with GFP or birds that were not injected showed no significant changes in the pitch or pitch distribution of targeted syllables during optical stimulation in Area X.

Though this experiment does confirm that terminal activation of VTA_x cells is sufficient to drive learning, it cannot answer whether dopamine receptors in Area X are mediating this effect. One could show that D1-type receptors are required for the effects produced by optogenetic stimulation by implanting ChR2-injected birds with microdialysis probes as well as fiberoptic cannulas and infusing D1-type receptor antagonists during optogenetic stimulation. Technically this would be quite challenging but would allow one to demonstrate that VTA terminals are indeed acting through D1-type receptors to drive learning.

5.4 Methods

5.4.1 Viral expression of channelrhodopsin and implantation of ferrules

Using surgical methods previously described, young adult male birds (60-90 dph) were bilaterally injected in the VTA with a virus containing a channelrhodopsin construct (2/9.AAV-CAG-ChR2-mCherry or 2/9.AAV-CAG-ChR2-YFP-neurexin). After waiting 3-6 months to allow for optimal viral expression, birds were anesthetized and placed in a stereotaxic apparatus and craniotomies were made over Area X bilaterally. All birds were then implanted bilaterally over Area X with fiberoptic ferrules at 2.7-3.0 mm ventral. Craniotomies were then sealed with melted bone wax and ferrules were secured in place with MetaBond and then covered with a layer of VetBond. After birds recovered from anesthesia under a heat lamp, fiberoptic cables (ThorLabs, 200 um core, 0.37 NA) were connected to the newly implanted ferrules by ferrule sleeves. The other ends of the fiberoptic cables were attached to a two-channel optical commutator (FRJ_1x2i_FC-2FC, Doric), allowing the bird to move about its cage freely. The commutator was then connected by a patch cable (ThorLabs) to a DPSS laser (BL473T3-100, Shanghai Lasers).

5.4.2 Pitch-contingent optogenetic manipulation

As described above for adult pitch learning experiments, we created a template that detected no less than 75% of the renditions of the targeted syllable with no more

than a 5 millisecond jitter in detection onset. After collecting two days of “baseline” song (i.e., produced when the bird was connected to the fiberoptic cables but the laser remained off), a threshold at the upper (or lower) 70th percentile of the target syllable’s pitch distribution was set and a 50 millisecond pulse of blue light (473 nm) was delivered to Area X whenever the program detected that the pitch of the targeted syllable was below (or above) this threshold. The bird’s pitch for the targeted syllable was measured in the late morning and early evening for the next four days (for 8 out of 10 syllables, see below) and the threshold was adjusted to the upper 70th percentile of their pitch accordingly. Out of the ten syllables targeted, eight were exposed to pitch-contingent optogenetic stimulation for four days, one for six days, and another for ten days. Light stimulation was then ended and song in the absence of stimulation was recorded for up to four days. Birds were then uncoupled from the fiberoptic cables and returned to the colony. 3-5 months after stimulation ended, birds were again recorded for 4-5 days before being perfused.

5.4.3 Electrophysiological and histological confirmation of channelrhodopsin expression

Six of the eight birds used for these experiments were tested for terminal field optogenetic responses in Area X with a 500 kOhm tungsten electrode (MicroProbes Inc.) coupled to a fiberoptic cable (ThorLabs, 200-um diameter core) through which 50-100

millisecond pulses of light were delivered and neural activity was recorded simultaneously (Differential A-C Amplifier 1700, A-M Systems).

Histology was performed as described above, with alternate sections stained against mCherry or GFP (Abcam) for visualization of fluorophore-tagged ChR2 in the terminal field in Area X as well as in cell bodies in VTA. A subset of tissue was co-stained for TH+ as well as for mCherry or GFP to examine colocalization of TH+ and ChR2 expression in cell bodies in the VTA. Only birds that had accurate placement of ferrules in the center of Area X and robust labeling of cell bodies in VTA and of axon terminals in Area X were included in our analysis.

6. Neither VTA_x cells nor D1-type receptors are necessary for pitch recovery in the adult

6.1 Introduction

After driving pitch learning with white noise to shift the pitch of a syllable away from its original pitch, songbirds are able to rapidly return to their original pitch range once white noise is discontinued (Tumer & Brainard, 2007). This recovery is not simply passive relaxation back to a former pitch but rather is considered a form of learning as it depends on both auditory feedback and song nuclei required for song learning. Since this process is internally rather than externally guided, recovery from pitch learning has long been likened to juvenile learning, which is also internally guided. Surprisingly I find that the same manipulations to VTA_x cells and dopaminergic signaling in Area X that impaired internally guided juvenile learning and externally reinforced adult pitch learning have no effect on recovery from pitch learning in the adult. Neither VTA_x ablation nor blockade of D1-type receptors in Area X is able to impair rates of recovery after pitch learning. The seemingly counterintuitive finding that two forms of internally guided learning are not dependent on similar mechanisms, at least in terms of striatonigral mechanisms, may be due to the fact that juvenile learning requires the animal to learn a new motor program while recovery from pitch learning requires the animal to return to an old motor program.

6.2 Results

6.2.1 VTA_x cell ablation does not impair recovery from pitch learning

After driving pitch learning in adult birds, birds will gradually return to their original pitch once WN playback ends. This process can take several days and scales with the amount of learning that occurs, that is if a bird is driven in one direction for ten days it would take him longer to recover back to his original pitch than a bird that was only driven for 5 days. After I drove pitch learning in birds I injected with a virally encoded Cre-dependent caspase (AAV2/1.EF1 α .FLEX-Casp3-2A-TEV) in the VTA and a retrogradely traveling virally encoded Cre in Area X (AAV2/9.CMV.HI.GFP-Cre.SV40) I recorded their songs for the next three days after discontinuing WN, both before and after viral expression (Figure 20).

Though birds were only able to learn roughly half as much as they had before ablation of VTA_x cells, all birds tested were able to recover to the same extent as before viral expression occurred (Percent of pitch recovered three days after discontinuing exposure to pitch contingent noise early and late in viral expression window: paired two tailed t-test: early: $75.30 \pm 8.67\%$; late: $83.89 \pm 10.24\%$; $n = 6$, $p = 0.577$) (Figure 49). We then compared the rate of recovery before and after viral expression by fitting an exponential to the daily pitch from the last day of white noise until the last day of recovery. This would allow us to see if, despite the smaller change in pitch after learning, the birds could recover at the same speed after viral ablation of VTA_x cells. Interestingly, there was no significant difference in the rate of recovery as measured by

the time constant before and after viral expression (time constant of recovery: paired two-tailed t-test: early: 81.95 ± 18.03 ; late: 117.59 ± 25.64 ; $n = 6$, $p = 0.26$). This implies that VTA_x cells, while critical for adult and juvenile learning, may not be required for recovery from pitch learning in the adult. However, several other possibilities could account for this that will be described in the discussion.

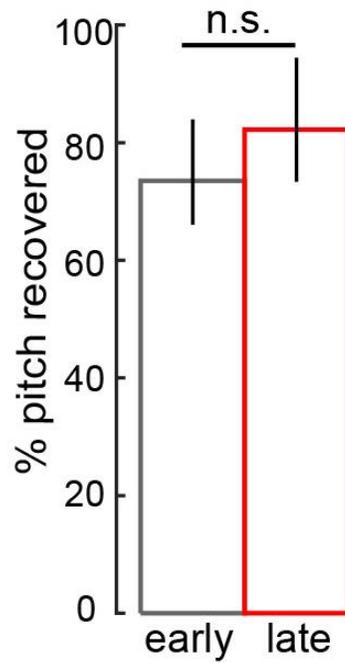


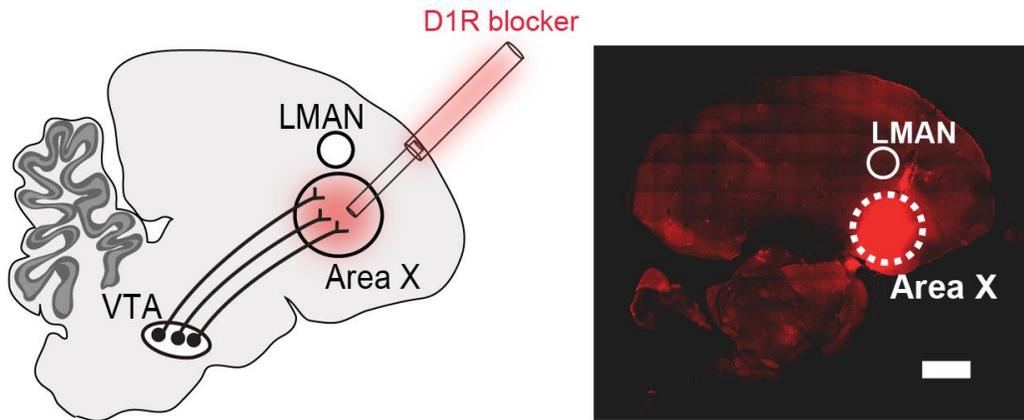
Figure 49: Ablation of VTA_x cells does not affect recovery.

Percent of pitch recovered three days after discontinuing WN early (grey) and late (red) in the viral expression window.

6.2.2 D1-type receptor blockade in Area X does not impair recovery from pitch learning

I also examined whether D1-type receptors were critical for recovery from pitch learning in adults. To do this I drove learning for an entire day and until ten A.M. the next morning when I turned off the WN. I then infused the bird with saline and recorded his song until 'lights out'. After the bird fully recovered to his baseline pitch I again drove learning for a day and into the next morning, turned off the WN then infused the bird with SCH23390 and recorded his song for the rest of the day (Figure 50). This paradigm, unlike the VTA_x cell ablation experiments, allowed me to compare recovery after equivalent amounts of learning.

Even though the birds learned equivalent amounts on the day of learning before SCH23390 infusion compared to the day of learning before saline infusion, they were still able to recover as much during SCH23390 infusion as during saline infusion (percent change in pitch during one day of recovery: paired two-tailed t-test: saline: $-1.727 \pm 0.322\%$; SCH23390: -0.87 ± 0.344 ; $n = 6$, $p = 0.256$) (Figure 51). This suggests that D1-type receptors are not critical for recovery though there are caveats that will be discussed later.



day	1	2	3	4
noise	+	-	+	-
saline	+	+	+	-
drug	-	-	-	+

Figure 50: Experimental design of adult recovery microdialysis experiments

Top left, sagittal section of zebra finch brain with location of microdialysis probe. *Top right*, infusion of muscimol-BODIPY through microdialysis probes to visualize drug spread. Scale bar, 1 mm. *Bottom*, experimental schedule.

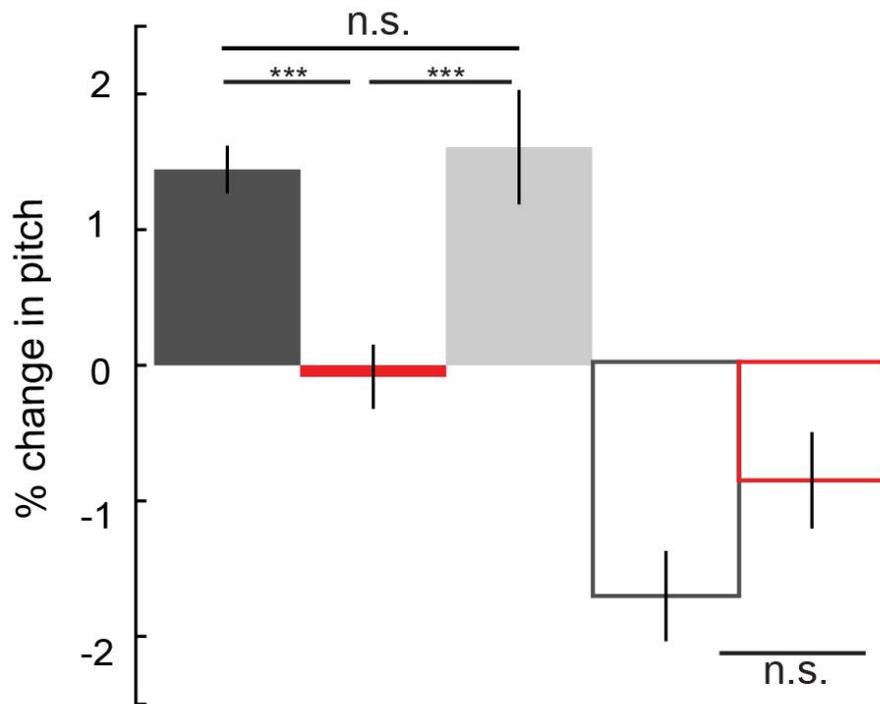


Figure 51: Blockade of D1-type receptors in Area X affects learning but does not affect recovery.

From left to right, percent change in target syllable pitch after WN during saline (dark and light grey) or SCH23390 (red) infusion. *P<0.05, **P<0.01, paired two tailed t-test, n = 6 birds. Percent of pitch recovered during saline (hollow bar with grey outline) or SCH23390 (hollow bar with red outline).

6.3 Discussion

Here I show that neither VTA_x cells nor D1-type receptors in Area X are necessary for recovery from pitch learning in the adult. Using a combination of intersectional ablation and microdialysis, I found that neither of the manipulations used previously to abolish juvenile learning and adult pitch learning affected recovery from pitch learning. This effect is surprising due to the fact that recovery relies on internally guided learning, as juvenile learning does, but does not appear to share similar mechanisms.

Though it may be that recovery does rely on mechanisms that are distinct from those involved in adult and juvenile learning, there are several possible reasons why recovery may have not been affected by my manipulations. In experiments in adults where I tested pitch learning before and after VTA_x cell ablation birds were able to learn significantly less after ablation. This would mean that they would have a “shorter” distance in terms of pitch to recover, making recovery easier for the bird to recover after ablation. To confidently say that recovery relies on VTA_x cells, I would want to perform experiments using a reversible, experimenter-activated technique such as DREADDs in order to drive learning to the same extent in two conditions. In one condition learning and recovery would occur in the absence of inactivation and in the other condition learning would be driven in the absence of inactivation but recovery would occur during inactivation of VTA terminals in Area X via local infusion of DREADDs. This

experiment, which will be described in greater detail in the later discussion section, would allow the experimenter to compare recovery with and without VTA terminal inactivation in Area X to equally large amounts of learning to see if birds could recover if the distance between newly learned pitch and original pitch was “farther” than in VTA_x ablation experiments.

Though the microdialysis experiments described did allow for the birds to recover from the same amount of learning these experiments may also have had similar constraints to those described above. Namely that perhaps recovery would be affected if the amount of learning driven was more substantial than the amount of learning that occurs in one day, which is the period of time that learning was driven over in these experiments. A simple solution to this would be to drive several days of learning, instead of only one, and then infuse a D1R antagonist on the first day of recovery to determine if recovery was affected when birds had a larger pitch distance to recover from. It may be that recovery is more robust to loss of VTA_x cells and dopamine signaling because it does indeed rely on mechanisms outside the striatonigral axis. However, more experiments are needed to rule out a role for VTA_x cells and striatal D1 signaling in recovery.

6.4 Methods

6.4.1 Intersectional ablation of VTA_x cells

Using identical procedures to those described in Chapter 3 and 4, male zebra finches (~100-110 dph for adult experiments) were anesthetized and placed in a stereotaxic apparatus. After making an incision in the skin, craniotomies were made with a smaller scalpel at a predetermined distance from the bifurcation of the midsagittal sinus using the stereotaxic coordinates described previously for VTA and Area X. Bilateral injections of a retrogradely transported Cre construct (AAV2/9.CMV.HI.GFP-Cre.SV40; Penn Vector, a total of 15 injections of 32.2 nl of Cre per hemisphere) were made into Area X at 3 different depths. A locally expressed Cre-dependent caspase construct was then injected into the VTA at 2 different locations along the medial-lateral axis (AAV2/1.Ef1 α .FLEXCasp3-2A-TEV; construct courtesy of Nirao Shah, UCSF, 15 injections of 32.2 nl of Casp3 per site per hemisphere i.e. a total of 4 caspase injection sites per bird). After these viral injections, the craniotomies and the incision site were sealed and the bird was allowed to recover from anesthesia under a heat lamp. At the endpoint of each experiment and 5 days prior to perfusion, birds were injected with AlexaFluor 594 in Area X to retrogradely label VTA_x neurons. Five days after these tracer injections, birds were perfused and tissue was processed as described above. Sections containing VTA were visualized and imaged under a confocal microscope (Zeiss Axioskop 2). The images were then examined in an image-processing

program and the number of fluorescent retrogradely labeled cells in VTA was counted in a semi-automated manner (ImageJ, CellCounter plug-in).

6.4.2 Microdialysis in Area X

As described in Chapter 3 and 4, young adult birds (> 80 dph) with clear tonal elements in their song were chosen for implantation of microdialysis probes. Using surgical procedures and stereotaxic coordinates described above, craniotomies were made over Area X and neural recordings were made to confirm its depth (Differential A-C Amplifier 1700, A-M Systems). Probes were then implanted with the tip of the semipermeable membrane placed at the most ventral part of Area X so that the membrane extended through the dorsal-ventral extent of Area X. The surgical site was covered with melted bone wax, and the probes were secured in place first using MetaBond and then a coating of VetBond. Birds were then removed from the apparatus and recovered under a heat lamp.

6.4.3 Pitch-contingent learning paradigm

VTA_X ablation experiments: As described in Chapters 3 and 4, after the birds recovered from surgery and began singing readily (typically 1-2 days after surgery), their songs were recorded and a template to detect to a tonal note of their song was

made in a custom program (EvTAF, Brainard lab, UCSF). The template was created to detect no less than 75% of the targeted syllables with no more than a 5 millisecond deviation in timing. After collecting two days of “baseline” song, a threshold at the upper 70th percentile of their pitch was set and a loud burst of white noise was played back to the bird whenever the program template detected that the pitch of the targeted note was below this threshold; this would effectively drive the bird’s pitch upwards as he learns to sing the targeted syllable higher to avoid the white noise burst. The bird’s pitch for the targeted note was measured in the late morning and early evening for the next four days and the threshold was adjusted to the upper 70th percentile of pitch to rapidly drive learning. After four days of white noise playback to drive pitch-contingent learning, the pitch-contingent white noise playback was turned off and the bird’s song was recorded for the next three to four days as the pitch of the targeted syllable recovered to its baseline pitch.

One month after viral injection, birds were again placed in the pitch-contingent learning paradigm; 2 days of baseline recordings were made, during which the template from the previous experiment was checked and adjusted to ensure that it could still detect the target syllable with the same level of accuracy as the previous experiment. The birds then experienced four days of pitch-contingent white noise playback to drive learning. The bird’s pitch for the targeted note was once again measured in the late morning and early evening for the next four days and the threshold was adjusted to the

upper 70th percentile of pitch to rapidly drive learning. The pitch-contingent white noise playback was turned off and the bird's song was recorded for the next three to four days. At the end of this recovery period, the birds were injected with dextran in Area X in order to allow for the number of VTA_X cells remaining to be quantified. Birds were perfused 5 days after dextran injection and brain tissue was processed as described above.

Microdialysis experiments: After recovery from surgery birds were placed in a sound isolation box and their first full day of song was recorded. Birds were recorded in the absence of white noise for two hours the morning after their first full day of song then infused with saline and recorded in the presence of pitch-contingent white noise for the entire day. White noise was turned off the next morning at 10 AM and birds were infused with saline. Their songs were then recorded for the rest of the day to provide a measure of their ability to recover with saline.

This protocol was repeated the following day (i.e. white noise on for 1 day then turned off at 10 AM the next day) but birds were instead infused with 5 mM SCH23390 and recorded for the rest of the day.

7. Discussion

Here I have shown that similar mechanisms underlie both internally and externally reinforced vocal learning. VTA_x cells and D1-type receptors in Area X are both critical for internally guided juvenile learning and for externally reinforced adult pitch learning. VTA_x cells are also required for context-dependent changes in song and deafening-induced song degradation. Pitch contingent activation of VTA terminals in Area X is sufficient to drive learning in targeted syllables and also reinforces singing more generally. Interestingly, though internally guided juvenile learning is dependent on both VTA_x cells and D1-type receptors in Area X, internally guided recovery from pitch learning does not appear to require either. Below I will summarize my results in greater detail (see Table 1 for a brief summary), suggest additional experiments to resolve questions unanswered by my current set of experiments, and discuss future directions.

Table 1: Summary of results. Green indicates the cell type or receptor was necessary for the form of learning/ song modification listed, magenta indicates the cell type or receptor was not necessary for the form of learning/ song modification listed. A diagonal line indicates that the role of the cell type or receptor was not tested. An asterisk indicates that the cell type or receptor was also found to be sufficient for the form of learning/ song modification listed.

form of learning/ song modification	necessity	
	VTA cells	D1Rs in Area X
juvenile learning	✓	✓
adult pitch learning	✓ *	✓
context-dependent changes in song	✓	
deafening-induced changes in song	✓	
adult recovery	✗	✗

7.1 Juvenile learning

I first examined the role of VTAx cells in sensorimotor learning. I used an intersectional genetic ablation approach to selectively lesion only the cells in VTA that project to Area X. The viruses I used allowed me to ablate this cell type at the height of sensorimotor learning when young birds are most extensively practicing their songs. I found that VTAx cells are required for accurate tutor song copying but not for motif stereotypy or a more general motivation to sing. The number of VTAx cells remaining also strongly correlates with the bird's ability to accurately copy the tutor song across all experimental birds, including those in which I failed to achieve robust ablation.

In order to confirm that the effects on learning were due to the absence of VTAx cells and not inadvertent damage to other collaterals of VTAx cells, I used a dual tracer strategy to retrogradely label cells from the VTA and from three other song nuclei known to be key players in juvenile learning. Upon histological examination of the VTA I found that less than five percent of cells that project to Area X also project to HVC, LMAN or Nif, in both juvenile and adult birds, allaying concerns that the effect on learning was due to damaging of collaterals to song nuclei other than Area X.

I also used *in vivo* microdialysis in young birds to examine whether D1-type receptors are necessary for juvenile song learning. I implanted birds just beginning the sensorimotor phase of song learning bilaterally with microdialysis probes over Area X and infused the birds with either saline or a D1-type receptor antagonist over the next ten days. In contrast to unimplanted birds or birds infused with saline, which both

showed a significant increase in song similarity to the tutor song, birds infused with a D1-type receptor antagonist showed no increase in similarity to the tutor song over the ten day period. However, several weeks after the end of drug treatment, birds infused with a D1-type receptor antagonist were able to “catch up” to control birds and sing songs that were statistically indistinguishable from those of control birds in terms of similarity to the tutor song. In total, I found that both VTAx cells and the receptors they presumably act on are necessary for juvenile song learning.

7.2 Adult learning

In order to examine if the same cells and receptors required for internally guided juvenile learning are also required for externally reinforced forms of vocal learning, I used a pitch learning paradigm in which adult birds learn to shift their pitch away from pitches that are paired with aversive bursts of white noise. When I ablated VTAx cells in adult birds with the same intersectional strategy I used in juveniles, I found that after robust viral expression occurred, birds were unable to learn as much as they did early in the time window for viral expression. Though this manipulation affected learning, pitch variability and the length and number of song bouts were unaffected.

I then used *in vivo* microdialysis to determine if externally reinforced pitch learning was also dependent on dopamine receptors in Area X. After bilaterally implanting adult birds with microdialysis probes, I infused the birds first with saline during pitch learning to establish a baseline measure of learning and then infused the

birds with a D1-type receptor antagonist during learning. I found that learning was markedly reduced both in terms of the mean pitch and the pitch distribution of the targeted syllable during D1-type receptor infusion. To determine if this effect was specific to a particular type of dopamine receptor, I then infused birds with a D2-type receptor antagonist and found that their rate of singing was greatly reduced both in comparison to their rate of singing during saline infusion and to the rate of singing of during D1-type receptor antagonist infusion. Once I corrected for the reduction in singing, I found that the change in pitch per syllable rendition was no different during D2-type receptor antagonist infusion than saline infusion. Overall I found that externally reinforced adult pitch learning was dependent on VTAx cells and specifically on D1-type receptors in Area X.

7.3 Modification of song in the adult

To determine if the same cell type involved in learning was also involved in active modification of adult song I again used an intersectional ablation strategy to examine the role of VTAx cells in context dependent changes in song. Though pitch variability during directed song was significantly lower than during undirected song before viral expression, after viral expression the pitch variability during directed song was not statistically different from the pitch variability during undirected song. Birds showed variable effects after VTAx cell ablation; in some birds, variability was decreased during undirected song but not affected during directed song while in others

variability was increased during directed song but remained the same during undirected song. The change in variability between directed and undirected song before and after ablation weakly correlated with the number of VTAx cells remaining.

Ablation of VTAx cells in another cohort of adult birds also impaired deafening-induced song degradation in comparison to controls. VTAx cell ablation did not entirely block, but rather delayed song degradation as more severe degradation appeared at later timepoints in experimental birds than in control birds. This result is not entirely surprising as cortical areas such as LMAN and HVC are also known to be involved in deafening-induced song degradation. These areas may be able to in part compensate for the absence of VTAx cells and suggest that VTAx cells are not the only player in adult song maintenance. Both deafening-induced song degradation and social context-dependent changes in song therefore are dependent, at least in part, on VTAx cells.

7.4 Optogenetic stimulation of VTA terminals in Area X

In addition to determining the necessity of VTAx cells I also was interested in determining if VTAx cells could alone drive learning. I injected birds with virally encoded channelrhodopsin in the VTA and after allowing several months for robust terminal field expression, I implanted the birds over Area X with fiberoptic cannulas. After coupling the birds to a laser with fiberoptic cables, I used the adult pitch learning

paradigm described above, replacing bursts of white noise with fifty millisecond pulses of laser light when the bird sang a pitch within a specified contingency.

Over the course of several days of stimulation, birds gradually shifted their pitch in the direction that received stimulation. I was able to drive syllables to higher as well as lower pitches and see significant changes in both the mean pitch and the pitch distribution after stimulation. I found that stimulation did not acutely affect the structure of the targeted syllable nor did it affect the structure of other non-targeted syllables in the song. This last observation was surprising to me as it suggests that VTAx cells, which presumably release dopamine, can act on a scale of less than one hundred milliseconds to precisely affect a single syllable.

One question that remains is if the effects I saw with channelrhodopsin stimulation were actually due to activation of dopamine receptors by the optically stimulated terminals. Below I will describe an experiment that could potentially address this question.

7.4.1 Simultaneous optogenetic stimulation and microdialysis

In order to show that the effects of optogenetic stimulation of VTAx terminals were mediated through a particular receptor type, I would need to combine the optogenetic experiments with microdialysis over Area X. A number of elements would have to come together to achieve this, including robust channelrhodopsin expression in the terminal field, correct placement of the microdialysis probes alongside the fiberoptic

cannulas, and the microdialysis probes staying useable and not clogging before the bird is acclimated to singing with cables which can take up to two weeks. If all these elements did indeed come together I could alternate days of driving learning with light in the presence of saline with days of driving learning with light in the presence of a receptor antagonist to determine if learning was reduced or even eliminated on days with antagonist infusion.

I would first want to infuse a D1-type receptor antagonist given that my results have shown that this receptor type is required for both juvenile song learning and adult pitch learning. Though it seems likely that the VTAx terminals would act on these receptors, given that D1-type receptor antagonists recapitulate the effects I see on both forms of learning after VTAx ablation, my experiments up to this point are unable to causally link the two. It could be that multiple receptor types are needed to drive learning and D1-type receptors, while necessary, are not sufficient to drive learning.

It would also be interesting to determine what neuromodulator or neurotransmitter the VTAx terminals actually release. Given that VTA cells positive for tyrosine hydroxylase can release GABA as well as glutamate into the striatum in mammals, it may be that VTAx terminals co-release several neurotransmitters along with dopamine. In the future, one could use fast scan cyclic voltammetry in Area X either in vivo or in slices from animals expressing channelrhodopsin in the VTA to detect which transmitters or modulators are released during VTAx terminal stimulation.

7.5 Adult recovery

Since its introduction to the birdsong field, adult pitch learning has been thought to be an externally reinforced form of learning while recovery from pitch learning has been thought of as an internally guided process. Under this assumption, I thought that recovery would rely on the same mechanisms as juvenile learning, another internally guided form of learning. To my surprise, in my experiments neither VTax cells nor D1-type receptors in Area X affected recovery from pitch learning.

After running adult pitch learning experiments as described above before and after ablation of VTax cells, I also recorded three days of recovery after the end of white noise playback. Neither the percent of pitch recovered nor the exponential of the recovery differed before and after VTax cell ablation. A potential confound to this experiment is that once VTax cells are ablated the birds are not able to learn as much and thus have a much shorter distance (in terms of pitch) to recover. Perhaps if birds were able to learn a larger amount after VTax cell ablation then recovery would be impaired since the birds would have a longer distance to recover.

In experiments to test the necessity of D1-type receptors in Area X to recovery from pitch learning I implanted birds bilaterally over Area X and drove learning for one day. The next day I infused either saline or a D1-type receptor antagonist and recorded song in the absence of white noise. Within-bird comparisons revealed no significant differences in recovery with saline and recovery with the D1-type receptor antagonist. Though these experiments allowed me to compare recovery with saline or with drug

after similar amounts of learning, unlike the ablation experiments which recovery after a large amount of learning was compared to recovery after a smaller amount of learning, the distance the birds needed to recovery was still minimal. To address this in the future, learning could simply be driven for three or four days instead of one day before saline and drug microdialysis to determine if larger amounts of recovery require the full complement of D1-type receptors in Area X. Testing the role of VTAx cells in recovery after a large amount of learning is a bit more tricky as the birds must have VTAx cells in order to learn; to get around this confound I would need to use a technique that would allow for rapid (i.e. within a day) inactivation of VTAx cells without compromising the cell type specificity I was able to achieve with VTAx cell ablation. I will describe an experiment below that attempts to address these issues.

7.5.1 VTA terminal field inactivation during recovery

In order to inactivate VTAx cells in a rapid, cell type specific manner, I would first drive viral expression of a DREADD linked to an inhibitory G protein in the VTA. After the injection I would run a four-day pitch learning experiment and then record the bird's recovery in the absence of white noise. After waiting two to three months for DREADDs to be strongly expressed in the terminal field I would implant the bird with microdialysis probes bilaterally over Area X. Once the bird recovered from the implant I would drive pitch learning for four days to achieve a similar amount of learning as I had several months ago. Before 'lights on' on the first day of recovery I would infuse the bird

with CNO, a designer drug that specifically activates DREADDs, into Area X to inactivate VTA terminals. I would then turn off the white noise and record the bird's recovery as I had previously. Terminal inactivation with DREADDs would allow me to compare recovery with and without VTAx terminals after identical amounts of learning.

After comparing this first day of recovery with VTAx terminal inactivation to the first day of recovery with normal VTAx terminal activity, I could attempt to block recovery for the next several days by infusing CNO for the next two days of recovery or I could infuse saline instead and test if the rate of recovery would increase once VTAx terminals are back online. If the probes were still intact at the end of the recovery period I could drive learning again for four days and record recovery during saline infusion to control for possible damage from the microdialysis implant.

7.6 Synthesis

Here I will use the data I have collected to speculate on the nature of reward prediction errors during internally and externally guided learning and general cellular and circuit level effects of dopamine on learning in the songbird.

7.6.1 Reward prediction errors during internally and externally reinforced learning

In classic work by Schultz and colleagues, dopaminergic cells in the midbrain were observed to fire in response to a reward and then, after several trials, in response to the cue that predicted the reward, referred to as a positive prediction error. These cells also showed suppression in their firing in response to the omission of an expected reward, referred to as a negative prediction error (Figure 52) (Schultz et al., 1997). Reward prediction errors have also recently been observed in VTA_x cells in singing birds (Vikram Gadagkar, 2016). When white noise (WN) is randomly played back to singing birds in a syllable, but not pitch-contingent paradigm, VTA_x cells suppress their firing, in the manner of a negative prediction error, when WN is played back during singing; these same cells also show a phasic increase in firing when WN is not played back, in the manner of a positive prediction error. This experiment however does not test for the role of VTA_x cells in a learning paradigm in which the bird can learn to predict and avoid WN by associating WN playback with a particular range of pitches.

Given that I know that VTA_x cells are necessary for both adult pitch learning and juvenile learning, I can speculate as to what the reward prediction errors (RPEs)

encoded by VTA_x cells might look like during each form of learning (Figure 52). During adult pitch learning before the bird has learned how to effectively avoid WN, VTA_x cells would at first show a negative RPE in response to WN playback immediately after playback of WN. As the bird learns to associate the pitch he sings before WN playback as predicting a subsequent “escape” or “hit”, the positive RPE will occur at the time when the bird sings the pitch that determines WN occurrence (milliseconds before WN playback) rather than at the time WN playback occurs during “hit” trials. If the positive RPE occurs when the bird sings the pitch that predicts WN but the pitch is actually in the “hit” range, VTA_x cell firing will be suppressed when WN playback occurs, creating a mismatch in expected and actual outcomes that can facilitate learning.

In the juvenile I predict that when the bird sings a part of song that matches the tutor song well there will be an increase in firing rate in VTA_x cells. Changes in firing rate could be graded as during the initial stages of learning birds are unable to produce song variants that closely match the tutor song; VTA_x cells could encode a “better” or “worse” outcome by scaling the size of their phasic response with the “goodness” of the match (Figure 52). As the bird’s song becomes a closer match to the tutor’s, activity in VTA_x cells would occur when a particular song element is produced rather than when the bird receives auditory feedback about that song element, with positive RPEs occurring when the bird produces the song element and VTA_x cell firing increasing if the element produced is indeed a good match with the tutor song. Changes in firing rate may become more binary as the bird’s song improves; instead of being graded, only

elements that are good matches to the tutor will result in an increase in VTA_x cell firing while elements that are not as good will result in a suppression of VTA_x cell firing.

Though the cell type-specific connectivity within the songbird VTA is not known, it may be that VTA interneurons, of which there are many, receive different inputs about various aspects of song at different time points and the interneurons that encode the different song features at a particular point in time converge on a single VTA_x cell whose output represents the “correctness” of the song at that particular point in time.

7.6.2 Effects of VTA_x cells on the song circuit as a whole

At a circuit level, VTA_x cells receive incoming error information from auditory areas as well as the ventral pallidum and send signals to the striatum about the correctness of song. Here I will speculate as to what occurs within the song system to make changes to song via an anterior forebrain loop that ultimately acts on a motor cortex analogue (see Figure 53 for a schematic). Many of these ideas have been explored in depth in a review by Fee and Goldberg (Fee & Goldberg, 2011) so I will integrate their hypotheses with observations I have made in my own research. Though it is not known whether VTA_x cells directly synapse onto medium spiny neurons (MSNs) or what neurotransmitters VTA_x cells do indeed release, I will assume here that the dopamine type 1 receptors (D1Rs) on MSNs are activated by dopamine released from VTA_x cells. For simplicity I will also describe only what occurs for one particular song feature which I will call ‘feature A’. It is known that the anterior forebrain loop from LMAN to Area X

to DLM, a thalamic nucleus, and back to LMAN, is organized in a topographic fashion so that each feature of song can be represented by a particular “channel” in the topography of the loop (Luo, Ding, & Perkel, 2001); while I will describe action through only one channel of the loop that corresponds to a particular song feature at a particular time, other channels that represent other song features will be active as well.

When the bird sings a version of ‘feature A’, LMAN and HVC inputs that are specific to ‘feature A’ will both release glutamate onto synapses on a specific MSN, strongly depolarizing the cell and tagging the cell with an eligibility trace, which here I will describe as a long-lasting depolarization though the nature of this eligibility trace is unknown. LMAN in this case carries copy of the motor bias signal that LMAN sends to RA, a motor cortex analog, and HVC carries a signal about the point in time when the song feature occurred. Once areas upstream of VTA, such as Aiv and VP, have evaluated whether ‘feature A’ is a good match to the tutor song, the VTA_x cells that respond to feature A will become active and phasically release dopamine and glutamate into Area X. The VTA_x cells would simultaneously release both dopamine and glutamate to activate D1Rs and NMDA receptors, respectively, onto a specific MSN. Activation of both D1Rs and NMDA receptors on a strongly depolarized MSN is known to be necessary for the expression of LTP (Ding & Perkel, 2004) so it is possible that the release of dopamine and glutamate from VTA_x can potentiate MSN activity.

Once the specific MSN becomes potentiated it will suppress the activity of pallidal cells that normally suppress activity in thalamic nucleus DLM. DLM will then

be released from inhibition and, as LMAN is known to represent different intensities and frequencies of syllables (Kao et al., 2005), can drive LMAN activity in the cells that represent 'feature A'. This will strengthen synapses from LMAN to RA that produce feature A and will also increase the strength of LMAN-to-Area X synapses that represent feature A so that when 'feature A' is sang correctly the next time both synapses will have greater weights than previously and will be able to more strongly drive the cells that produce the correct 'feature A'.

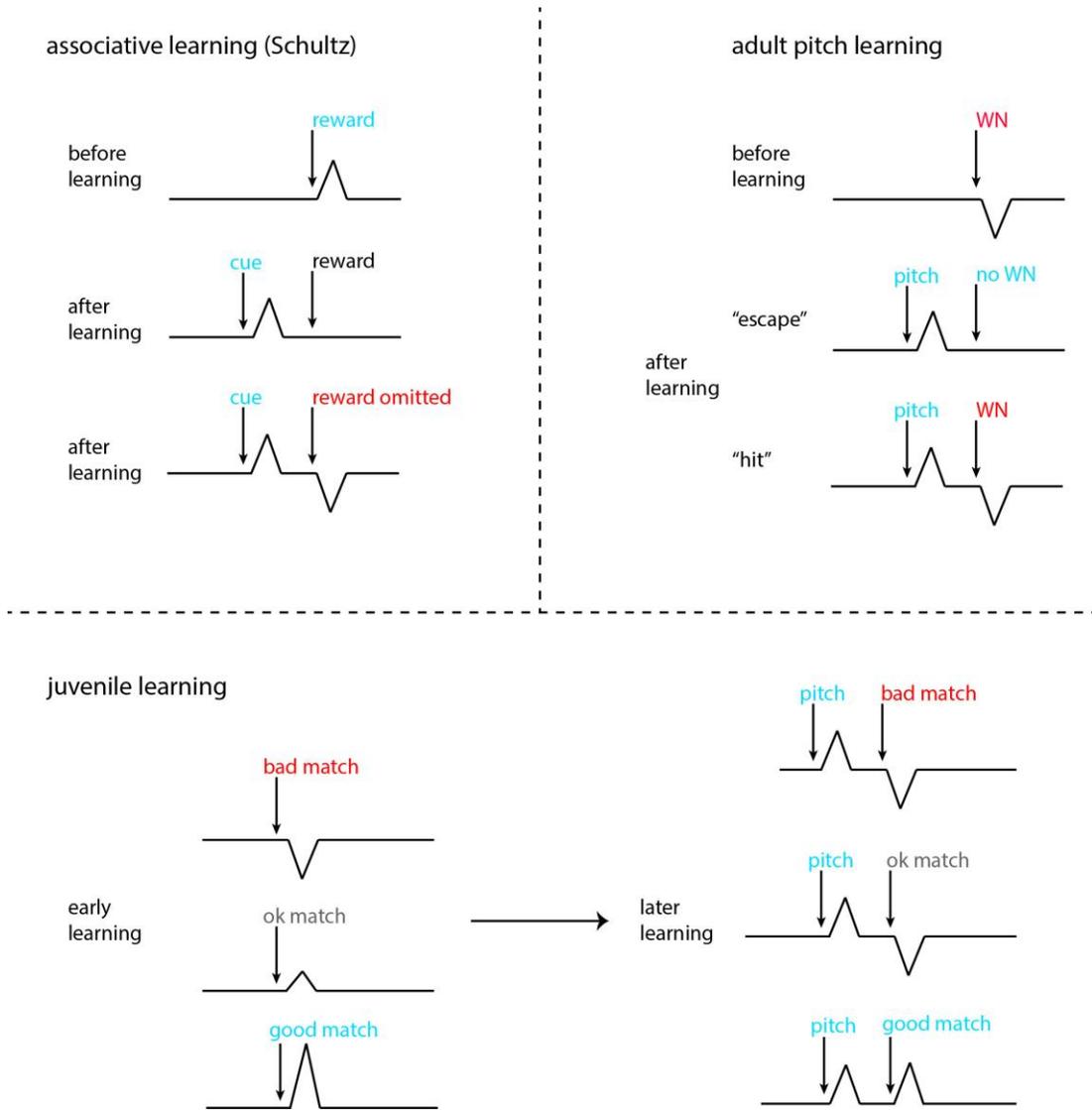
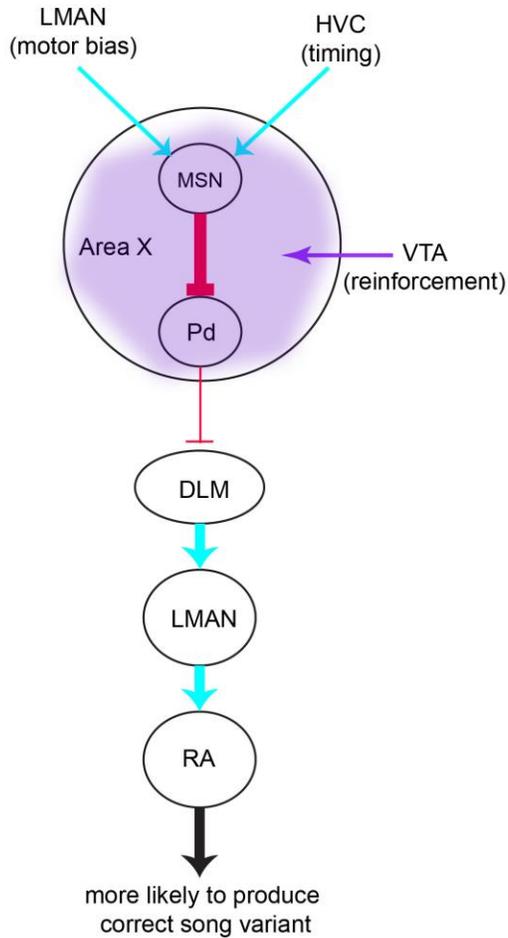


Figure 52: VTAx cell activity during learning.

Toy diagrams of VTAx cell activity during associative learning based off of experiments by Schultz and colleagues as well as hypothesized activity during adult pitch learning and juvenile learning.

correct variant



incorrect variant

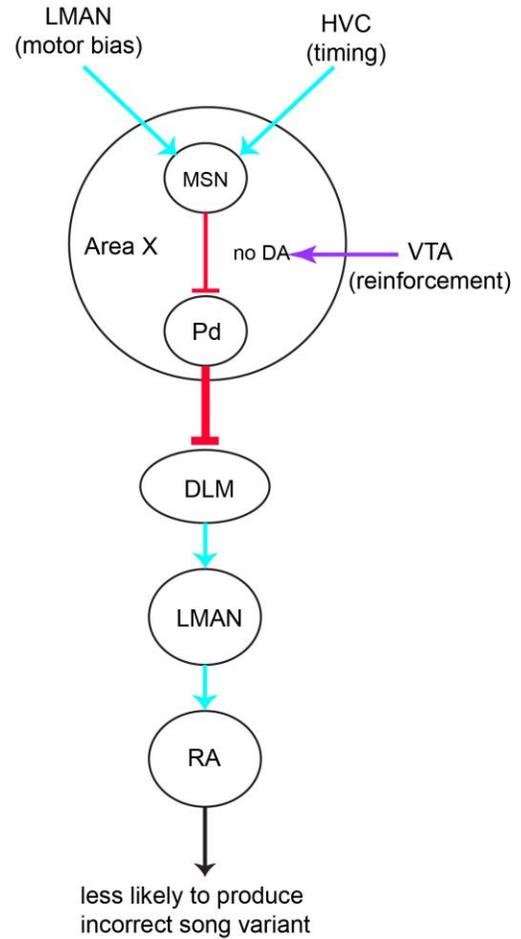


Figure 53: Hypothesized circuit activity during singing

Circuit diagram of activity propagating through the anterior forebrain pathway when the bird sings correct (left panel) or incorrect (right panel) variants of song. For correct variants, dopamine is released from the VTA into Area X (purple shading) while dopamine release is suppressed when the bird sings incorrect variants, eventually resulting in a strengthening or weakening in the LMAN-RA synapses that are needed to produce that particular variant. Red lines indicate inhibitory synapses, blue lines indicate excitatory synapses.

7.7 Future directions

The results of my thesis work are most exciting to me when I think of the future experiments they pave the way for. Yes, VTax cells are necessary for internally and externally reinforced learning but how, as a population, do they act to drive learning? How do their responses change over the course of learning in a juvenile bird? What inputs do VTax cells receive and what kind of signals do these multiple inputs convey? Below I will describe experiments that begin to address these exciting questions.

7.7.1 Population activity of VTax cells during learning

While single unit recordings of antidromically identified VTax cells have been made during random distorted auditory feedback, the activity of these cells individually and as a population during learning is unknown, in part because of the technical challenges of “holding” a cell over the course of learning. The advent of fiber photometry allows for the long term imaging of activity of a specific population of cells. Though this technique lacks single cell resolution, it appears to be a more tenable first approach to imaging structures deep brain structures as it causes minimal tissue compression and damage.

With this technique, the activity of VTax cells could be monitored over learning to gather information for how the activity of the population changes as young birds learn to copy the tutor song. This could reveal whether or not, as a whole, VTax cells encode a reward prediction error and more generally, if reward prediction errors are

used in learning a naturalistic, internally guided skill. Though decidedly more challenging, one photon deep brain imaging could then determine if VTAx cells are homogeneous in the signals they encode or if, as seen in mammals, different cells signal inverse reward prediction errors or alertness or novelty.

7.7.2 Exploring inputs to the VTA

The VTA receives two inputs that are known components of the song system; Aiv, a large swath of the arcopallium that receives auditory inputs from across the cortex (Mandelblat-Cerf et al., 2014), and the ventral pallidum (VP), which is an intermediary in the disinhibitory loop between Area X and VTA (Gale & Perkel, 2010). Single unit recordings in antidromically identified VTA-projecting Aiv cells suggest that these cells encode an error signal as they are active only when noise is played to the bird during singing but not when noise is played back to the bird or when noise is imposed on playback of the bird's own song (Mandelblat-Cerf et al., 2014). In mammalian systems VP has long been known as a "pleasure center"; enriched with a variety of different opioid receptors, animals implanted with stimulating electrodes in VP will repeatedly self-stimulate (Smith et al., 2009). Thus these two areas may provide negative (Aiv) and positive (VP) signals to the VTA, which then presumably integrates the signals and releases dopamine if the produced motor variant is deemed to be correct by the two upstream structures (Figure 54).

Experiments in which channelrhodopsin-expressing Aiv or VP terminals in the VTA are stimulated with light in a pitch contingent manner could reveal not only the valence of each input but also whether these inputs act on different timescales. These experiments could also determine whether the inputs positively or negatively reinforce song more generally. By varying the time window of stimulation to either slightly before the targeted syllable or in the middle of the syllable during white noise pitch learning one could also determine whether the stimulated terminals carry a premotor or auditory feedback signal, respectively.

Given that these two areas receive a variety of inputs that are not only from the song system, it will be interesting to determine if they are involved in directing other behaviors than song. Conditioned place preference experiments or two-choice key peck experiments in which birds receive stimulation when they are on a particular side of the cage or if they peck the appropriate key could reveal whether terminal activation of either input could drive reinforcement of behaviors other than singing. Imaging retrogradely labeled Aiv or VP cell that project to the VTA with fiber photometry in young birds learning to sing and also learning an operant task could reveal whether activity differed from singing versus task learning.

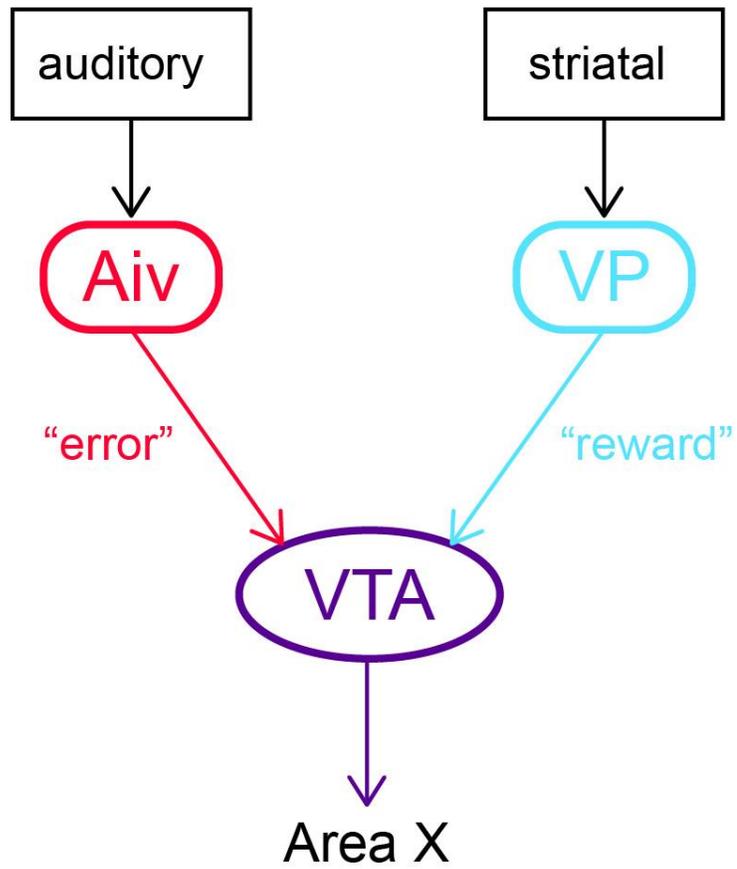


Figure 54: Schematic of inputs to the VTA.

Appendix A

Here I will include the summary and experimental section of a manuscript I co-authored that has just now been accepted. The manuscript is titled “A novel cell type mediates motor to auditory interactions important for vocal learning”. The first two years of graduate school I worked closely with a former postdoctoral associate, Todd Roberts, to characterize the structure and function of a novel cell type that we found to be necessary to vocal learning and vocal timing. Masashi Tanaka, Matthew Kearney and Gaurav Chattree also performed experiments included in this manuscript. I have highlighted my contributions to the manuscript in bold font, presented below.

Summary

Learning to vocalize depends on the ability to adaptively modify the duration and sequencing of simpler vocal elements. Neurons that convey motor-related signals to the auditory system are theorized to facilitate this form of learning, but the identity and function of such neurons remain unknown. We identified a novel neuron type in the songbird that transmits vocal motor signals to the auditory cortex. Genetically ablating these neurons in juveniles disrupted their ability to imitate features of an adult bird’s song. Ablating these neurons in adults had little effect on previously learned songs, but interfered with their ability to adaptively modify the duration of vocal elements and largely prevented the degradation of song’s temporal features caused by

deafening. These findings identify a motor to auditory circuit essential to vocal learning and the adaptive modification of vocal timing.

Results

A novel neuron type connects the vocal motor system to the auditory system

To identify the neurons that extend axons from HVC to Av, we injected different color fluorescent retrograde tracers into Av, RA, and Area X, which were identified using a combination of stereotaxic coordinates and multi-unit electrophysiological recordings (Fig. 1a-b, n = 7 adult birds (> 100 days post hatch), n = 3 juvenile birds (60 days post hatch), Av (dextran, Alexa Fluor 594), RA (dextran, Alexa Fluor 488), and Area X (fast blue); all three regions were accurately targeted in 70% of cases, 9/14 hemispheres in adult birds, 5/6 hemispheres in juvenile birds). All injections that successfully targeted Av, as determined by retrograde label in two of its other known afferents (the thalamic nucleus uvaefomis (Uva) and NIf), also retrogradely labeled a sparsely distributed population of neurons in HVC. In contrast, injections targeting regions immediately dorsal or ventral to Av did not result in retrograde labeling in HVC in either juvenile or adult animals (n = 6 hemispheres from 6 animals). Notably, HVC neurons that were labeled by tracer injections in Av were not co-labeled by tracers injected either in Area X or RA (Fig. 1b; n = 14 hemispheres from 10 animals). These findings are consistent with the

notion that the axonal projection from HVC to Av arises from a previously unrecognized and distinct PN type (i.e., the HVC_{Av} neuron) and not from the axon collaterals of HVC_{RA} or HVC_X neurons.

To better characterize HVC_{Av} neuronal morphology, we injected fluorescent retrograde tracers or a retrogradely transported GFP-expressing virus (AAV2/9-GFP) into Av in a separate cohort of male zebra finches (n = 20 birds). Confocal analysis of labeled cells in fixed tissue revealed that HVC_{Av} neuronal morphology is characterized by stellate dendrites with sparsely distributed spines, a large diameter cell body, and an axon that exits HVC along its rostral – ventral border (Fig. 1c, radial extent: $140.21 \pm 4.98 \mu\text{m}$, n = 28 cells from 3 birds; 0.16 spines/ μm , n = 460 spines across 2929 μm of dendrite, measured from 10 cells in 2 birds; cell body diameter: $21.19 \pm 0.842 \mu\text{m}$, n = 28 cells from 3 birds). The HVC_{Av} cells have roughly the same cell body diameter as HVC_X and fall in between HVC_X and HVC_{RA} cells in terms of radial extent (Mooney, 2000). Cell counts indicate that HVC_{Av} neurons constitute only a very small percentage of HVC PNs (130 ± 69.8 neurons/hemisphere, n = 26 hemispheres from 17 birds; $\leq 1\%$, based on estimates of total HVC neuron numbers that vary between 50,000 and 100,000 per hemisphere (Alvarez-Buylla, Kirn, & Nottebohm, 1990; Wild, Williams, Howie, & Mooney, 2005), and appear to be evenly distributed throughout HVC, resulting in a tiled appearance in confocal images (Fig. 1b). Unlike HVC_X and HVC_{RA} neurons, which elaborate extensive axonal collaterals within HVC, we did not

find anatomical evidence that HVC_{Av} extend axon collaterals within HVC. Therefore, HVC_{Av} neurons may function solely to transmit activity from HVC to Avalanche.

Morphological differences between neuron types often are paralleled by physiological differences that correspond to their different functional roles (Dutar, Petrozzino, Vu, Schmidt, & Perkel, 2000; Mooney, 2000). Using visually targeted whole-cell current clamp recordings in brain slices, we found that HVC_{Av} neurons exhibited no spontaneous action potential activity but fired action potentials at high rates with little spike rate accommodation in response to positive current pulses (Fig. 1d). The intrinsic electrophysiological properties of HVC_{Av} neurons were distinct from those of HVC_X or HVC_{RA} neurons in several respects, including spike adaptation, spike gain, input resistance, and the amplitude of hyperpolarization-activated cation currents (I_h) (Fig. 1d-h). Taken together, our findings demonstrate a novel HVC_{Av} PN type that is morphologically distinct and whose electrical properties may also be capable of sustaining higher firing frequencies in response to synaptic drive than the other two types of HVC PNs, which fire sparsely during song.

HVC_{Av} neurons transmit motor-related signals during song production

To begin to characterize the functional properties of HVC_{Av} neurons, we monitored the activity of small populations of HVC_{Av} neurons in freely singing adult zebra finches using *in vivo* calcium imaging methods. We selectively expressed genetically encoded calcium indicators in HVC_{Av} neurons by injecting a retrogradely

transported virus (AAV2/9-CAG-GCaMP6s; n = 3 birds) into Av or, in another bird, by injecting a Cre-dependent virus (AAV2/1-FLEX-CAG-GCaMP6s) into HVC and a retrogradely transported Cre-recombinase containing virus (AAV2/9-CMV-Cre) in Av. A 1 mm diameter GRIN lens was implanted over HVC and calcium transients were detected using a miniaturized head-mounted fluorescent microscope (Fig. 2a, b; n = 4 adult birds). We observed significant increases in neuronal calcium signals associated with song production and little or no changes in fluorescence when birds were not vocalizing (49/49 songs in 4 birds showed a greater than 2 S.D. increase in fluorescence over baseline in relation to song onset, Fig. 2c; Supplementary Fig. 1). During singing bouts, HVC_{Av} neurons exhibited significant increases in activity prior to song onset (135.74 +/- 54.93 milliseconds before song onset for 49 songs from 4 birds where activity onset is defined as the time point at which delta F/F increases 2 S.D. above baseline). Although individual neurons displayed slightly different temporal patterns of activity, their activity patterns were consistently dependent on singing and exhibited stereotyped profiles from one motif to the next (Supplementary Fig. 1). In addition, region of interest (ROI) analysis revealed that bulk changes in fluorescence from HVC were able to capture song-locked activity patterns, including significant increases in activity prior to song onset and stereotyped activity patterns locked to individual motifs (Fig. 2d).

The pattern of increased activity prior to song onset is consistent with the idea that HVC_{Av} neurons convey a motor-related signal. To further test this idea, we imaged

singing-related activity in HVC_{Av} neurons in a subset of birds before and after deafening (**n = 2 birds; deafening was achieved by bilateral removal of the basilar papilla**). We found that singing-related activity of HVC_{Av} neurons persisted after deafening (29/30 songs in 2 birds showed a greater than 2 S.D. increase in fluorescence over baseline in relation to song onset) and displayed patterns that were qualitatively similar to those measured previously with intact hearing, including activity prior to song onset (112.11 +/- 61.72 milliseconds before song onset for 30 songs from 2 birds where activity onset is defined as the time point at which delta F/F increases 2 S.D. above baseline, Fig. 2e). These findings support the idea that HVC_{Av} neurons transmit motor-related activity during song production.

HVC_{Av} neurons are selectively innervated by song premotor neurons

A variety of evidence indicates that HVC_{RA} neurons are a source of premotor signals that contribute to song timing and that HVC_X neurons convey song motor-related information to the basal ganglia. As local axon collaterals of both HVC_{RA} and HVC_X neurons form excitatory synapses with other HVC neurons (Kosche, Vallentin, & Long, 2015; Mooney & Prather, 2005), either or both neuron types could provide motor-related information to HVC_{Av} neurons. To clarify which of these PN types provide synaptic input onto HVC_{Av} neurons, we made visually targeted whole-cell recordings from **retrogradely labeled HVC_{Av} neurons** in brain slices and antidromically stimulated HVC_{RA} and HVC_X axon collaterals (Fig. 3). Antidromic stimulation of HVC_{RA} axon

collaterals routinely elicited excitatory and inhibitory synaptic currents in HVC_{AV} neurons (Fig. 3a, left inset; n = 10/11 cells; IPSC: 9/11 (Supplementary Fig. 2); no input: 1/11; 5 birds). In contrast, antidromic stimulation of HVC_X cells never evoked a synaptic response of any kind in HVC_{AV} neurons (Fig. 3a, right inset; n = 0/8 cells; 4 birds), even though the same stimulation could elicit antidromic spikes in HVC_X neurons (Fig. 3b, right inset; n = 2/5 cells; 2 birds) and also readily evoked synaptic responses in both HVC_{RA} (Fig. 3b, left inset; EPSC: n = 2/5 cells; IPSC: n = 2/5 cells; no input: n = 1/5; 3 birds) and HVC_X neurons (Supplementary Fig. 2; IPSC: n = 3/5 cells; no input: n = 0/5 cells; 2 birds). Furthermore, although axons from two of HVC's afferents, the thalamic nucleus Uva and the telencephalic nucleus interface (Nif), enter HVC along its rostroventral border, electrical stimulation along this border also failed to evoke synaptic responses in HVC_{AV} neurons (Supplementary Fig. 2; n = 0/8 cells; 4 birds). Therefore, HVC_{AV} neurons are specifically positioned within the HVC microcircuit to convey premotor information important to song timing from HVC_{RA} neurons to the auditory system.

Genetically ablating HVC_{AV} neurons in juveniles disrupts song learning

Juvenile male zebra finches memorize a tutor song between 20 and 60 days after hatching and then copy the syllables and syllable sequences of this memorized song model in a process of sensorimotor learning that spans from 45 to 90 days (L. A. Eales, 1985; Immelmann, 1969). To determine the role of HVC_{AV} neurons in song learning, we

used an intersectional genetic method (Yang et al., 2013) to selectively ablate these cells in juvenile birds after they had an opportunity to memorize a tutor song, but before they had formed an accurate copy of the tutor song model (Fig. 4a, b). We injected a virus expressing a Cre-dependent form of caspase 3 (AAV2/1-FLEX-taCasp3-TEVp), which triggers cellular apoptosis (Yang et al., 2013), into HVC and injected a retrogradely transported virus expressing Cre-recombinase (AAV2/9-CMV-Cre) into Av of juvenile male zebra finches (Fig. 4a,b; n = 5 male zebra finches; 35-40 days at the time of the viral injection; viral expression requires 2-4 weeks (see Methods)). Siblings that received either no virus injections or that received only injections of AAV2/1-FLEX-taCasp3-TEVp into HVC served as control groups. To quantify the effects of this genetic lesioning method we injected a retrograde tracer into Av in adult birds that had been subjected to intersectional HVC_{Av} lesions as juveniles and quantified the number of surviving HVC_{Av} neurons. Cell counts confirmed that the intersectional expression of Cas3 in HVC_{Av} neurons of juveniles significantly reduced the adult number of HVC_{Av} neurons compared to either of two control groups (Fig. 4c; the two control groups had similar numbers of HVC_{Av} neurons to each other and thus were subsequently treated as a single group).

All birds were raised to adulthood and their adult songs were compared to their tutors' songs to estimate the quality of copying (see Methods). Genetically ablating HVC_{Av} neurons in juveniles severely disrupted their ability to copy a tutor song (Fig. 4

d-e; $n = 5$ experimental birds, 46.3% similarity to tutor song; $n = 8$ control siblings, 72.9% similarity; Mann-Whitney $U = -3.8$, $Z = 2.56$, $P = 0.01$). Within-bird comparisons indicated that similarity to the tutor song correlated with the number of surviving HVC_{Av} neurons (Fig. 4 f-h). Juveniles were able to copy at least some syllables from their tutor (Fig. 4e and g, Supplementary Fig. 3, 4/5 birds copied at least one syllable from their tutor, with syllable copying determined by >70% accuracy, see Methods), suggesting that reducing the numbers of HVC_{Av} neurons does not block the capacity or motivation to imitate individual syllables. Nonetheless, as a group lesioned birds failed to faithfully imitate the song of their tutor, indicating that HVC_{Av} neurons play an important role in tutor song learning.

A remaining issue is whether the behavioral effects we detected were specifically related to ablation of HVC_{Av} neurons, and not to incidental cell death of other HVC neurons. In a separate group of birds, we tested the possibility that the intersectional methods used to target HVC_{Av} cells also kill HVC_X cells, some of which extend axons through or near Av en route to the basal ganglia and might plausibly take up the AAV 2/9 Cre virus from the injection site in Av. However, we found that the number of HVC_X neurons was unaffected by the intersectional method we used to kill HVC_{Av} neurons (Supplementary Fig. 4; $n = 2$ birds; see Methods). Therefore, deficits in song copying likely arise from the selective loss of HVC_{Av} neurons, reinforcing the idea that this cell type plays an important role in song learning.

Ablating HVC_{Av} neurons protects song's temporal features in deafened adults

Adult zebra finches use auditory feedback to maintain stable songs, as revealed by peripheral deafening, which triggers spectral degradation of individual syllables and also destabilizes syllable sequences (Brainard & Doupe, 2000a; K. W. Nordeen & Nordeen, 1992) over a period of days to several weeks (Tschida & Mooney, 2012). Furthermore, much of the neural circuitry implicated in song copying in juveniles also plays a role in feedback-dependent song maintenance in adults. For example, lesions placed in LMAN, a premotor output of a cortico-basal ganglia pathway important to juvenile song learning, have little effect on adult song performance but largely prevent the degradation of an adult song's spectral and temporal features that deafening normally triggers (Brainard & Doupe, 2000a, 2000b; Lombardino & Nottebohm, 2000). To test whether HVC_{Av} neurons are also important to song maintenance, we first selectively ablated HVC_{Av} neurons in adult zebra finches and recorded their songs for six to ten weeks. We found that birds continued to sing highly stereotyped songs following ablation of HVC_{Av} neurons (Fig. 5a, b; two-sample $t(22) = -1.4$, $P = 0.16$ for HVC_{Av}-lesioned birds ($n = 12$) versus HVC_{Av} intact birds ($n = 12$)). Although we did find a slight, yet significant decrease in song self-similarity and motif duration (Supplementary Fig. 5), the trial-to-trial variability in spectral features of individual syllables and timing of inter-syllable gaps were unaffected by bilateral ablation of HVC_{Av} neurons (syllable frequency: two-sample paired $t(4) = 0.2$, $P = 0.8$; inter-syllable gaps: two-sample paired $t(4) = 1.3$, $P = 0.27$; $n = 5$

birds in each group). These findings indicate that a normal complement of HVC_{Av} neurons is not critical to adult song production or to song maintenance in the presence of normal auditory feedback. In contrast, unilateral HVC lesions, made by injecting AAV2/1-FLEX-taCasp3-TEVp and AAV2/9-CMV-Cre into HVC, disrupted song production in a manner similar to the effects of unilateral lesions of HVC (Williams, Crane, Hale, Esposito, & Nottebohm, 1992). In summary, in adult birds with intact hearing, selectively ablating HVC_{Av} neurons exerted little effect on acute song performance, trial-to-trial song variability, or longer-term song maintenance.

We then deafened a cohort of adult male zebra finches in which we had previously bilaterally ablated HVC_{Av} neurons and also deafened another cohort of animals in which HVC was left intact ($n = 7$ animals in each group). Visual inspection of sonograms indicated that the songs of birds with HVC_{Av} lesions remained more stable following deafening than the songs of deafened birds with an intact HVC (Fig. 5c). To better characterize the nature of these prophylactic effects, we calculated differences in syllable transition matrices before and 10 weeks after deafening to detect temporal changes and we used a self-similarity score to detect spectral changes (Fig. 5d, e; see Methods). Selectively ablating HVC_{Av} neurons largely prevented deafening-induced destabilization of syllable sequences, as revealed by lower difference matrix scores in deafened birds with HVC_{Av} lesions (Fig. 5d, e) (two-sample $t(12) = -4.8$, $P = 0.0004$ for changes in song syllable transition matrix before and

after deafening from lesioned ($n = 7$) versus intact birds ($n = 7$)). In contrast, HVC_{Av} lesions did not seem to prevent spectral degradation of syllables following deafening, as self-similarity scores before and 10 weeks after deafening were similar in the experimental and control groups (Fig. 5e; two-sample $t(12) = 1.9$, $P = 0.07$ comparing self-similarity scores from HVC_{Av} - lesioned versus control birds). Indeed, in birds with HVC_{Av} lesions, within-syllable comparisons before and after deafening revealed significant increases in entropy, a measurement that is highly sensitive to song's spectral features (Fig. 5f; Supplementary Fig. 5; two-sample $t(11) = -4.0$, $P = 0.0019$ for changes in entropy measured in single syllables in HVC_{Av} - lesioned birds before and 10 weeks after deafening). These findings indicate that ablating HVC_{Av} neurons protects temporal but not spectral features of song from deafening-induced degradation, consistent with the idea that these neurons serve a specialized role in mediating feedback-dependent changes to song timing.

A specialized role for HVC_{Av} neurons in adult plasticity of song timing

To further explore the role of HVC_{Av} neurons in feedback-dependent song plasticity, we used singing-contingent playback of white noise to induce adult birds to modify either the spectral (i.e., fundamental frequency, or "pitch") or temporal (i.e., song element duration) features of their songs before and after we bilaterally ablated HVC_{Av} neurons (Ali et al., 2013). These experiments exploit slight trial-to-trial variations in syllable pitch and song element timing: targeting white noise playback to pitch or

timing variants that fall above or below a user-set threshold drives the bird to adaptively modify pitch or timing, subsequently reducing the number of renditions that trigger noise playback. Notably, adult finches can independently modify either syllable pitch or song element duration using these protocols and also rapidly recover their original pitch and timing once noise playback is discontinued (Ali et al., 2013; Tumer & Brainard, 2007).

We measured rates of pitch and song element duration learning and subsequent recovery before and after genetic ablation of HVC_{Av} neurons. We found that rates of pitch learning and recovery did not differ before and after genetic ablation of HVC_{Av} neurons (Fig. 6a-c, $n = 5$ birds). In contrast, almost all (4/5) birds in which HVC_{Av} neurons had been ablated displayed much slower rates of song element duration learning and all (5/5) were severely impaired in their ability to recover their original song element timings after noise playback was discontinued (Fig. 6d-f). Taken together with the results of adult deafening studies, these findings indicate that HVC_{Av} neurons play a specialized role in the feedback-dependent modification and recovery of song timing in adult zebra finches.

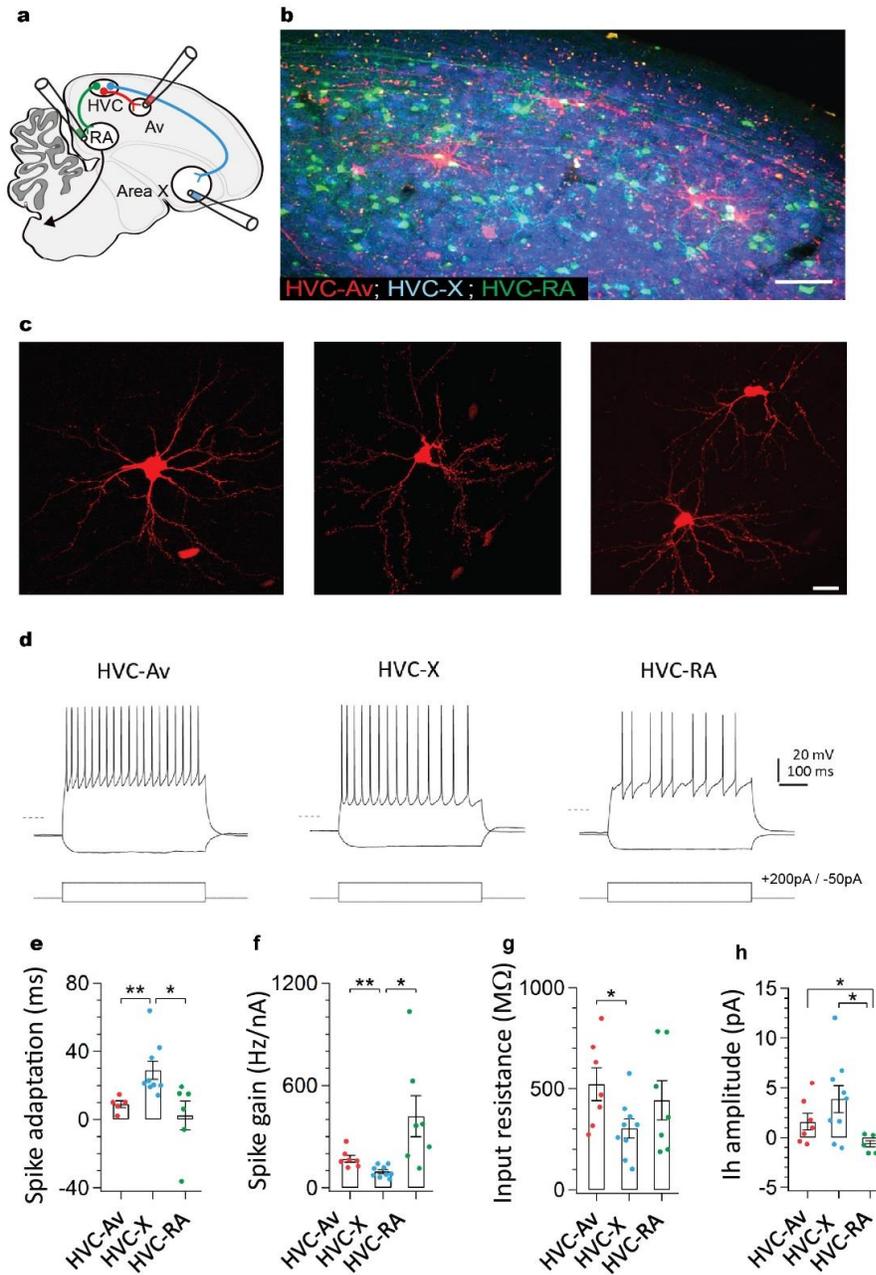


Figure 1. A novel cell type links the song premotor nucleus HVC to the auditory region Avalanche. **a)** Schematic showing the design of retrograde labeling experiments. Different fluorescent retrograde tracers were injected into Av (dextran, Alexa Fluor 594), RA (dextran, Alexa Fluor 488), and Area X (fast blue). **b)** Parasagittal section through

retrogradely labeled HVC (left panel) reveals that neurons projecting into Avalanche (red) are distinct from neurons projecting to Area X (blue) or RA (green). Inset shows magnified image outlined by white box. **c**) Images of individual HVC_{Av} neurons retrogradely labeled by AAV2/9 GFP injection into Av. **d**) Visualized whole-cell current clamp recordings from retrogradely-labeled HVC projection neurons in brain slices in response to 500ms current injection (-50 and +200 pA; dashed line = -80mV). HVC_{Av} neurons differ from HVC_X neurons in their intrinsic properties, including their **e**) spike adaptation rate (two-sample $t(10) = 3.5$, $P = 0.005$ for HVC_{Av} neurons ($n = 5$) versus HVC_X neurons ($n = 9$); two-sample $t(9) = 2.6$, $P = 0.02$ for HVC_X neurons ($n = 9$) versus HVC_{RA} neurons ($n = 6$)), **f**) spike gain (two-sample $t(10) = 3.3$, $P = 0.007$ for HVC_{Av} neurons ($n = 7$) versus HVC_X neurons ($n = 9$); two-sample $t(6) = 2.7$, $P = 0.03$ for HVC_X neurons ($n = 9$) versus HVC_{RA} neurons ($n = 7$)), **g**) input resistance (two-sample $t(10) = 2.3$, $P = 0.04$ for HVC_{Av} neurons ($n = 7$) versus HVC_X neurons ($n = 9$)). **h**) HVC_{Av} neurons differ from HVC_{RA} neurons in their I_h amplitude (two-sample $t(7) = 2.5$, $P = 0.04$ for HVC_{Av} neurons ($n = 7$) versus HVC_{RA} neurons ($n = 7$); two-sample $t(9) = 3.2$, $P = 0.01$ for HVC_X neurons ($n = 9$) versus HVC_{RA} neurons ($n = 7$)). Scale bar, 100 μ m in B (left panel) and 15 μ m in B (right panel). Scale bar, 100 μ m in B and 15 μ m in C.

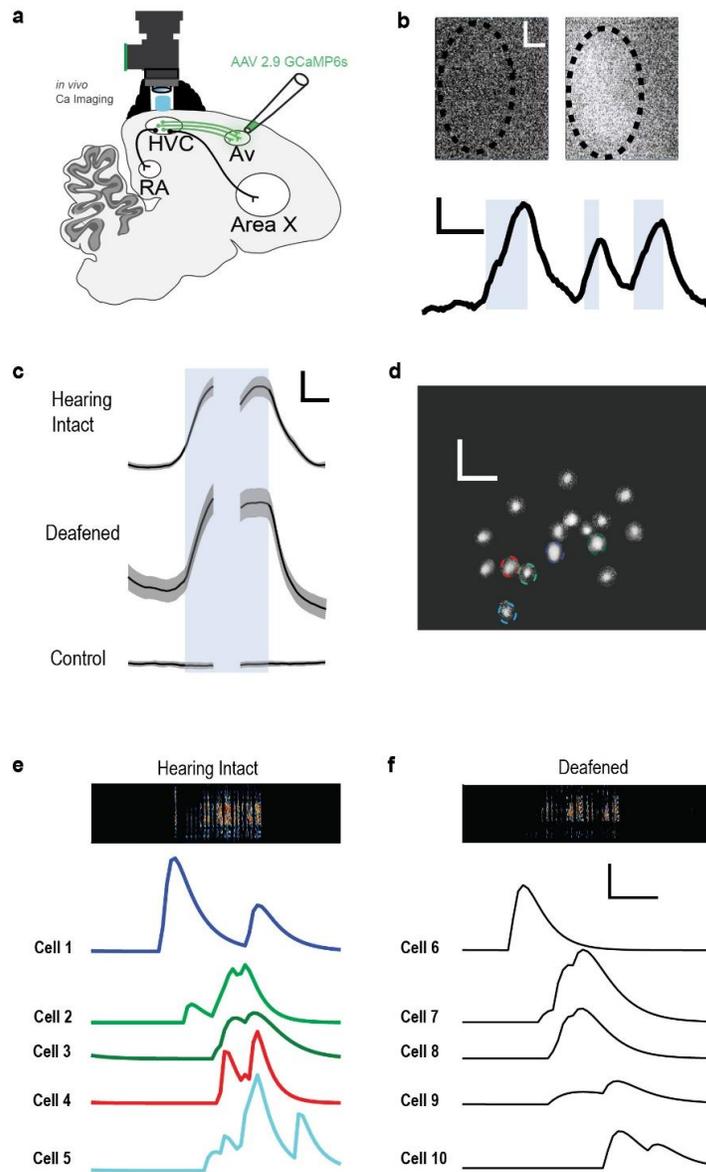


Figure 2: HVC_{Av} neurons transmit motor-related signals during song production. a) Schematic of imaging experiments employing a miniature epifluorescence microscope (Inscopix) for *in vivo* calcium imaging of HVC_{Av} neurons expressing GCaMP6s. b) Bulk fluorescent signal measured in HVC at rest (top left) and during singing (top right). Bottom panel shows bulk change in fluorescent signal during singing of multiple song bouts (song onset to song offset for each bout indicated by blue shading). Scale bars, 100 μm \times 100 μm (top panels) and 1% and 2s (bottom panel). c) Average change in bulk fluorescence signals measured during singing in birds expressing GCaMP6s in HVC_{Av} neurons with their hearing intact (top panel, n=4) and

after deafening (middle panel, n=2), and in a bird not injected with GCaMP (bottom panel, n=1). Onset and offset of song bout indicated by blue shading. Scale bars, 2% and 1s. **d)** Example field of GCaMP-labelled HVC_{AV} neurons imaged through miniature microscope in a singing zebra finch. Putative individual cells, which were identified with CNMF analysis, are indicated by colored outlines. Scale bars, 100 μm \times 100 μm . **e)** Activity of five putative HVC_{AV} neurons indicated in panel d) during a single song bout comprising two motifs (sound spectrogram at top; color in the sound spectrogram indicates relative power with black indicating the lowest power (during silent intervals) and red indicating the highest relative power within the vocal range). **f)** Singing related activity of five different putative HVC_{AV} neurons from the same bird from which d) and e) were collected but several days after deafening; activity is aligned to a single bout comprising two motifs. Scale bars in e) and f), 20 arbitrary activity units and 500 ms.

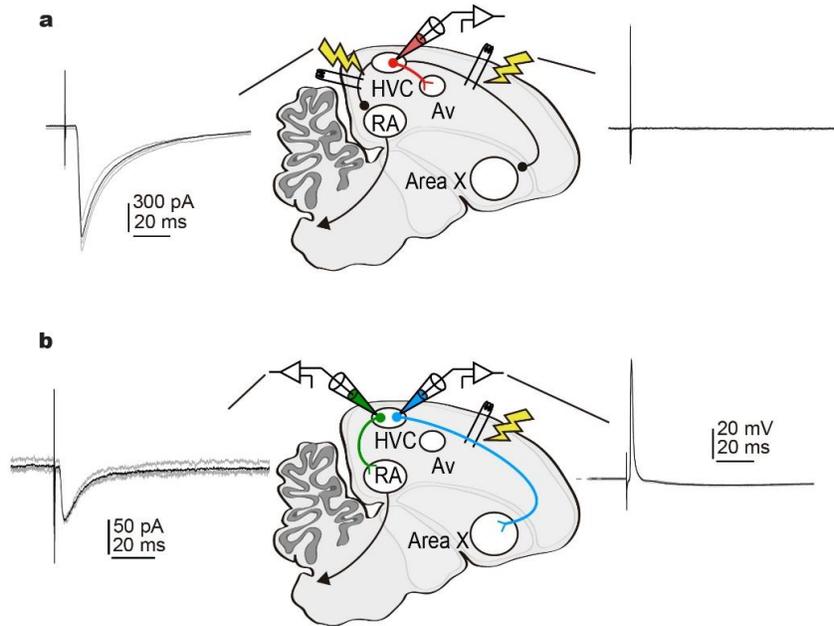


Figure 3. HVC_{Av} neurons receive selective input from premotor HVC_{RA} neurons.

a) Schematic of *in vitro* brain slice preparation in which whole cell voltage clamp recordings were made from retrogradely-labeled HVC_{Av} neurons while either HVC_{RA} or HVC_X axons were electrically stimulated to evoke antidromic propagation of activity into the HVC local circuit (HVC_{RA} : 40 - 80 μA ; HVC_X axons: 40 - 300 μA). Left inset, excitatory synaptic currents evoked in an HVC_{Av} neuron in response to HVC_{RA} axon bundle stimulation (80 μA). Right inset, absence of synaptic currents recorded in the same HVC_{Av} neuron following HVC_X axon bundle stimulation (160 μA ; $V_h = -70$ mV in both examples). **b)** Schematic of whole cell voltage and current clamp recordings made from retrogradely labeled HVC_{RA} or HVC_X cells while electrically stimulating HVC_X axon bundles. Left inset, excitatory synaptic currents evoked in an HVC_{RA} cell following HVC_X axon bundle stimulation (80 μA ; $V_h = -70$ mV). Right inset, antidromic action potential evoked in an HVC_X cell following HVC_X axon bundle stimulation (80 μA ; $V_{rest} = -80$ mV). In all inset panels, individual currents or potentials are shown in gray and mean current or potential is shown in black.

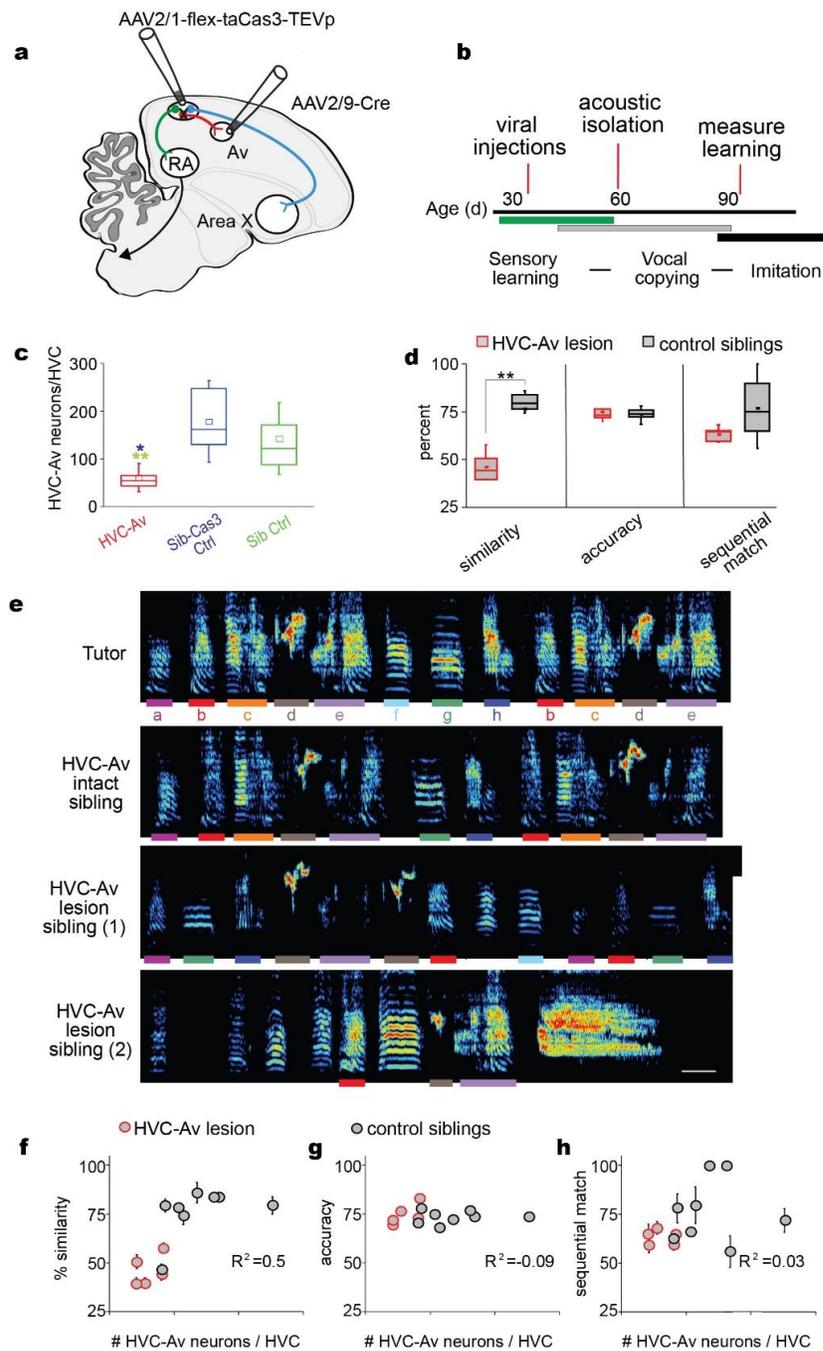


Figure 4. Intersectional genetic ablation of HVC_{Av} neurons in juvenile zebra finches impairs their ability to copy a tutor song. **a)** Schematic showing viral strategy for genetically ablating HVC_{Av} neurons with a Cre-dependent form of caspase 3 (Cas3). **b)** Timeline of experiments. Viral injections of AAV2/1-FLEX-taCas3-TEVp into HVC

and AAV2/9-Cre-GFP into Av are made in juvenile male zebra finches between 35-40 days posthatch (dph). Birds are separated from their parents and other siblings starting at 60 dph and the quality of song copying is measured at >90 dph, when song copying is complete. **c**) The number of retrogradely labeled HVC_{Av} neurons (HVC_{Av} neurons per hemisphere) in adult birds that were injected as juveniles with both AAV2/1-FLEX-taCasp3-TEVp into HVC and AAV2/9-Cre-GFP into Av (red, HVC_{Av}) is significantly lower than the number of HVC_{Av} neurons found in adult siblings injected as juveniles only with AAV2/1-FLEX-taCasp3-TEVp into HVC (blue, Sib-Cas3 Ctrl) or adult siblings that were not injected with any virus (green, Sib Ctrl) (Mann-Whitney U(9) = 86, Z = -3.31, P = 0.0009 for HVC_{Av} lesioned (red) versus Sib-Cas3 (blue); Mann-Whitney U(16) = 16, Z = 3.44, P = .0006 for HVC_{Av} lesioned (red) versus Sib Ctrl (green)). **d**) Adult birds injected with both Cas3 and Cre at ~ 35-40 dph sang poorer copies of their tutors' songs compared to their control siblings (combined group of Sib-Cas3 and Sib Ctrl birds), as measured by song similarity% scores (see Methods) but show no difference to control siblings in measures of percent accuracy and sequential match percentage (see Methods). Tukey boxplots (**c** and **d**) show the first and third quartile of the data; the median, mean and 1.5 interquartile range are shown by the band, the dot and whiskers respectively. **e**) Example sound spectrograms from the tutor (top), from one adult pupil (control) with a normal complement of HVC_{Av} neurons, and from two adult siblings (siblings 1 and 2) in which HVC_{Av} neurons were genetically ablated during juvenile life. Color in the sound spectrogram indicates relative power with black indicating the lowest power (during silent intervals) and red indicating the highest relative power within the vocal range. Individual syllables in the tutor's song and the copied versions, defined by >70% accuracy scores, of these syllables in the pupil's adult songs are color coded by lines under each of the tutor's syllables. The control sibling's song is 78.5% similar to the tutor and this bird had 106.5 HVC_{Av} neurons per hemisphere (neurons averaged from the two hemispheres). Sibling 1's song is 50.6% similar to the tutor and this bird had 42.5 HVC_{Av} neurons, and sibling 2's song is 39.5% similar to tutor and this bird had 41.5 HVC_{Av} neurons. Scale bars: 100 msec, ordinate spans 0 - 10 kHz. Colored bars correspond to individual syllables copied from the tutor's song. **f**) Similarity of the adult bird's copy to its tutor's song plotted as a function of the average surviving numbers of HVC_{Av} neurons counted in the two hemispheres in each bird (R² = 0.5, P = 0.004, n = 13 birds). **g**) Accuracy of the adult song copy as a function of average number of HVC_{Av} neurons counted in the two hemispheres from each bird (R² = -0.09, P = 0.9, n = 13 birds). **h**) Sequential match of the adult copy to its tutor song as a function of average surviving number of HVC_{Av} neurons counted from the two hemispheres in each bird (R² = 0.03, P = 0.25, n = 13 birds).

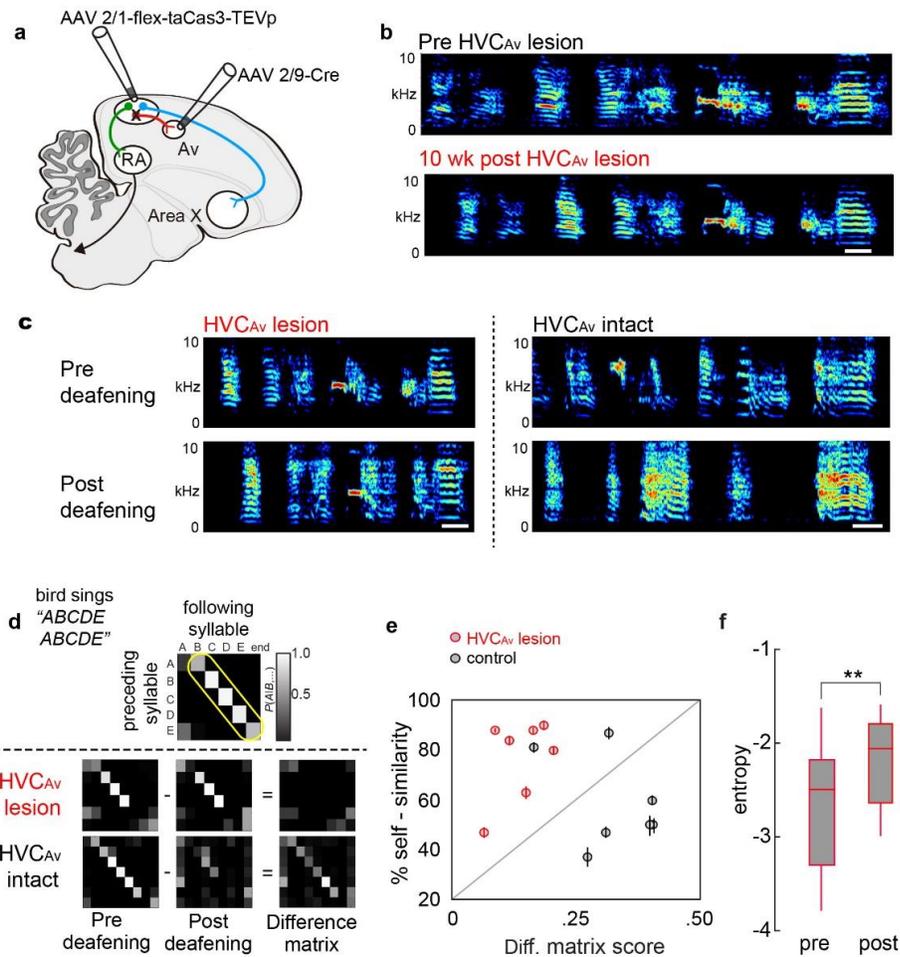


Figure 5. Intersectional ablation of HVC_{Av} neurons in adult birds does not disrupt song production but does attenuate deafening-induced degradation of song's temporal features. **a)** Schematic showing viral strategy for genetically ablating HVC_{Av} neurons with a Cre-dependent caspase 3 in adult birds. **b)** Sonograms recorded from an adult zebra finch before and 10 weeks after bilateral ablation of HVC_{Av} neurons reveal no evidence of song degradation (two-sample $t_{22} = -1.4$, $P = 0.16$ for comparisons in song self-similarity from HVC_{Av} lesioned birds ($n = 12$) versus control adult birds ($n = 12$ birds)). Color in the sound spectrogram indicates relative power with black indicating the lowest power (during silent intervals) and red indicating the highest relative power within the vocal range. **c)** Example sonograms recorded before and 10 weeks after deafening in adult zebra finches in which HVC_{Av} neurons had been previously ablated (*left*) or were intact (*right*). **d)** Song transition matrix scores, which serve as a primary measure of the linearity and stability of syllable sequences, are calculated by summing probability scores along the boxed region (*yellow* indicates core syllable transition

sequence before deafening) within the matrix. Subtracting the song matrix at 10 weeks post deafening from the pre-deafening song matrix reveals little change in syllable sequence linearity and stability in birds with bilateral HVC_{Av} lesions, whereas the difference matrix from deafened controls showed marked changes. **e)** Ablating HVC_{Av} neurons reduces the magnitude of changes to syllable sequences but not syllable features following hearing loss, as revealed by group data plotting difference matrix scores and self-similarity scores for syllables pre- and 10 weeks following deafening ($n = 7$ HVC_{Av} -lesioned birds, $n = 7$ HVC_{Av} intact birds; two-sample $t(12) = -4.8$, $P = 0.0004$ comparing difference matrix scores from lesioned versus intact birds; two-sample $t(12) = 1.9$, $P = 0.07$ comparing self-similarity scores from lesioned versus intact birds). **f)** Within-syllable comparisons reveal a significant increase in entropy before and 10 weeks after deafening in HVC_{Av} -lesioned birds (two-sample $t(11) = -4.0$, $P = 0.0019$).

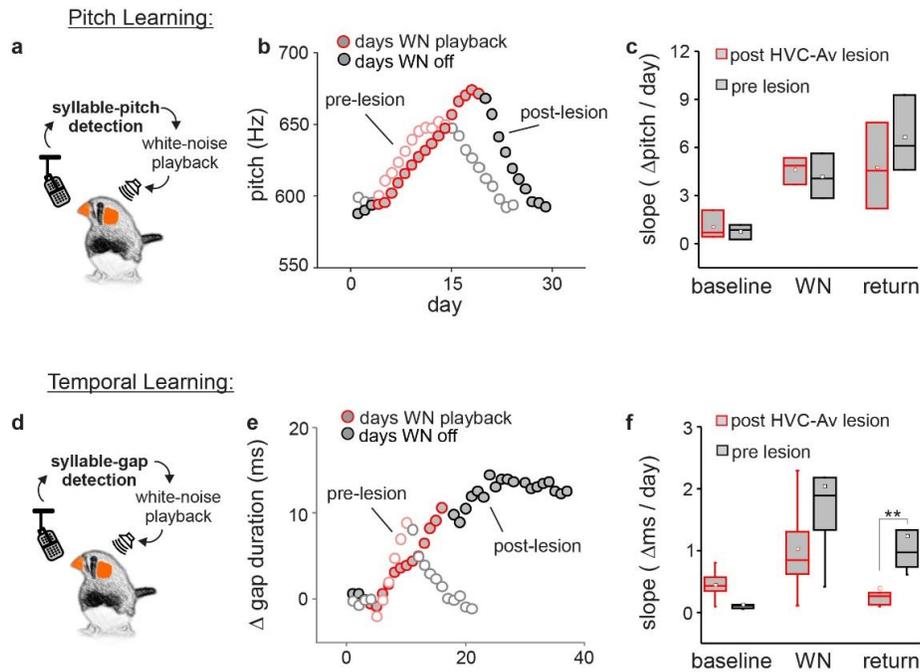
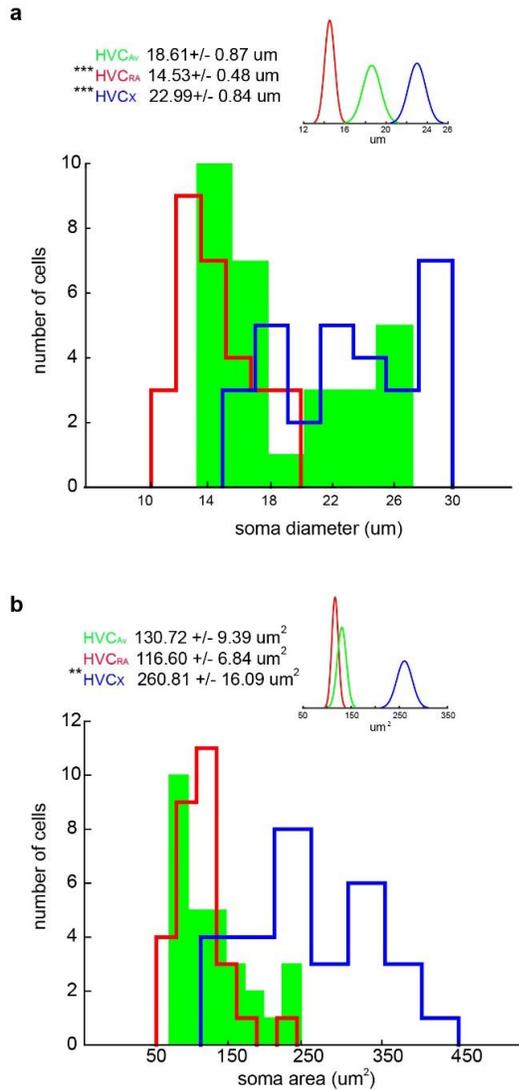
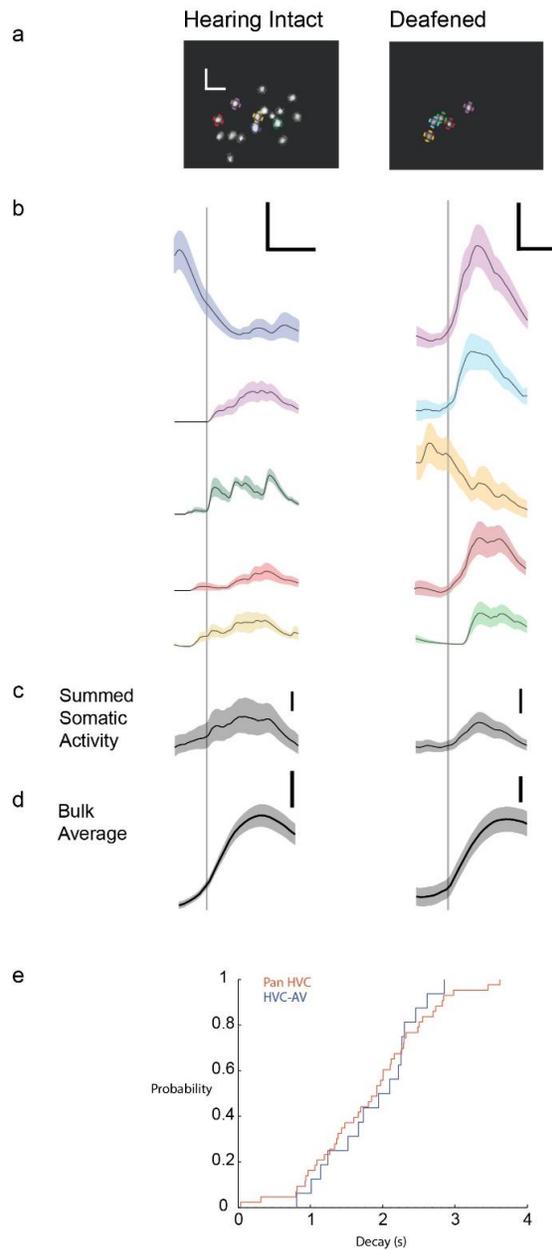


Figure 6. Intersectional ablation of HVC_{Av} neurons in adult zebra finches interferes with feedback-dependent plasticity of song element timing but not syllable pitch. **a)** Schematic of pitch learning paradigm. **b)** Syllable pitch (i.e., fundamental frequency) measured during pitch-contingent playback of white noise (WN; red) and subsequent recovery of original pitch after noise was discontinued measured in an adult male zebra finch before (unfilled circles) and after (grey filled circles) genetic ablation of HVC_{Av} neurons (average standard deviation of pitch during baseline, WN and recovery days was 9.2Hz, 9.2Hz and 10.6Hz pre-lesions and 13.0Hz, 10.9Hz and 11.3Hz post-lesion). **c)** Change in syllable pitch per day (Hz/day) before, during, and after WN playback across birds before (black) and after (red) genetic ablation of HVC_{Av} neurons. **d)** Schematic of song element duration learning paradigm. **e)** Change in song element timing in response to song element duration-contingent WN playback (red) and subsequent recovery of timing measured in an adult male zebra finch before (unfilled circles) and after (grey filled circles) genetic ablation of HVC_{Av} neurons (average standard deviation of song element duration during baseline, WN and recovery days was 3.9ms, 4.1ms and 3.7ms pre-lesions and 3.3ms, 3.8ms and 5.2ms post-lesion). **f)** Change in song element duration per day (ms/day) before, during, and after WN playback across birds before (black) and after (red) genetic ablation of HVC_{Av} neurons reveals a significant disruption in recovery of song timing ($n = 5$ birds, paired t-test $t(4) = 4.8$, $P = 0.008$). Tukey boxplots (**c** and **f**) show the first and third quartile of the data, the median, mean and 1.5 interquartile range are shown by the band, the dot and whiskers respectively.

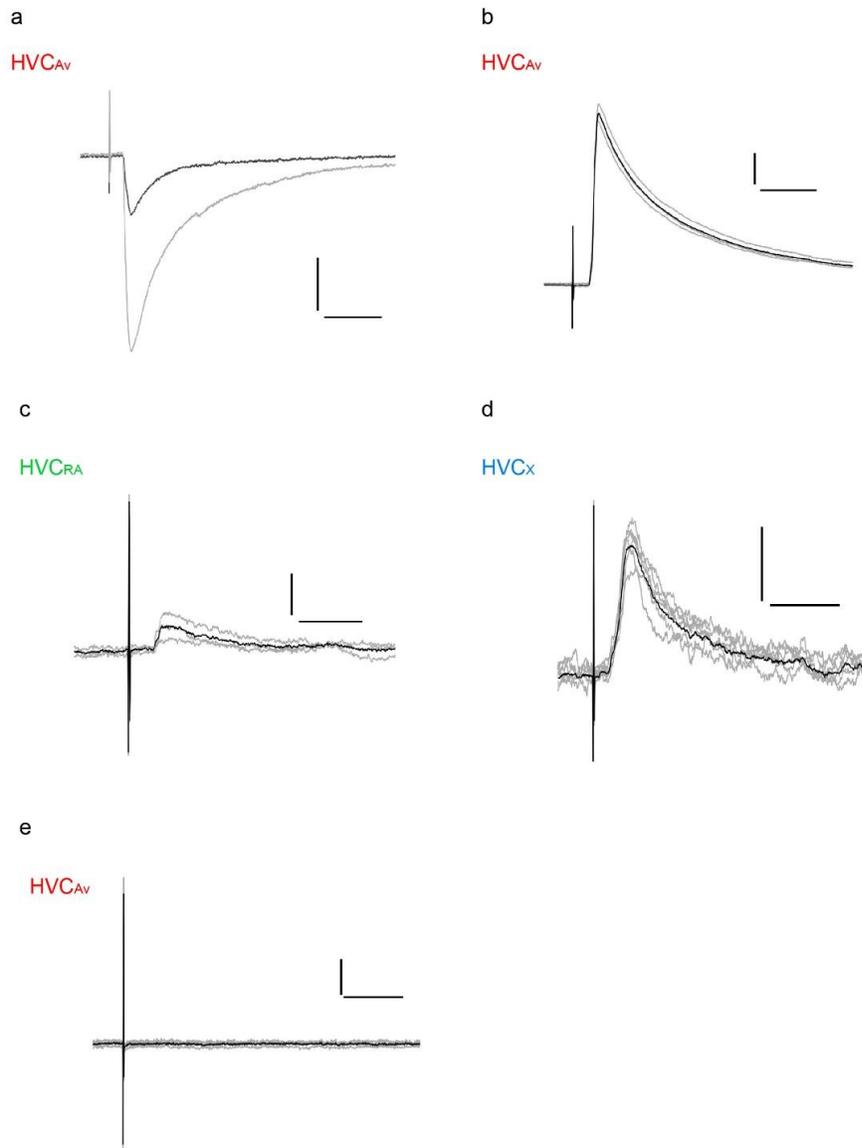


Supplementary figure 1. Comparisons of GFP-expressing HVC_{AV} , HVC_{RA} and HVC_X cell bodies. a) Soma diameter measured at the long axis of the cell body for HVC_{AV} (green), HVC_{RA} (red), and HVC_X (blue) cells (two-tailed t-test: HVC_{AV} versus HVC_{RA} , $n = 29$ for both groups, $p = 0.0001$; HVC_{AV} versus HVC_X , $n = 29$ for both groups, $p = 0.0006$). b) Soma area for HVC_{AV} (green), HVC_{RA} (red), and HVC_X (blue) cells (two-tailed t-test: HVC_{AV} versus HVC_{RA} , $n = 29$ for both groups, $p = 0.229$; HVC_{AV} versus HVC_X , $n = 29$ for both groups, $p < 0.0001$). Numbers listed on top left corner of each panel indicate the mean and S.E.M. for each cell type. Inset shows Gaussian fits to data shown in histogram. HVC_{RA} and HVC_X cell body measurements derived from data published in Tschida and Mooney, 2012.

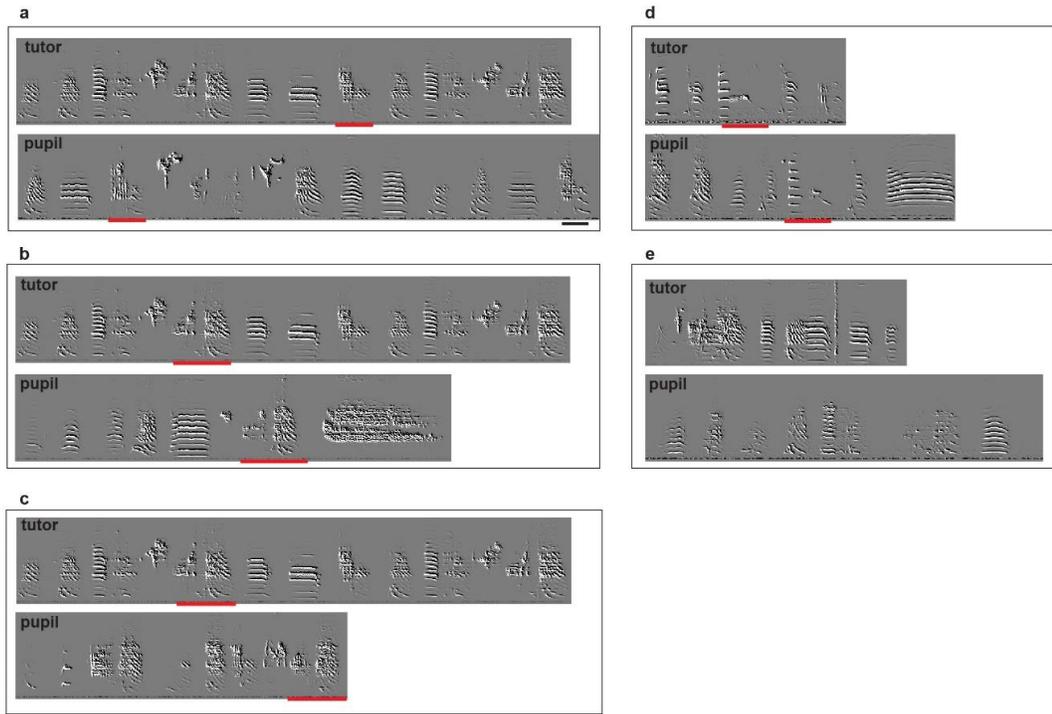


Supplementary figure 2. Different HVC_{AV} neurons show different activity patterns during singing in hearing-intact and deafened birds. **a)** Field of GCaMP-labelled HVC_{AV} neurons imaged through miniature microscope in a singing zebra finch in a single bird with hearing intact (left) and after deafening (right). Individual cells are indicated by colored outlines. Scale bars, 100 μm \times 100 μm . **b)** Mean singing-related activity traces of five putative HVC_{AV} neurons identified by CNMF analysis with hearing intact (left, $n = 24$ songs) and after deafening (right, $n = 25$ songs). Black lines represent

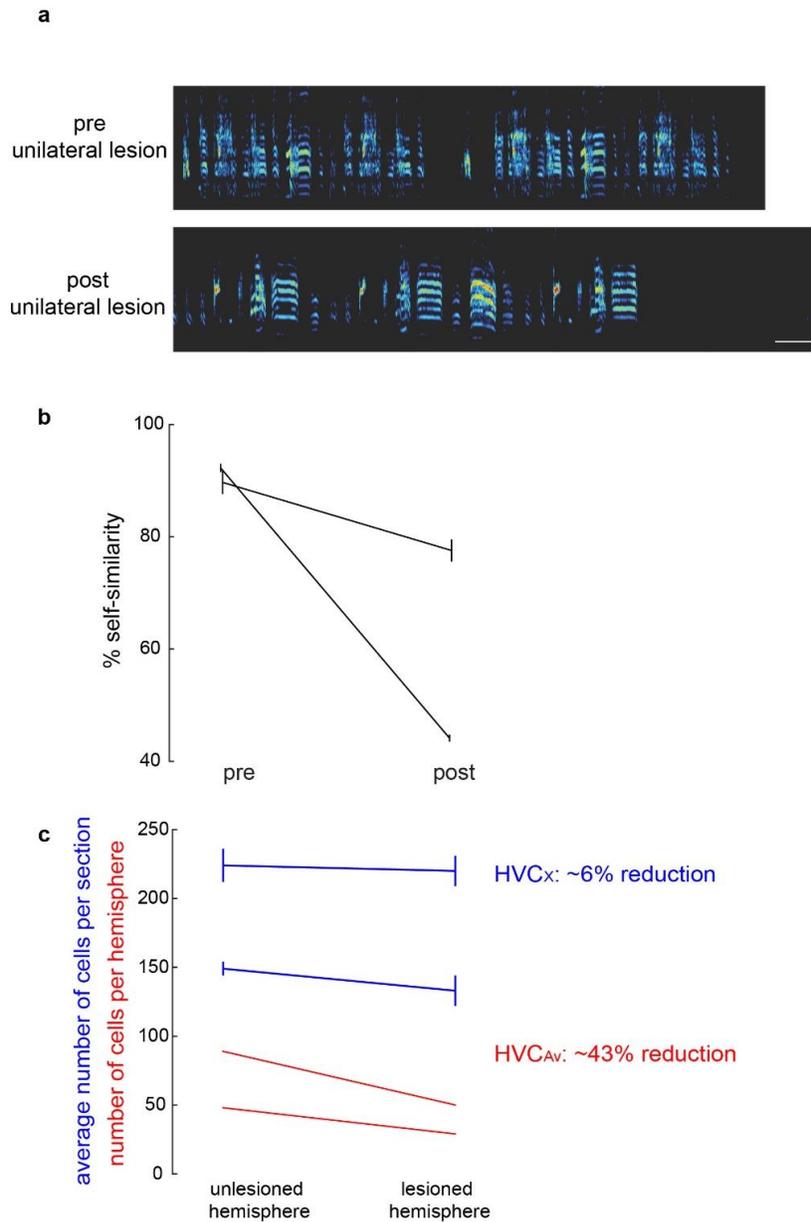
mean activity traces for each neuron and the shaded area denotes \pm one standard error of the mean response. Vertical scale bars, 10 (left) and 5 (right) arbitrary activity units; horizontal scale bars = 1 s and apply to traces in b – d; song motif onset is marked by vertical grey line. **c)** The summed activity from individual putative cell bodies shown in panel b). Vertical scale bars: 25 (left) and 20 (right) arbitrary activity units. **d)** Mean change in bulk fluorescence signals measured during singing from all birds with hearing intact (left, n = 4 birds) and after deafening (right, n = 2 birds)(same data as plotted in Figure 2c; scale bar 2% df/f). **e)** Cumulative probability distributions of mean decay time constants measured in singing zebra finches for GCaMP6s-expressing HVC_{AV} neurons (blue, n = 16 ROIs) and a mixed population of GCaMP6s-expressing HVC neurons (red, n = 43 ROIs). The KS test p value is 0.9914 and the KS statistic is 0.1221. Raw imaging files to which the CNMF algorithm was applied are provided as Supplementary Movies 1 and 2.



Supplementary figure 3. **a)** Evoked EPSC in HVC_{AV} cell after HVC_{RA} stimulation at 80 (black trace) and 160 (grey trace) μA ; scale bar 20 ms, 200 pA. **b)** Evoked IPSC in HVC_{AV} cell after HVC_{RA} stimulation at 80 μA ; scale bar 20 ms, 300 pA. **c)** Evoked IPSC in HVC_{RA} cell after HVC_X stimulation at 80 μA ; scale bar 20 ms, 50 pA. **d)** Evoked IPSC in HVC_X cell after HVC_X stimulation at 80 μA ; scale bar 20 ms, 100 pA. **e)** Recording in HVC_{AV} cell after electrical stimulation of LaM at 160 μA ; scale bar 20 ms, 100 pA.

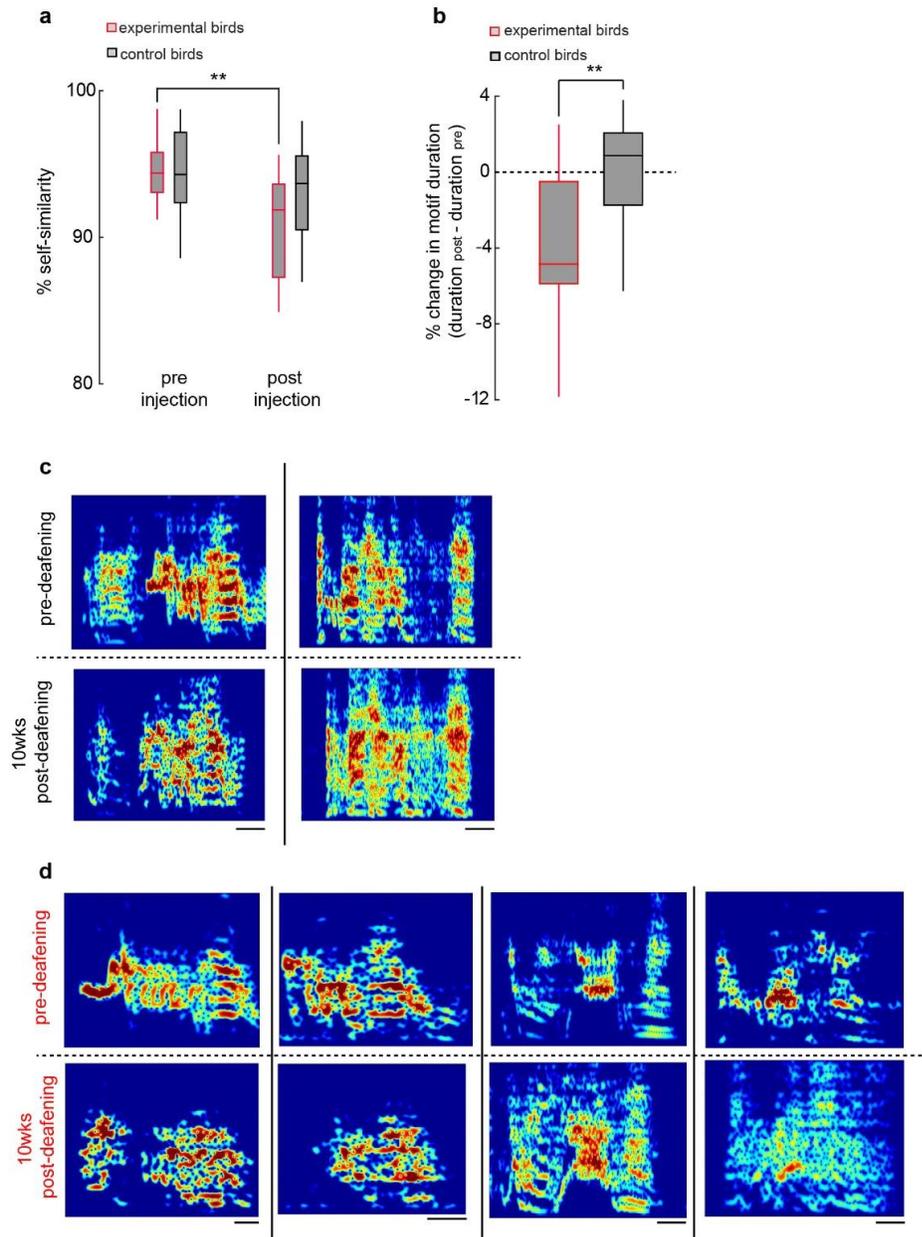


Supplementary figure 4. Spectral derivatives illustrating learning outcome for all 5 (a-e) HVC-Av lesioned birds. Birds in panels a-c were tutored by the same adult male zebra finch in separate experiments. In panels a-d examples of copied syllable(s) are marked by a red line. The fifth zebra finch, in panel e, did not copy either of the two complex syllables in the tutors' song. Birds and in panels a-b are the same birds illustrated in Figure 3 of the manuscript. Scale bar = 100ms (illustrated in bottom right of panel a).



Supplementary figure 5. Cre-dependent caspase3 is efficacious and specific to targeted population. a) Example of song from one bird before and after unilateral lesion of HVC from co-injection of Cre-dependent caspase 3 and Cre into HVC. **b)** Percent similarity to bird's own song (self-similarity) before and after unilateral lesions of HVC for 2 birds after co-injection of Cre-dependent caspase 3 and Cre into HVC unilaterally. **c)** Average number of cells per 50 micron section of HVC in 2 birds in which HVC_{AV} cells were unilaterally lesioned. HVC_x cells, which were not targeted with the intersectional ablation strategy (blue), were comparable in number between unlesioned and lesioned

hemispheres while HVC_{Av} cells, which were targeted (red), showed a reduction by nearly half in lesioned hemispheres.



Supplementary figure 6. Ablation of HVC_{Av} in adult birds causes slight decreases in motif similarity and duration. **a**) Experimental birds show a significant but slight decrease in percent similarity to bird's own song (self-similarity) after HVC_{Av} ablation (n = 12 HVC_{Av} lesioned birds, paired two-sample $t(11)=4.34$, $P = 0.001$ comparing percent self-similarity before lesioning to percent self-similarity after lesioning in experimental birds.) **b**) Experimental birds show a significant decrease in motif duration after HVC_{Av} ablation (n = 12 HVC_{Av} lesioned birds, n = 12 HVC_{Av} intact birds; two-sample

$t(22) = 2.49, P = 0.02$ comparing percent change in motif duration from lesioned versus intact birds). **c)** Example sonograms of single syllables from two control birds before (top row) and after (bottom row) deafening. Scale bars, 50 ms. **d)** Example sonograms of single syllables from four experimental birds before (top row) and ten weeks after (bottom row) deafening. Scale bars, 50 ms.

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Biography

I was born in Oklahoma City, Oklahoma on August 11, 1988. I attended the University of Chicago and graduated with an A.B. with Honors in the College in June 2011. I am the first author of two manuscripts, “A novel cell type mediates motor to auditory interactions important for vocal learning” and “A common neural circuit mechanism for internally guided and externally reinforced forms of motor learning”, the first of which was recently accepted and the second which is currently under review. I was selected to be a Grass Fellow at the Marine Biological Lab in Woods Hole, Massachusetts for the summer of 2017.