Cortical circuit dynamics during vocal learning in a songbird

A DISSERTATION
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

Nancy Forward Day

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Dr. Teresa A. Nick, Adviser

December 2011

Acknowledgements

Perhaps more than anything else, I learned that having a supportive and generally awesome adviser is a major asset. For that, I extend a huge “Thank You” to my supportive and generally awesome adviser, Teresa Nick. Teresa has given me many opportunities to broaden my scientific and intellectual horizons while in the lab and has encouraged (and challenged) me every step of the way. Without her guidance and input, the science from the last five years would not be nearly as comprehensive or as exciting. Thank you, T, for keeping me on a flexible leash – for letting me rove and wander at times to explore new ideas, but always reining me back in to keep me focused on the task at hand. I wouldn’t have benefitted nearly as much without the opportunity to wander a bit.

The second most important thing I learned in graduate school is that no PhD is obtained without the support from friends and family. Mom and Dad were always a phone call away and ready to share in the excitement, disappointment, or frustrations of the day, week, or month. Stacy Decker, a most tolerant friend, provided me with a Minnesota family and helped keep life outside of the lab fun and interesting: agreeing to raise several litters of kittens, undertaking crazy long bike rides, and introducing me to other wonderful friends. I wouldn’t have remained nearly as positive these last five years without my fabulous friendship with Elizabeth Gibbs, who is an excellent sounding board for all things in life, and will be a treasured colleague in the

future. And finally, Mai Lan Leong made non-experiment days fun and was a great office mate.

I was equally fortunate to have a great group of lab buddies (Vanessa Carels, Benjamin Best, Tim Balmer, and Jill Frisch) who were there in the trenches with me and from whom I learned so much. Without these folks, coming into lab each morning wouldn't have been nearly as rewarding.

The Graduate Program in Neuroscience comprises a wonderful group of people. The Class of 2006, in particular, is an intelligent and fun-loving group of comrades with whom I'm grateful to have shared this experience with from Day 1. In addition, Virginia Seybold was an incredibly supportive DGS.

Dedication

To Daphne & Sebastian –

for being a great joy to come home to after a long day at work and

for making no morning or evening routine.

Abstract

Vocal learning in humans and songbirds occurs during a sensitive period in development. Oscine songbirds, such as zebra finches, memorize a tutor song during the sensory phase and then, using auditory feedback, match their own vocalizations to the tutor song memory during the sensorimotor phase. Songbirds possess a set of anatomically distinct brain nuclei that are dedicated to vocal learning. HVC is a telencephalic song nucleus with auditory and motor activities.

The cellular and neural circuit determinants of sensitive periods, which are characterized by enhanced plasticity, are not well understood. Activity in the sensorimotor song control area HVC changes during song learning (Crandall et al., 2007b). For example, neurons in the HVC of juveniles have longer, weaker bursts than those in adults. Changes in the circuitry of HVC that may underlie these developmental changes are not known.

We have examined how population bursts in HVC change during sensorimotor learning in the zebra finch. First, we found that bursts of activity in HVC predict stability in song during singing. This led to the hypothesis that bursts in HVC were stronger in the afternoon when song is known to be more stable (Deregnaucourt et al., 2005). We found that bursts in HVC increase each day and during development.

To examine changes in HVC burst activity during song learning, we recorded ensembles of HVC neurons using multiple tetrode recording in

anesthetized juvenile and adult zebra finches. Arrays of tetrodes, such as those we have used, enable simultaneous recording of many neurons and analysis of their functional interactions. To identify specific cells in the ensemble, we antidromically stimulated projections from HVC to other song nuclei and co-clustered antidromic and spontaneous spikes. This method enables the study of identified HVC projection neurons in the context of the functioning circuit.

With combined tetrode and antidromic methods, we have begun to investigate interactions among HVC neurons. We have found that both efferent projections of HVC are active in population bursts, and that the class of neurons that project to a brain pathway known to generate song variability increases its bursting activity in adults. We also found that a population of functionally inhibitory neurons exhibits prolonged bursting in juveniles, which are active in developing sensory systems during a sensitive period. Collectively, these data implicate HVC in song learning. We speculate that bursts of activity in HVC, by virtue of a change in inhibition, limit song variability over time during sensorimotor learning.

Table of Contents

List of Figures

Chapter 1:

Chapter 2:

Chapter 3:

Chapter 4:

List of Abbreviations

AFP	Anterior forebrain pathway
BOS	Bird's Own Song
DLM	Dorsolateral thalamus (thalamic song nucleus)
HVC	Used as the proper name (cortical song nucleus)
HVC_{INT}	HVC interneuron
HVC_{RA}	HVC – RA projecting neuron
HVC_{X}	HVC – Area X projecting neuron
LMAN	Lateral magnocelluar nucleus of the anterior nidopallium (cortical song nucleus)
Nif	Interfacial nucleus of the nidopallium (sensorimotor song nucleus)
nXIIts	Hypoglossal nucleus (12^{th} nerve)
PNN	Perineuronal net
PV	Parvalbumin-positive interneuron
RA	Robust nucleus of the arcopallium (cortical song nucleus)
Uva	Nucleus uvaeformis (thalamic song nucleus)

Contributions

The following contributions were made by individuals other than Nancy F. Day or Teresa A. Nick:

Chapters 2 & 3: *Amanda K. Kinnischtzke, Murtaza Adam,* and *Shane Crandall* collected the chronic electrophysiological data and performed initial analyses.

Chapter 4: *Stephen J. Kerrigan* and *Naoya Aoki* helped optimize automatic and manual cluster cutting.

Chapter 1

General Introduction

Communication between animals can manifest itself using a variety of different modalities: e.g. visual, olfaction, electric, and/or acoustic. The signals that animals use to communicate can indicate their reproductive fitness, protect their territory or offspring, and/or influence social interactions with conspecifics or heterospecifics. Vocal communication is one form of acoustic signaling between individual animals that can greatly impact the nuances of an organism's life, including survival, attachment to others to improve the chances of survival, and in some communities, development of social structure, and culture. In many animals, vocal communication is unlearned and consists of innately generated sounds to reflect the current state of the animal. However, in a subset of mammalian and avian species, vocal signals, such as words or songs, are learned. In these cases, mature vocalizations develop over several months or years following a period of trial-and-error learning using auditory feedback. The development of speech, and later language, in humans is unique amongst primates; language is a defining characteristic that separates man from other animals. How the brain acquires such a complex behavior such as speech can be investigated using other species that have similar neural architecture and learn complex, sequential motor patterns.

The Songbird

Learned vocal communication in the animal kingdom is relatively rare. Humans, cetaceans, elephants, pinnepeds (e.g. seals), and bats are the only known mammals known to shape their vocalizations through learning (Hauser et al., 2002; Seyfarth and Cheney, 2003). These mammalian vocal learners

comprise fewer than 300 species (Williams, 2004). However, many species of birds learn their vocalizations, including parrots and hummingbirds (comprising roughly 700 species total), and oscine songbirds (~4,500 species) serve as excellent models for unraveling the neural mechanisms of human speech acquisition. Oscine songbirds are members of the avian order of perching birds, Passeriformes; these species of birds learn their vocalizations from a tutor animal. In contrast, one genus in the Passeriformes suborder, suboscines (~1,150 species), do not learn vocalizations, and produce only innate, unlearned calls (Williams, 2004).

One member of the oscine family, the male zebra finch (*Taeniopygia guttata*), has emerged as a superior model for determining the neural mechanisms of vocal learning. Its ability to readily reproduce in captivity, rapid maturity, and near-constant singing behavior are useful for laboratory investigations. The single and repetitive nature of its song facilitates the study of normal song acquisition, and sequence learning. For example, song learning can be analyzed motif-by-motif, syllable-by-syllable as a bird progressively shapes his vocalizations into a mature song (Deregnaucourt et al., 2005). In addition to wild-caught birds, such as song or swamp sparrows, other songbirds are also routinely used in laboratory studies. The Bengalese finch is an attractive model for syntactic development, and the canary is widely studied because it is an open-ended learner (repeated, seasonal learning). In addition to producing innate, unlearned calls, each of these species produces robust singing behavior that results from learning a song from a tutor animal. Songs from different

species can vary dramatically in spectral and temporal information, but all songs serve similar functions: to attract mates, and to defend and establish territory (Williams, 2004). Calls are produced by all species of bird and serve a variety of functions including intimidation, mating, alarm, and food solicitation (Zann, 1996); they are short, monosyllabic vocalizations that are less complex and more spectrotemporally variable than songs. In many species, only the male produces singing behavior, using it to attract the attention of females for the formation of a pair bond and reproduction.

Song Structure

In the zebra finch, only males produce song. Songs consist of multiple syllables that are repeated in a specific pattern to form motifs, the repeated unit of a song (Sossinka and Bohner, 1980) (Figure 1). Syllables are the smallest component of song and are surrounded by brief periods of silence (~ 15 – 20 ms). Syllables are composed of harmonics and may be rapidly modulated in amplitude and frequency. The hearing range of the zebra finch is concentrated in the 2-5 kHz range; accordingly, most syllables have greatest power in that frequency (Woolley, 2004). A song bout is formed from multiple, nearly identical motifs. In adults, each motif consists of the same pattern of syllables and has high stereotypy. Typically, songs are composed of 2 - 8 motifs (Sossinka and Bohner, 1980).

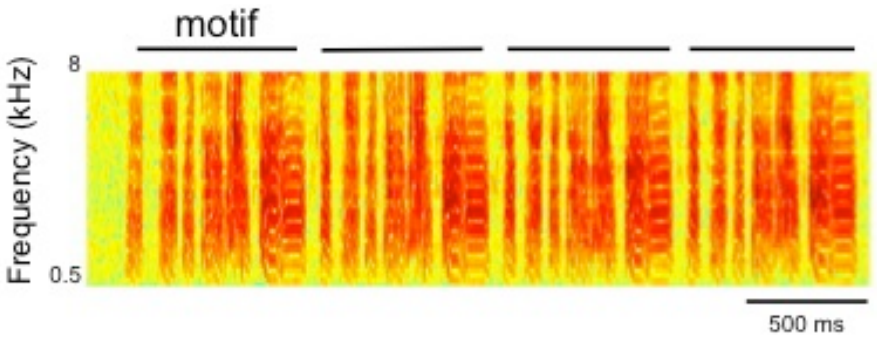

Figure 1. Structure of a crystallized zebra finch song.
An example of a mature zebra finch song shows four repeated motifs. Each motif is composed of different syllables that are repeated in the same order. Note the low variability in the temporal and spectral properties of the song. Color indicates the power at each frequency (red = higher power; yellow = lower power).

Song learning

The process that a zebra finch uses to learn his song is very similar to how humans acquire speech (Doupe and Kuhl, 1999). It consists of several phases during a critical period. Sensory critical periods (Hubel and Wiesel, 1970; Hensch, 2004; Knudsen, 2004) provide excellent windows in time to study the characteristics of neural plasticity. Though song acquisition varies depending on the species, the stages of learning are consistent across all known songbird species (Williams, 2004). Figure 2 schematizes the process of song learning in the zebra finch. In normal song development, juveniles interact with a tutor, usually their father, who provides them with a song model they learn to copy. Two types of experiments revealed how song learning is achieved. Isolation from a tutor clarified the time window of the sensory phase; birds reared in isolation developed abnormal song and were unable to sculpt their vocalizations to a mature song following exposure to a tutor (Konishi and Nottebohm, 1969). Deafening experiments highlighted the importance of auditory feedback (Konishi, 1965). Song learning begins with a sensory phase and is followed by a

sensorimotor phase of vocal exploration (Thorpe, 1958; Marler, 1970; Marler and Peters, 1977). Song learning concludes when the song crystallizes and becomes resilient to further change. The template-matching theory (Konishi, 1965) suggests that songbirds form a memory of a tutor song during a sensory phase that serves as a template to guide song during sensorimotor learning to its crystallized state.

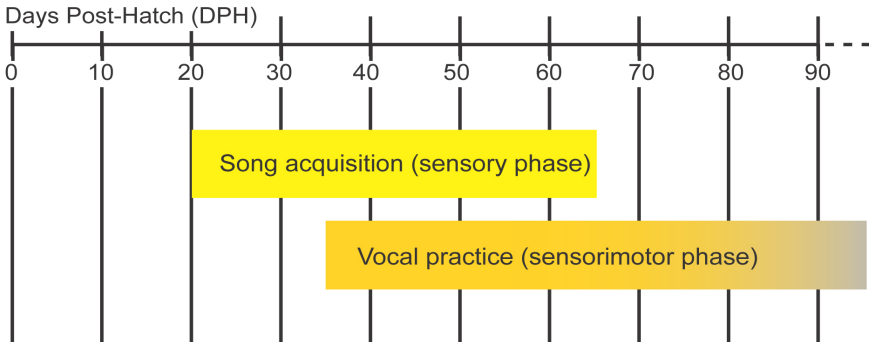

Figure 2. Phases of song learning in the zebra finch.
Juvenile male zebra finches learn a mature song in two overlapping stages. The sensory phase begins ~20 days post hatch and concludes around Day 65. During the sensory phase, the juvenile memorizes a song from his tutor that he will later copy. The sensorimotor phase is characterized by the onset of vocalization at ~35 days. The bird progresses from "subsong", a babbling state, to crystallized song by ~90 days using trial & error learning. Adapted from (Thorpe, 1958; Konishi, 1965; Marler, 1970; Marler and Peters, 1977).

In both humans and songbirds, the sensory phase is a silent period of sensory acquisition, in which the animal is exposed to the acoustic features of speech or song. Isolation experiments have shown that the sensory phase begins at roughly 25 days post hatch (dph) and concludes around 60 days (Nottebohm, 1968; Immelmann, 1969; Eales, 1985). During this phase, juvenile zebra finches are exposed predominately to their father's song, and form a memory of his song (the "tutor song"). They can also acquire components from neighboring males, particularly following fledging from the nest at 18 days in the wild, or in laboratory colonies (Williams, 1990). Zebra finches require interaction

to best acquire their songs (Adret, 1993); however, inanimate models of male zebra finches can also serve as sufficient social partners using operant conditioning paradigms (Deregnaucourt et al., 2005).

Zebra finches have overlapping stages of sensory and sensorimotor learning. At ~35 dph, while the juvenile is still acquiring his sensory template, he begins to actively vocalize, which initiates the sensorimotor phase of vocal learning. Initial vocalizations are low-volume and have variable spectral and temporal components; these vocalizations are commonly referred to as "subsong" and are considered equivalent to babbling in human infants. These vocalizations allow the juvenile to explore the range of his vocal abilities and constitute the first forays in his matching his vocalizations to his tutor song memory.

Throughout the sensorimotor phase, the juvenile actively sculpts his vocalizations to match his stored template. This stage consists of trial-and-error learning, in which the juvenile must selectively stabilize individual components of his vocalizations over time to perfect his song. The strategies that juvenile finches use to learn song are diverse, and not well understood, but include repeating the same syllable to perfection or sculpting the motif as a whole and varying changes to syllables with each rendition (Tchernichovski et al., 2001; Liu et al., 2004). By approximately 100 days, the animal has developed a mature song that is resistant to change, and bears close resemblance to the tutor song.

Auditory feedback is not only critical for the acquisition of speech or song, but also for the maintenance of these vocalizations. Young children deafened

during speech learning show substantial degradation in speech (Plant and Hammarberg, 1983). Similarly, zebra finches deafened during sensorimotor learning develop highly abnormal song (Price, 1979). In addition, birds deafened in adulthood show marked deterioration of their songs within one year; songs become more “scratchy” and are prone to syllable addition or deletion (Nordeen and Nordeen, 1992). Auditory perturbation also shows that feedback influences human speech (Houde and Jordan, 1998; Jones & Munhall, 2000) and adult song (Leonardo and Konishi, 1999; Sober and Brainard, 2009). Birds actively shift the pitch of song renditions when they receive distorted auditory feedback that alters their perception of their vocalization. These studies underscore the importance of auditory feedback in sensorimotor learning and maintenance of song.

The Song System

Oscine songbirds possess a series of anatomically discrete brain nuclei in the telencephalon and basal ganglia that are dedicated to song learning and production. These seven brain regions are collectively known as the song system (Nottebohm et al., 1976). The song system (diagrammed in Figure 3) is composed of two primary pathways: the descending motor pathway and the anterior forebrain pathway (AFP). The descending motor pathway is composed of two pallial (cortical) song nuclei: HVC (used as its proper name) (Reiner et al., 2004), and its efferent target the robust nucleus of the arcopallium (RA) (Nottebohm et al., 1976; Katz and Gurney, 1981; McCasland and Konishi, 1981; Margoliash, 1983; Bottjer et al., 1989; Theunissen and Doupe, 1998; Mooney,

2000). HVC receives projections from the auditory areas CM (caudal mesopallium) and Nif (interfacial nucleus of the nidopallium) (Nottebohm et al., 1982; Bottjer et al., 1989; Bauer et al., 2008). RA projection neurons synapse (Karten, 1991) onto motor neurons in the tracheosyringeal portion of the nucleus of the twelfth nerve (nXIIts) and on respiratory premotor neurons in the nucleus retroambigualis (Ram), which controls expiration, and the nucleus parambigualis (PAm), which controls inspiration (Nottebohm et al., 1982; Wild and Arends, 1987; Wild et al., 2000). The second primary pathway, in the songbird brain, the AFP, is network of nuclei that are similar to mammalian cortical-basal ganglia pathways (Doupe et al., 2005). The AFP is composed of three brain regions: Area X (a striatal/pallidal area), DLM (medial nucleus of the dorsolateral thalamus) and LMAN (lateral magnocellular nucleus of the anterior nidopallium). RA receives dual cortical input from HVC and LMAN. These three song nuclei (HVC, RA, and LMAN) are pallial (cortical) and are evolutionary and developmentally related to mammalian cortex (Reiner et al., 2005). Though the organization of the song system is radically different from the layered mammalian cortex and produces one very specific behavior – singing – there are many similarities between its neural circuitry and mammalian systems, even those not dedicated to vocal learning (Farries and Perkel, 2002). These similarities allow for comparison between the neural circuits in the song system and mammalian systems to elucidate mechanisms of learning and memory.

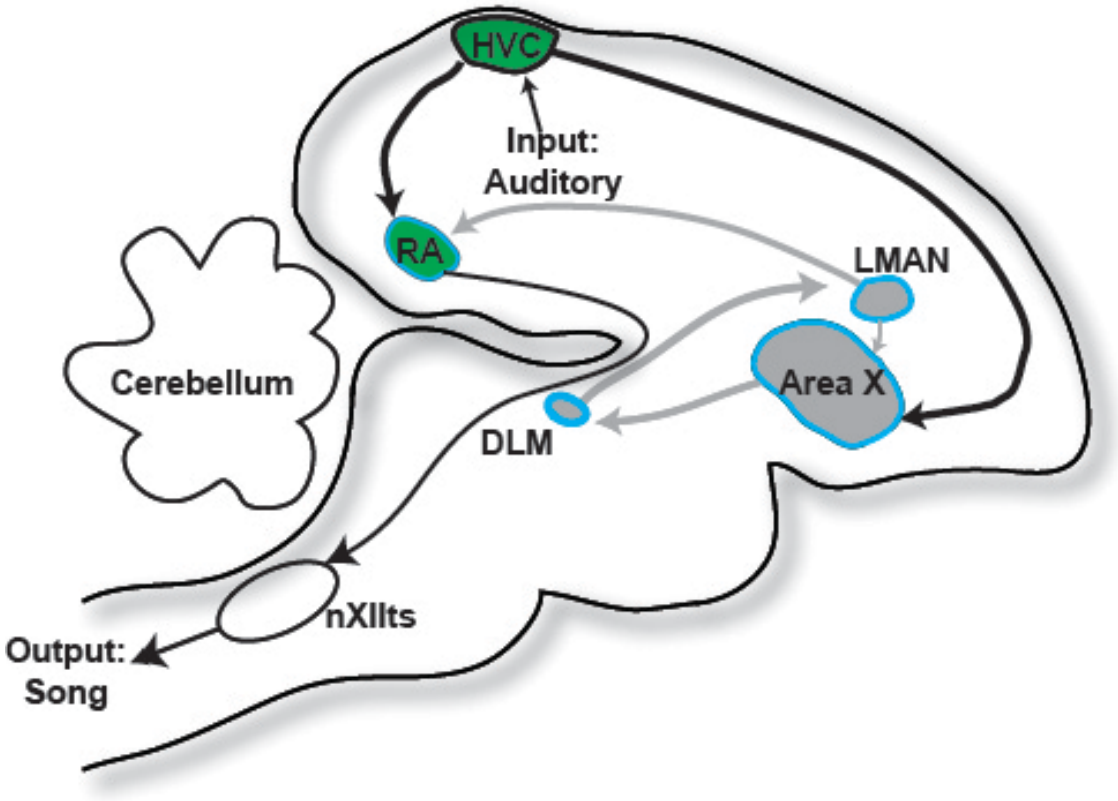

Figure 3. Simplified schematic of the zebra finch song system nuclei.
The oscine song system is composed two primary pathways: the motor pathway (green), and the anterior forebrain pathway (AFP; gray). The motor pathway is critical for song production, and the AFP is necessary for song learning. In the motor pathway, the sensorimotor song nucleus HVC (its proper name) receives auditory input from primary auditory cortex and thalamus. It sends an excitatory projection to motor nucleus RA (robust nucleus of the arcopallium), which innervates motor neurons in the brainstem to drive singing behavior. RA also receives excitatory input from LMAN (lateral nucleus of the anterior nidopallium), the output nucleus of the AFP. Area X (analog to the basal ganglia) has inhibitory projections to DLM (medial portion of the dorsolateral thalamic nucleus). DLM sends excitatory projections to LMAN.

The Motor Pathway

Lesioning studies have revealed the contribution of each song nucleus to vocal production and learning. HVC and RA are required for vocal production; if either of these song nuclei is lesioned, all vocalizations cease (Nottebohm et al., 1976; Aronov et al., 2008). Both HVC and RA show premotor neural activity (McCasland and Konishi, 1981; Yu and Margoliash, 1996; Hahnloser et al., 2002) and low current electrical stimulation of RA or HVC in awake zebra finches elicits vocal patterns (Vicario and Simpson, 1995). The forebrain nucleus HVC has both motor and auditory activities in the song system and is required for normal song perception (Brenowitz, 1991; Gentner et al., 2000). HVC receives efferents from a thalamic song nucleus, Uva (nucleus uvaeformis), which codes motor signals and is important for song production (Bottjer et al., 1989; Williams and Vicario, 1993) and from auditory brain regions. Auditory signals in HVC are from CM (Bauer et al., 2008), a homologue of secondary auditory cortex; and the forebrain song nucleus Nif (interfacial nucleus of the nidopallium) (Katz and Gurney 1981; Bottjer et al., 1989; Wild, 1994), which is also innervated by Uva. Neurons in HVC respond preferentially to playback of the Bird's Own Song (BOS) and have stereotyped responses to BOS and other auditory stimuli (Margoliash, 1983, 1986; Fortune and Margoliash, 1995; Lewicki and Arthur, 1996; Volman, 1996; Theunissen and Doupe, 1998), including tutor song as juveniles (Nick and Konishi, 2005a). Selective BOS responses are first observed in Nif (Coleman and Mooney, 2004) in the ascending auditory pathway.

In addition to its auditory properties, mounting evidence suggests the temporal properties of song are patterned in HVC (Vu et al., 1994; Yu and Margoliash, 1996; Long and Fee, 2008). For example, electrically stimulating HVC during singing resets the motif; the animal must restart his motif from the beginning. Stimulating song nuclei outside of the motor pathway, such as Area X in the AFP, does not elicit similar patterns of vocal activity (Ashmore et al., 2005; Vu et al., 1994). In addition, specific bilateral cooling of HVC (but not RA) with a Peltier device slowed neural activity resulting in intact spectral properties of the song, but lengthened motifs relative to the degree of cooling (Long and Fee, 2008). These data are also consistent with a model of distributed coding of song patterning.

The Anterior Forebrain Pathway

Efferent neurons from HVC are not confined to the motor pathway; the projection from HVC to Area X, a nucleus in the avian basal ganglia, positions HVC as a sensorimotor area at the nexus between the motor and anterior forebrain pathways. The latter pathway is a specialized cortical-basal ganglia-thalamic-cortical loop that is common to all mammalian and avian vocal learners (Perkel and Farries, 2000; Jarvis et al., 2005 Jurgens, 2009), which suggests that there are common neural mechanisms for learned vocal production.

The analog of the basal ganglia in songbirds is Area X, one of two projection targets from HVC. In general, the basal ganglia are critical for learning sequential movement (Middleton & Strick, 2000). For example, the AFP plays an important role in vocal exploration necessary for song learning (Kao et al., 2005;

Andalman and Fee, 2009). There are many similarities between avian and mammalian basal ganglia components. First, the striatum and globus pallidus in mammals are comprised of inhibitory spiny and aspiny neurons. Similarly, in songbirds, spiny and aspiny neurons in Area X release the inhibitory neurotransmitter, GABA; they also have similar electrophysiological properties to those seen in mammalian basal ganglia systems (Farries and Perkel, 2000, 2002). Similar to mammalian systems, four major cell classes in the striatum are found in Area X: medium spiny neurons that are immunopositive for either enkephalin or substance 2, fast-spiking interneurons, cholinergic neurons that are tonically active, and low threshold spiking interneurons that are active in vitro and in the awake, behaving animal (Farries and Perkel, 2002; Goldberg et al., 2010). Combined with pallidal-like projections that are distinctly similar to the direct and indirect pathways found in the mammalian pallidum, it is clear that Area X has structural and functional similarities to mammalian basal ganglia circuits (Reiner, 2009).

The output nucleus of the anterior forebrain pathway is the pallial song nucleus, LMAN, which receives input from DLM. LMAN sends an excitatory projection to RA (Nottebohm et al., 1982). Though they are not considered a part of the motor pathway, LMAN and Area X show premotor activity (Hessler and Doupe, 1999; Leonardo and Fee, 2001). Activity in LMAN is influenced by social interactions and modestly influences adult song. In directed singing conditions (a male singing to a female), song becomes more stereotyped and less variable than undirected (song not directed at an audience) stereotyped, crystallized

song (Kao et al., 2008). LMAN exhibits precise firing during directed singing compared to undirected singing. In addition, lesioning LMAN in adults reduces the variability of undirected song (Kao et al., 2005). These studies suggest that LMAN influences song output in adult animals (albeit modestly compared to the motor pathway); its role in song learning is more substantial.

Lesions to AFP nuclei in juveniles impair vocal learning, but do not disrupt song production (Bottjer and Arnold, 1984; Scharff and Nottebohm, 1991; Brainard, 2004). Bilateral ablation of Area X disrupts song learning by stalling vocal production in a permanently variable state. Juvenile finches with Area X lesions are unable to match the tutor song model and retain highly variable song with low stereotypy (Bottjer and Arnold, 1984). In contrast, bilateral lesions to LMAN, the output nucleus of the AFP, result in immediate crystallization of an immature song (Bottjer and Arnold, 1984). Subsequent song has very high stereotypy, but vocalizations never mature into a typical zebra finch song. These data, combined with data from adult animals (Kao et al., 2005) suggest that LMAN is responsible for “injecting” variability into the song – enhancing the range of vocalizations that the juvenile produces – which may manifest itself in trial-and-error learning necessary for mature vocalizations. Therefore, during development, LMAN is crucial for providing plasticity in song. LMAN activity is from excitatory projections from DLM, which receives inhibitory projections from Area X; this is one pathway through which Area X is capable of reducing activity in LMAN. The developmental changes in the AFP, and other brain regions (such

as the ventral tegmental area) that may directly influence LMAN activity during song learning are still unknown.

HVC and song learning

The AFP is critical for song learning (e.g. Bottjer et al., 1984; Kao et al., 2004). Despite having both auditory and motor properties, the role of HVC is song learning is not well understood. Simple lesion experiments were adequate for initially assessing the role of AFP song nuclei in song learning and maintenance (Bottjer et al., 1984; Kao et al., 2004), but lesions to HVC prevent singing (Nottebohm et al., 1976). Therefore, determining the contribution of HVC to song learning, and how its circuitry is remodeled during sensorimotor trial-and-error learning is important to elucidate the neural mechanisms of vocal learning.

Chronic, multi-unit studies have been used to analyze changes in HVC during sensorimotor learning in juveniles, and in adults. These studies have shown that premotor activity is tightly correlated with vocalization in the adult (McCasland and Konishi, 1981). Longitudinal recordings have shown that there is a stronger auditory response to the most current version of a juvenile's song during sleep (Nick and Konishi, 2001) and a response to the tutor song in HVC during waking in juveniles (Nick and Konishi, 2005a). These observations provided evidence that HVC and/or its inputs change during song learning, and that HVC processes signals that code for the most current version of the song and the tutor song memory (Nick and Konishi, 2005a).

Neural activity in HVC is developmentally regulated (Crandall et al., 2007a). In the juvenile, multiunit population activity in HVC is observed before the

onset of vocalization and outlasts the vocalization (Crandall et al., 2007a). During song learning, activity bursts in HVC decrease in duration (Crandall et al., 2007a). The change in HVC burst duration is correlated with song maturation (Crandall et al., 2007a), which suggests that shorter bursts of population activity during singing reflect more mature song. Collectively, these data indicate that HVC may have a role in vocal learning.

Chronic, multi-unit recordings are used to examine how a population of neurons changes over time, in awake, behaving animals. Despite their long-term stability, one drawback is that the identity of individual neurons in the recording is unknown. However, multiple studies from the birdsong field suggest that interneurons are the primary cell type that is recorded using multi-unit techniques (Crandall et al., 2007a; Yu & Margoliash, 1996; Rauske et al., 2003). Therefore, other recording techniques, such as intracellular recording, or single-unit extracellular recordings, are required to determine the firing patterns and activity of different classes of neurons within HVC during auditory playback and during singing.

Classes of HVC projection neurons

There are three primary types of neurons in HVC (Kirn et al., 1991; Johnson and Bottjer, 1993; Mooney, 2000): There are two non-overlapping populations of projection neurons, HVC_X (project to the basal ganglia nucleus HVC), HVC_{RA} (project to the motor nucleus RA), and a local population of HVC_{INT} (interneurons). Interneurons do not project outside of HVC, which may allow for local synaptic processing (Katz and Gurney, 1981; Mooney, 2000). The synaptic

connectivity between HVC neurons has been determined using paired intracellular recordings (Mooney and Prather, 2005). Briefly, they found that HVC_{RA} may inhibit HVC_X neurons via multiple parvalbumin-positive interneurons, HVC_{INT} innervate both types of projection neurons, and HVC_{RA} neurons and HVC_X neurons are synaptically coupled. The interconnectivity among neurons within HVC is particularly interesting because the output of HVC is to both motor cortex (RA) and the basal ganglia (Area X).

Neurons in HVC that project to Area X (HVC_X) may play an important role in song learning due to their input to the vocal variability-inducing (AFP) circuit. Area X is important for learning and perception (Bottjer and Arnold, 1984; Scharff and Nottebohm, 1991; Scharff et al., 1998; Kao et al., 2005). One hypothesis is that HVC_X neurons relay an error signal to the AFP based on auditory feedback from the BOS. Previous studies have shown that HVC (Schmidt and Konishi, 1998; Nick and Konishi, 2005a), Area X, and LMAN (Solis and Doupe, 1999) have selective responses to BOS in sleeping or anesthetized birds than to other auditory stimuli; the response is less robust in awake-behaving finches (Schmidt and Konishi, 1998). These auditory representations in the AFP likely originate from HVC_X projections (Prather et al., 2008; Prather et al., 2009). Intracellular HVC_X recordings revealed that they have strong auditory-selective properties (Mooney 2000). HVC_X neurons fire in bursts at precise times in the motif during singing (Kozhevnikov and Fee, 2007; Prather et al., 2008) and their activity is likely shaped by HVC_{INT} (Mooney, 2000). The firing patterns of HVC_X neurons

were not altered when distorted auditory feedback was presented to a juvenile songbird during learning (Kozhevnikov and Fee, 2007).

Projections from HVC to motor area RA (HVC_{RA}) do not show precise auditory responses (Katz and Gurney, 1981; Lewicki and Arthur, 1996), but do show precise firing during singing (Hahnloser et al., 2002). HVC_{RA} cells fire ultra sparse, high-frequency bursts that are time-locked to the fine structure of a syllable. These bursts typically occur once per motif, further implicating the role of HVC in coding pattern and timing in song production (Hahnloser et al., 2002). This timing may influence the activity of neurons in RA, which produce relatively sparse burst time-locked to multiple syllables with 0.2 ms of precision (Yu and Margoliash, 1996; Chi and Margoliash, 2001).

HVC_{RA} neurons synapse with multiple neurons in RA (Nixdorf, 1989), and in HVC with interneurons and HVC_X neurons (Mooney and Prather, 2005). Synapses from HVC_{RA} cells form within RA at approximately ~25 days, prior to the onset of vocalization, which is ~10 days after initial synapses from LMAN form in RA (Mooney, 1992). In addition, HVC_{RA} neurons are replaced by adult neurogenesis, and are functionally active in circuits (Alvarez-Buylla et al., 1990; Kirn et al., 1991).

The final class of HVC neurons is HVC_{INT}, local inhibitory interneurons, which arborize within HVC (Nixdorf, 1989; Mooney, 2000). Compared to projection neurons, interneurons have higher spontaneous firing rates, shorter action potential durations, and high frequency, nonadapting action potential bursts in response to depolarizing stimulation (Dutar et al., 1998; Kubota and

Taniguchi, 1998; Mooney, 2000; Rauske et al., 2003). Interneurons also fire tonically during singing and during BOS playback, compared to the more sparsely firing projection neurons (Margoliash, 1997; Mooney, 2000; Hahnloser et al., 2002; Rauske et al., 2003). Interneurons in HVC are all immunoreactive for one or more of the calcium-binding proteins: parvalbumin, calbindin, or calretnin (Wild et al., 2005). Differences in morphology and immunoreactivity suggest there are at least nine separate subclasses of local HVC interneurons (Wild et al., 2005).

A role for interneurons in senstive periods in development

The role of interneurons in shaping HVC activity is of special interest not only with regard to song learning (in HVC and/or other song nuclei), but also more broadly to critical period plasticity (Hensch, 2005). Evidence that interneurons are crucial for regulating critical period plasticity is predominately from research on the critical period for ocular dominance (Hensch, 2005). Interneurons are typically inhibitory and are thought to play crucial roles in regulating neural activity during development and throughout life. GABAergic interneurons detect changes in sensory input (Fagiolini 2004), regulate excitability of glutamatergic pyramidal neurons (Rudolph 2007), refine local cortical circuits (Hensch 1998), influence cortical development (Wang & Kriegstein, Zheng & Knutsen 1999), and synchronize brain regions (Galaretta & Hestrin 2001; Gonzalez-Burgos & Lewis 2008, Tamas 2000).

Prolonged bursting of interneurons has been observed in developing sensory systems with critical periods, such as ocular dominance plasticity

(Fagiolini and Hensch, 2000). Prolonged bursting diminishes as the system matures (Hensch, 2005). In the birdsong system, neural activity in HVC is characterized by prolonged bursts in juvenile animals (Crandall et al., 2007a). A group of fast-spiking, putative interneurons were shown to have activity both before and after vocalization in the juvenile. These data further implicate interneuron development as an important factor in critical period plasticity, specifically, in this case, in the songbird. Understanding how interneurons may modify neural ensembles in the song system is an important step in elucidating the neural mechanisms of vocal learning.

One class of interneurons that is particularly relevant to the study of critical period plasticity contains the calcium-binding protein, parvalbumin (PV). Parvalbumin-positive (PV+) interneurons, which are less than 8% of mammalian neocortical neurons (Xu et al., 2010), control cortical rhythms and, thus, circuit activity and function (Engel et al., 2001; Womelsdorf and Fries, 2007; Cardin et al., 2009). In addition, PV+ interneurons are fast spiking and are preferentially surrounded by extracellular matrix structures called perineuronal nets (PNN) that are composed of chondroitin-sulfate proteoglycans (Hartig et al., 1999; Hensch, 2005). PNNs have been found in experience-dependent sensory (and sensorimotor) systems that have critical periods including the visual system (Pizzorusso et al., 2002), mouse barrel cortex (McRae et al., 2007), and the song system (Balmer et al., 2009). In the visual system, destruction of PNNs using the Chondroitinase-ABC (ChABC) enzyme reopens the critical period for ocular dominance plasticity (Pizzorusso et al., 2002). These data suggest that the

formation of PNNs is a hallmark of a mature sensory system at the end of its critical period; elimination of the nets reverses the system to a more immature state.

In the song system, PNNs are found in the seven primary nuclei (Uva, Nif, HVC, RA, Area X, LMAN, and DLM), in addition to primary sensory areas such as the visual Wulst, Field L (auditory), and nucleus basalis (somatosensory) (Balmer et al., 2007). PV+ interneurons and PNNs are developmentally regulated, and net expression is highest in adults with crystallized song. These data suggest that the presence of PNNs in the song system may be important for the closure the critical period for vocal learning, which is similar to the role that PNNs have in ocular dominance plasticity (Hensch, 2005). The appearance of perineuronal nets is experience-dependent, and may serve as a physical marker for mature sensory and/or sensorimotor systems. PNNs are also known to stabilize sensory circuits and affect neuronal excitability (Hockfield and McKay, 1983; Zaremba et al., 1989). Collectively, these data suggest that interneurons are critical for neural development. Therefore, it is important to elucidate the role of interneurons in a developing sensory and sensorimotor systems.

The current project

This dissertation project explores changes in HVC neural activity that occur in tandem with vocal learning, introduces a new method that permits analysis of intact forebrain circuits that may be important for vocal learning, and describes changes in populations of antidromically and functionally identified single units. The experiments contained within this dissertation were designed to

understand how neural activity in HVC correlates with song behavior, and to ask how circuit dynamics change in a forebrain region (HVC) during experience-dependent learning.

Song learning is achieved by sculpting variable sounds into mature copies of a learned tutor song. This process is critical for song learning, but it is unclear how neural activity in the song system relates to restricted vocal plasticity as song matures. Chapters 2 and 3 analyze multiunit activity in juveniles at the end of the sensorimotor learning period to monitor population changes in HVC activity during song learning. Chapter 2 investigates the role of premotor activity in regulating song on a millisecond timescale. We found that millisecond-by-millisecond, HVC activity predicts stability in song behavior. Specifically, bursts in HVC stabilize individual song features such as frequency, and decrements in HVC activity predict variability of specific parts of the motif.

HVC burstiness (the tendency to fire short duration, high rate bouts of action potentials) during singing increases with development (Chapter 3). This finding suggests that weakening of HVC activity might enable profound daily regulation of vocal plasticity observed in juveniles (Deregnaucourt et al., 2005). Chapter 3 directly tests the hypothesis that less activity in HVC results in greater vocal plasticity each morning in juveniles during song learning. The data show that HVC activity is weaker in the morning when song is more plastic and that activity is greater in the evening when song is more stable.

The changes that were observed in the pattern of HVC activity led to the development of a new method to record single units in ensemble recordings.

Chapter 4 details how antidromic stimulation and tetrode recording can be combined to identify projection neurons in an active circuit, and how dozens of pairs of neurons can be recorded simultaneously to analyze functional network connectivity. Finally, Chapter 5 implements the novel technique described in Chapter 4 and shows developmental changes in identified units in HVC. In addition to antidromically identified neurons, we characterized two populations of cells in HVC using coherence to identify a subset of cells 'coactive' with the population, and a subset that fires independently of the population. These 'anticorrelated' cells have longer burst durations in the juvenile, which is consistent with Crandall et al. (2007). These data also support the hypothesis that inhibition of HVC_X neurons during the sensorimotor phase may be important for song development. We propose that HVC entrains the AFP via HVC_X cells to promote song learning. Consistent with this hypothesis, we report that HVC_X units have longer burst durations in adults than in juveniles. We did not observe changes in HVC_{RA} cells. Together, these changes provide insight into the neural mechanisms that underlie vocal learning in the zebra finch.

Chapter 2

Top-down regulation of plasticity in the birdsong system: “premotor” activity in the nucleus HVC predicts song variability better than it predicts song features

Day NF*, Kinnischtzke AK*, Adam M, Nick TA (2008)

J Neurophys 100: 2956-2965

*These authors contributed equally to this work.

We studied real-time changes in brain activity during active vocal learning in the zebra finch songbird. The song nucleus HVC is required for the production of learned song. To quantify the relationship of HVC activity and behavior, HVC population activity during repeated vocal sequences (motifs) was recorded and temporally aligned relative to the motif, millisecond by millisecond. Somewhat surprisingly, HVC activity did not reliably predict any vocal feature except amplitude and, to a lesser extent, entropy and pitch goodness (sound periodicity). Variance in “premotor” HVC activity did not reliably predict variance in behavior. In contrast, HVC activity inversely predicted the variance of amplitude, entropy, frequency, pitch, and FM. We reasoned that, if HVC was involved in song learning, the relationship of HVC activity to learned features would be developmentally regulated. To test this hypothesis, we compared the HVC song feature relationships in adults and juveniles in the sensorimotor “babbling” period. We found that the relationship of HVC activity to variance in FM was developmentally regulated, with the greatest difference at an HVC vocalization lag of 50 ms. Collectively, these data show that, millisecond by millisecond, bursts in HVC activity predict song stability on-line during singing, whereas decrements in HVC activity predict plasticity. These relationships between neural activity and plasticity may play a role in vocal learning in songbirds by enabling the selective stabilization of parts of the song that match a learned tutor model.

INTRODUCTION

Experience permanently alters the brain through cellular and circuit changes during critical or sensitive periods of sensory development (Wiesel and Hubel, 1963; Knudsen, 2004; Hensch, 2005). We do not yet understand the mechanisms that define sensitive periods in the development of motor circuits that control behavior. Vocal learning in songbirds provides an ideal model of a sensitive period in the development of a complex, sequential behavior. Zebra finch songbirds, like humans, learn their vocalizations (Marler, 1970; Doupe and Kuhl, 1999). During the sensorimotor period, finch vocalizations progress over an ~55-day period from simple prototypes to a mature song motif, which is an ~1-s series of complex sounds emitted in a stereotyped pattern (Bolhuis and Gahr, 2006). Sounds separated by silence are termed “syllables.” Syllables are concatenated to form motifs, and motifs are concatenated to form songs. Emitted sounds are progressively matched to a learned tutor song using auditory feedback (Konishi, 1965). The process of learning can be observed motif-by-motif, syllable-by-syllable, as the bird slowly sculpts his song toward the mature form (Deregnaucourt et al., 2005).

The neural song system is a series of anatomically distinct clusters of neurons (nuclei) in the thalamus, basal ganglia, and pallium (cortex) that are dedicated to the production and plasticity of song (Nottebohm et al., 1976; Bottjer et al., 1984). The nucleus HVC (Reiner et al., 2004) is a pallial brain region that controls song behavior (Nottebohm et al., 1976; Vu et al., 1994). During anesthesia (Volman, 1993) and sleep (Nick and Konishi, 2005b), HVC always

responds preferentially to the bird's own song, even very early in song development. HVC activity during singing changes as song matures, most dramatically demonstrated by prolonged afterdischarges or bursts in juveniles that are rarely observed in adults (Crandall et al., 2007a). During the sensitive period for vocal learning in the wake state, a neural signal that is selective for the tutor song has been recorded in HVC (Nick and Konishi, 2005a), which suggests that instructive auditory signals have access to the juvenile HVC during waking and, perhaps, during singing. In addition, HVC activity during sleep is positively correlated with overnight song stability in juveniles (Crandall et al., 2007b), which suggests that HVC sleep activity may have a role in song learning. Collectively, these data indicate that HVC activity is dynamically regulated during song learning and suggest that it is a locus of vocal plasticity. The simplest test of the role of HVC in song plasticity would be to lesion it. However, lesioning HVC prevents production of learned song (Nottebohm et al., 1976). Instead, male finches with HVC lesions produce primitive vocalizations that are song-like (Simpson and Vicario, 1990; Aronov et al., 2008). Thus direct neural–behavioral comparisons during song learning have been used to ascertain the role of HVC in plasticity (Crandall et al., 2007b; Crandall et al., 2007a).

HVC seems to code song behavior at the motif level, as opposed to the level of syllables or songs (Vu et al., 1994; Yu and Margoliash, 1996; Hahnloser et al., 2002). Analysis of song development indicates that shaping of song occurs at the level of the motif, because syllables mature in the temporal context of the motif, with no transpositions (Tchernichovski et al., 2001). For these reasons, our

laboratory has focused on neural–behavioral comparisons during motifs to study the role(s) of HVC in song plasticity (Crandall et al. 2007b). Bursts of population activity during motifs decrease in duration and increase in rate during vocal development (Crandall et al., 2007a). How might these bursts relate to behavior?

A basal ganglia-thalamic-cortical loop, the anterior forebrain pathway (AFP), is necessary for song plasticity (Bottjer et al., 1984; Brainard and Doupe, 2000). Recent studies have clarified the role of the AFP in plasticity (Kao et al., 2005; Olveczky et al., 2005; Kao and Brainard, 2006; Thompson et al., 2007): the AFP seems to induce variability in song behavior that is necessary for vocal learning through trial-and-error (Fig. 1). Furthermore, two of these studies indicate that gross anatomical lesioning or inactivation of one of the nuclei of the AFP [the lateral magnocellular nucleus of the anterior nidopallium (LMAN)] correlates with an immediate decrement in moment-by-moment song variability (Olveczky et al., 2005; Kao and Brainard, 2006). Collectively, these data suggest that the role of the AFP role in song plasticity is to induce variability on short time scales and thus enable trial-and-error. What selects the sounds that are stabilized? What restricts motor plasticity during development? If the AFP alone controls song variability, why does activity in the afferent nucleus HVC change with development (Volman, 1993; Nick and Konishi, 2005b; Crandall et al., 2007b; Crandall et al., 2007a) and why is auditory information routed through HVC? Because a memory of the tutor song guides song learning through auditory feedback (Konishi, 1965), a neural representation of the tutor song and auditory signals must play a role in song stabilization. If tutor song–selective

neural auditory signals stabilize the bird's own song (BOS), where is the memory of the BOS stored? HVC responds selectively to both the BOS and the tutor song (Margoliash, 1983; Margoliash and Konishi, 1985; Volman, 1993; Nick and Konishi, 2005a) and projects directly to Area X, which is part of the AFP. In addition, the HVC neurons that project to Area X do not distinguish between hearing and production of the BOS in the mature adult (Prather et al., 2008), which suggests that the HVC neural signal driven by BOS auditory playback may be sensorimotor, as opposed to purely sensory, in nature. Consistent with a role for HVC in sensorimotor integration, HVC activity during singing (Crandall et al. 2007b) and in response to auditory playback of the BOS (Volman, 1993; Nick and Konishi, 2005b) and the tutor song (Nick and Konishi, 2005a) changes with developmental song learning. Does HVC have a role in the auditory-guided restriction of plasticity during vocal learning?

To assess the relationship of HVC activity to song plasticity and stability, we examined HVC population activity millisecond by millisecond during singing in juvenile and adult zebra finches. Instead of profoundly altering the song system using gross inactivation and lesioning, we used developmental song learning to naturally change behavior over thousands of motifs and show underlying neural mechanisms. These experiments are only possible in longitudinal recordings. Using established methods, we extracted motifs and corresponding neural activity across entire days and weeks (Crandall et al., 2007b). We used multiunit recordings that are very stable and more informative (better at predicting behavior) than any other intracortical signal, including single unit activity and

local field potentials (Stark and Abeles, 2007). We found that peaks in HVC activity predicted song stability, whereas activity troughs predicted plasticity. These data suggest that, in addition to its well-know role in song production (Nottebohm et al., 1976; Vu et al., 1994), HVC may also regulate song variability and, consequently, trial-and-error learning.

METHODS

Subjects

Forty-one juvenile (age 61–90 days; in the late sensorimotor stage of song development) and 22 adult (>90 days) male zebra finches (*Taeniopygia guttata)* were surgically implanted with chronic population recording electrodes. Of the juveniles, 17 had high-quality neural recordings (premotor RMS signal:noise >2), but only 10 sang ~100 motifs in at least 1 day and were used for this study. Of the adults, five had high quality neural recordings and sang ~100 motifs. Three of the finches implanted as juveniles (Blue-70, Blue-15, and Blue-81) were also recorded after day 90 (~100 motifs) and thus included in the adult group for those days. All song recordings were made in the absence of a female (undirected). Undirected song is thought to reflect song practice (Jarvis et al., 1998). All juvenile and three of the adult finches were reared in our facility on a 12:12 light cycle. Two adult birds were obtained from a commercial supplier. Although experience and not age is probably the strongest predictor of song system maturity, age is correlated with experience and presents a more quantifiable parameter. None of the finches used in these experiments were ever exposed to auditory playback. The finches were allowed to hear their own vocalizations. All

procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Chronic physiological recording

Basic methods have been previously described (Crandall et al., 2007a). For population recordings, finches were implanted with a set of recording electrodes: one or two 50-μm nichrome-formvar electrodes in HVC (not plated, 1.1–1 MΩ, AM Systems, Carlsborg, WA), a 50-μm nichrome-formvar electrode adjacent to HVC for use as a reference electrode, and 75-μm silver ground and EEG electrodes. The headstage and recording environment were previously described (Schmidt and Konishi, 1998; Nick and Konishi, 2001).

All data were acquired with custom-written (Datafleet, Minneapolis, MN) LabView software (National Instruments, Austin, TX) at a sampling frequency of 44.1 kHz. During recording, a lightweight operational amplifier was attached to the bird and connected to a mercury commutator via a flexible cable. HVC neural activity was amplified 1,000 times and filtered 300–10,000 Hz. Song was monitored with a microphone (Earthworks, Milford, NH), high-pass in-line filtered at 100-Hz (Shure, Chicago, IL), and recorded. Localization of electrodes to HVC was confirmed with premotor activity in all cases and cresyl-violet histology in 7 of 16 finches.

Song behavior analysis

All data were analyzed with custom-written Matlab functions. Initial song analysis consisted of the sorting of sound data and exclusion of movement noise. Sound data were further sorted according to temporal properties. Preliminary

songs were defined as sounds lasting ~500 ms with time gaps of no more than 20 ms.

To specifically study activity during learned song behavior, a canonical motif was identified by a skilled observer from the oldest day available from each finch and used to extract motifs and corresponding neural activity throughout the recording period. Multiple motif forms could have been selected depending on the developmental stage. However, we were most interested in how HVC activity changed once the overall rhythm had been established and thus selected the most mature motif available. Because perfusion and histology were performed as soon as the electrode recording declined, the final mature motif may not have been achieved by some subjects.

We focused our analysis on behaviorally relevant vocalizations (instead of, for example, movements) by band-pass filtering our vocal data (1–8 kHz). Amplitude envelopes of the canonical motif and all preliminary songs were constructed by filtering the rectified sound recording with a Savitzky-Golay smoothing filter (4th-order polynomial fit; 20-ms frame size). Amplitude envelopes of the canonical motif and preliminary songs were cross-correlated (Crandall et al., 2007a). Sharp peaks in the cross-correlation indicated the onset of a motif that matched the canonical motif. Behavioral song motifs and corresponding neural activity were excised and saved for further analysis.

Song was quantified based on six features: amplitude, Weiner entropy, frequency, pitch, FM, and pitch goodness (a measure of sound periodicity). These features were calculated from the raw motif in 9.3-ms bins with sliding 1-

ms steps with a subprogram of Sound Analysis Pro (Tchernichovski et al., 2000), ported to Matlab by S. Saar.

Multiunit physiology analysis and comparisons to song features

To examine patterns of population spiking activity during the motif, amplitude envelopes of neural activity during motifs were created by band-pass filtering 300–6,000 Hz, rectifying, and smoothing with a Savitzky-Golay smoothing filter (4th-order polynomial fit; 50-ms frame size).

For each bird, neural data for all available motifs were temporally aligned, and the mean and variance for each millisecond was taken across all motifs for each day. Likewise, data for each song feature were aligned, and the mean and variance for each millisecond was taken across all motifs. For the three birds that spanned the 90-day age, data preceding and on/following day 90 were analyzed separately and classed as “Juvenile” or “Adult,” respectively.

Statistical analysis and data presentation

Vectors representing the mean or variance for neural activity or vocal features were compared using multiple linear regression. Neural activity preceding song features by 30–70 ms was used for comparisons. Pearson’s correlation coefficients (Rs) were compared between adults and juveniles using unpaired Student’s *t-test* (2-tailed). For linear regression comparisons of juveniles and adults, ideally each bird would contribute only one Pearson *R* value for each feature and time lag, even though multiple days were recorded. Thus for each bird, single vectors for each feature and for neural activity were calculated by taking the mean of each parameter for each millisecond across multiple days.

All bars represent means and all error bars represent SE. Significance was defined as p= 0.05. A Bonferroni correction for multiple comparisons was applied to the five time-point analyses (30 –70 ms neural preceding song) of the same vocal feature. The criterion for significance in these cases was P α/5 = 0.01.

RESULTS

Lulls in HVC activity occur during plastic song syllables

Qualitative comparison of song behavior and smoothed HVC population activity showed that lulls in HVC bursting were correlated with increased plasticity in the corresponding song syllable. The example shown in Fig. 2 shows data from a relatively mature finch (99 days) that had a partially crystallized song. There was strong HVC population burst rhythm except during a syllable that was still quite variable with regard to duration (Fig. 2; other examples in Supplementary Figs. 1 and 2). Note the decrease in HVC activity during the variable syllable (solid arrowheads and yellow boxes). These exemplar data suggest that song plasticity increases when HVC activity decreases. The remainder of the study quantifies and elaborates this idea.

Temporal alignment of song motifs shows reliable patterns in HVC activity and song features

To quantitatively test the hypothesis that HVC oscillations relate to song plasticity, it is important to identify temporal song segments or syllables that are plastic and compare them over multiple renditions. However, if the song segment is plastic, it is difficult to identify it based on its own song features because they

are inherently unstable. Therefore we used the temporal context of the motif to align song elements, both stable and plastic.

We measured HVC activity during singing across multiple days. Zebra finches were maintained in the same recording chamber for many days before and during recording. Finches were only disturbed for food and water replenishment. The electrodes were never experimentally adjusted. These efforts resulted in stable recordings over many days and, in a few cases, for several weeks. For all data shown, the finch had recovered from surgery for ~6 days, been in the recording chamber for ~3 days, and sang in the chamber at least 1 day. All HVC activity in this manuscript occurred during undirected song motifs. “Juveniles” were 61–90 days of age (in the late sensorimotor stage of song development) and “adults” were >90 days of age (Supplementary Table 1).

As in a previous study (Crandall et al., 2007a), cross-correlation of the amplitude envelopes of each bird’s relatively mature motif and his entire song database enabled precise extraction of song motifs and corresponding neural activity. For analysis, HVC activity was rectified and smoothed. Song motifs were analyzed using six song features calculated by a subprogram of Sound Analysis Pro (Tchernichovski et al., 2000): amplitude, entropy, frequency, pitch, FM, and pitch goodness (sound periodicity). Temporal patterns relative to the motif (Fig. 3A) were found in both the neural activity (Fig. 3B) and song features (Fig. 3, *C* and *D).* The reader may note the bars of red (high levels) and blue (low levels). Although the overall pattern of HVC activity was stable, there were small changes in some bars (e.g., they shifted in time relative to the motif and/or

changed in duration). To quantify the pattern of HVC activity across each day, we took the mean and variance of the HVC activity for each millisecond. Similar to this millisecond level analysis of HVC activity, daily patterns of song features were quantified by taking the mean and variance across all motifs for each millisecond of the motif.

HVC activity correlates with the mean and variance of some song features

To directly assess potential effects of HVC activity on song features, we compared the mean and variance vectors computed for each millisecond of the motif. We used linear regression to compare the relationship of song features at each millisecond to the neural activity preceding that millisecond. Figure 4 shows the comparison of mean HVC activity (50 ms prior) to respective song features: the mean and variance of amplitude and FM. As would be expected for a premotor area, HVC activity was correlated with the mean amplitude of song (Fig. 4A). Mean HVC activity inversely predicted amplitude variance (Fig. 4B). In contrast to amplitude, HVC did not directly predict FM (Fig. 4C). More interestingly, HVC weakly predicted FM variance.

Although HVC activity predicts some song features, the predictive capacity is not developmentally modulated or temporally precise

To quantitatively assess the effects of HVC activity on song behavior across development, we compared the Pearson *Rs* for mean HVC activity versus the mean of each song feature using unpaired *t-test* (Fig. 5). The relationship of song features at each millisecond was compared with the neural activity preceding that millisecond by 30–70 ms. Increased HVC activity directly

predicted increased amplitude at all time points examined (Fig. 5, row 1). The peak relationship was with HVC activity preceding the corresponding song millisecond by 40 ms, although the relationship in the time range 30–50 ms was fairly stable. This suggests that HVC's prediction of amplitude is not temporally precise. Mean HVC activity also weakly predicted decreased entropy and increased pitch goodness in juveniles (Fig. 5, rows 2 and 3). However, these effects were not temporally precise (compare 30 – 60 ms) or significantly different between juveniles and adults. The reader may note the near-significant comparison at 50 ms for pitch goodness (significance with a Bonferroni correction is $P < 0.01$). Collectively, these data indicate that HVC activity levels do not directly specify song features with temporal precision or developmental regulation.

Variance of HVC activity predicts variance in some song features, but the predictive capacity is not developmentally modulated

One might predict that variance in the activity of a premotor area would be reflected in the variance of behavior. Surprisingly, this is not what we generally found in the time range typically defined as "premotor" in HVC (40 – 60 ms; Fig. 6; Supplementary Fig. 3) (McCasland and Konishi, 1981; Troyer and Doupe, 2000b). HVC activity variance 50 ms before the corresponding song feature is shown in Fig. 6. The variance of HVC activity weakly predicted variance in song amplitude but little else. These relationships were not significantly developmentally modulated. These data suggest that a great deal of HVC activity preceding song does not directly specify spectral aspects of vocalizations.

Mean HVC activity predicts variance in five song features, three with temporal precision and developmental regulation

Unlike the variance in HVC activity, which had little predictive value for song variation, mean HVC activity did predict variance in most song features. In juveniles, the variances of all features except pitch goodness were inversely predicted by HVC activity [note the nonzero *R* values for juveniles (black) in Fig. 7]. The variance of amplitude and Weiner entropy were equally well predicted by HVC activity in juveniles and adults (Fig. 7, rows 1 and 2). In contrast, FM was developmentally regulated (Fig. 7, row 6). In addition, the developmental regulation of frequency and pitch was near significant (Fig. 7, rows 4 and 5). Notably, the developmental modulation was significant for FM variance when it was compared with HVC activity 50 ms prior, but not other time points, indicating that the developing HVC–FM variability relationship was temporally precise (Fig. 7, row 6, column 3). Likewise, the relationship of the variance of frequency and pitch to mean HVC activity peaked at 50 ms in the juvenile. Thus the strongest relationships between neural activity and song variance were with neural activity leading behavior by 50 ms. These results confirm prior reports that have estimated an ~50-ms neurobehavioral delay (McCasland and Konishi, 1981; Troyer and Doupe, 2000b).

DISCUSSION

This study provides the first evidence that the song nucleus HVC may restrict plasticity during sensorimotor learning. Evidence indicates that HVC is involved in the production of learned song (Nottebohm et al., 1976; Simpson and

Vicario, 1990; Vu et al., 1994). This would suggest that HVC activity should be correlated with song. However, direct comparison of HVC activity and emitted sounds during singing showed that the mean HVC activity did not correlate well with the mean of most song features, nor did the variance of HVC activity correlate well with the variance of most song features. In contrast, mean HVC activity levels correlated with decreased song variance, millisecond by millisecond (Fig. 8). In addition, the relationship of mean HVC activity levels and song variance was temporally precise and, for frequency components, developmentally modulated. These data strongly suggest that HVC activity is actively and dynamically involved in real-time song learning. Furthermore, they showed an additional, novel mechanism through which premotor brain areas may shape vocal behavior through selective stabilization of specific time windows of sequentially modulated sounds.

Does HVC activity affect song stability through the AFP?

We analyzed the HVC song nucleus in a behavioral context and found that, in addition to its well-known role in song production (Nottebohm et al., 1976; Simpson and Vicario, 1990; Vu et al., 1994), HVC also seems to decrease song variability on moment-to-moment time scales. Potentially, increased HVC activity could more strongly drive the HVC projection to the nucleus robustus arcopallialis (RA; song “motor cortex”) and thereby reduce behavioral variability. However, the relationship between HVC activity levels and the variance of song was differentially regulated during development based on song feature, suggesting a more interesting model. Increased HVC activity could entrain the activity of the

AFP via HVC's only other efferent connection, to Area X (song basal ganglia). Previous correlative lesioning and inactivation studies have argued that the AFP, which is not in the direct motor pathway, drives song variability (Fig. 1; Kao and Brainard 2006; Olveczky et al. 2005). Lesioning the output nucleus of the AFP (LMAN) correlates with decreased song variability and impaired vocal learning (Bottjer et al. 1984; Kao et al. 2005; Olveczky et al. 2005). If the HVC-to-RA motor pathway alone could induce song variability and enable trial-and-error learning, why would lesioning the AFP block song learning (Bottjer et al. 1984)? It is possible that the memory of the learned tutor song is stored or compared with ongoing behavioral feedback in the AFP. However, studies from many laboratories using a variety of techniques indicate that the locus of the tutor memory and the site of memory-feedback comparison are found upstream of HVC (Mello and Clayton, 1994; Jarvis and Nottebohm, 1997; Nick and Konishi, 2005a; Bolhuis and Gahr, 2006; Phan et al., 2006). Although some neural processing loops could place the AFP upstream of HVC, synaptic delays and coding efficiency support the more parsimonious explanation that auditory areas and/or the nuclei that project to HVC (uva, nucleus interfacialis, and the medial magnocellular nucleus) contain the tutor song memory and compare it to auditory feedback. In light of the data presented here, we propose that modulation of HVC activity level controls song variability and trial-and-error learning by controlling the AFP. If the effect of HVC on song variability is orchestrated through the AFP, it cannot be considered direct motor control but higher order planning or prediction. If the effects of HVC on song variability are direct through RA, the

proposed role of the AFP in inducing song variability and thereby enabling vocal experimentation (Olveczky et al., 2005) should be reassessed.

Cellular model of motor control in the song system

How might HVC population activity affect song variability? Population activity is relatively uniform across the nucleus HVC (Schmidt, 2003; Nick and Konishi, 2005b). Inhibitory interneurons maintain spatially broad oscillations (Buzsaki and Chrobak, 1995) and promote synchronous activity in cortex/pallium (Galarreta and Hestrin, 2001; Hasenstaub et al., 2005b). The most active neurons in HVC are fast-spiking neurons, which dominate multielectrode single-unit recordings (Crandall et al. 2007b) and which may become more synchronous with development (N. Aoki, D. Q. Nykamp, and T. A. Nick, unpublished observations). Collectively, these data led us to propose that faster oscillatory bursting activity characteristic of the adult HVC (Crandall et al. 2007b) is caused by the synchronous firing of interneurons. Interneurons shape the activity of projection neurons (Cobb et al., 1995; Klausberger et al., 2003). We propose that synchronous activity in HVC interneurons entrains the activity of Area X–projecting HVC neurons and thus entrains the activity of the AFP. Control of the AFP would control variability in the behavior, because the output nucleus of the AFP (LMAN) seems to induce variability in vocal output (Kao et al., 2005; Olveczky et al., 2005). We hypothesize that as HVC interneuron bursts become stronger in development, the activity of Area X–projecting neurons becomes more entrained, the AFP becomes more entrained, the song becomes less variable, and plasticity becomes more difficult to induce. A companion hypothesis

predicts that HVC interneurons entrain RA-projecting neurons as well to produce more stable behavior. It is also possible that the effects on song variability that we observe result at least partially from the introduction of synaptic “noise” directly onto the HVC neurons that project to RA, particularly when HVC activity is dominated by lower-frequency activity in juveniles (Crandall et al. 2007b). However, the fact that lesions of the AFP block song plasticity (Bottjer et al., 1984; Williams and Mehta, 1999; Brainard and Doupe, 2000) suggests that control of song variability and plasticity involves the AFP (see above). We have examined correlations between neural activity and behavior in finches that have been relatively unperturbed by our experiments but strongly perturbed by the developmental processes that naturally occurred during our long-term recordings. Our findings were only possible because we allowed the system to develop naturally without experimental manipulation. The next set of experiments will test the cellular hypotheses that arose from these longitudinal neurobehavioral studies using technically easier, shorter-term recording and acute perturbation with pharmacological and electrical methods.

Song learning in the context of top-down motor control

All areas of the cerebral cortex are subject to top-down control, through which complex information at higher processing stages shapes lower-level processes (Gilbert and Sigman, 2007). However, the developmental assembly of top-down control is completely uncharted. Furthermore, potential roles of top-down control in the development of complex behaviors are not understood. Top-down control in the song system may represent an expectation of the bird’s own

song based on prior experience. Such feed-forward predictions alter the influence of bottom-up sensory inputs (Driver and Frith, 2000). As a consequence, strong top-down control may be anathema to sensory-guided plasticity (Engel et al., 2001). If so, initially weak top-down influences may be crucial for sensorimotor learning (Engel et al., 2001; Buschman and Miller, 2007). During song development, we speculate that release of top-down control may be necessary for bottom-up influences to affect network activity and shape the vocalization. After song maturation, increased top-down control of song through increased oscillatory activity may explain the lack of immediate effects of perturbation of sensory feedback (Zevin et al., 2004).

The oscillatory pattern in HVC may represent an expectation or prediction of the learned song that is driven by auditory signals. In the mature adult, the prediction could be driven by both the motor program, contained in the pattern of firing of HVC RA-projecting neurons and/or extrinsic inputs, and by auditory stimuli that match the product of the motor program, the bird's own song. In the juvenile, the sensorimotor expectation should be weaker, according to this hypothesis. Previous work has shown that the auditory response to playback of the bird's own song increases with development (Nick and Konishi, 2005b). A tutor song matching signal (Nick and Konishi, 2005a) could strengthen the bird's own song prediction based on the degree of the match between the song produced and the tutor song. Because the prediction would not directly drive behavior, Hebbian processes would be released from the constraints of synaptic motor and auditory feedback delays (Troyer and Doupe, 2000b). Prolonged

bursting in juvenile, but not adult, HVC neurons (Crandall et al., 2007a) provides a potential mechanism through which the delay between premotor activity that predicts sound *A* and auditory feedback signals from sound *A* could be bridged during a sensitive period in development and thereby increase interconnections of effective functional networks.

The correlations that we report are relatively weak. This may be due to the fact that HVC is inherently sensorimotor, not just motor, and thus sensory feedback signals during singing may cloud the purely premotor–behavioral relationship. The weak correlations may be necessary for song learning, which proceeds over an ~55-day period. Stronger effects of HVC activity on song variability may not allow enough plasticity or stability for optimal song learning. Whatever the case, no correlation between song system activity and song variance has ever been reported, and thus the effects of differing degrees of correlation have no context for evaluation.

Neuroethological implications

Why would a premotor area selectively modulate plasticity in frequency components during a vocal sensitive period? Song is an indicator trait that conveys the fitness of a male zebra finch to prospective female mates. Such a trait can only be reliable if it is costly (Zahavi, 1975). There seem to be trade-offs between song frequency bandwidth and syllable rate, with an upper performance limit defined by the highest bandwidth and rate (Podos, 1997; Ballentine et al., 2004). Female swamp sparrows and canaries prefer males performing nearest this upper performance limit (Draganoiu et al., 2002; Ballentine et al., 2004). HVC

seems to have a role in song timing and thus rate (Hahnloser et al., 2002; Solis and Perkel, 2005). We have provided the first evidence that HVC also may have a role in regulating song FM. Furthermore, the relationship of HVC to variance of frequency components changes with development. This finding is consistent with the “developmental stress” hypothesis that proposes that, for song indicator traits, the costs occur during development rather than production (Nowicki and Searcy, 2005). Because the relationship of HVC to FM variance is developmentally regulated and the burst rate of HVC activity during singing increases during song learning (Crandall et al., 2007a), it is possible that HVC is the site of bandwidth rate optimization during developmental song learning.

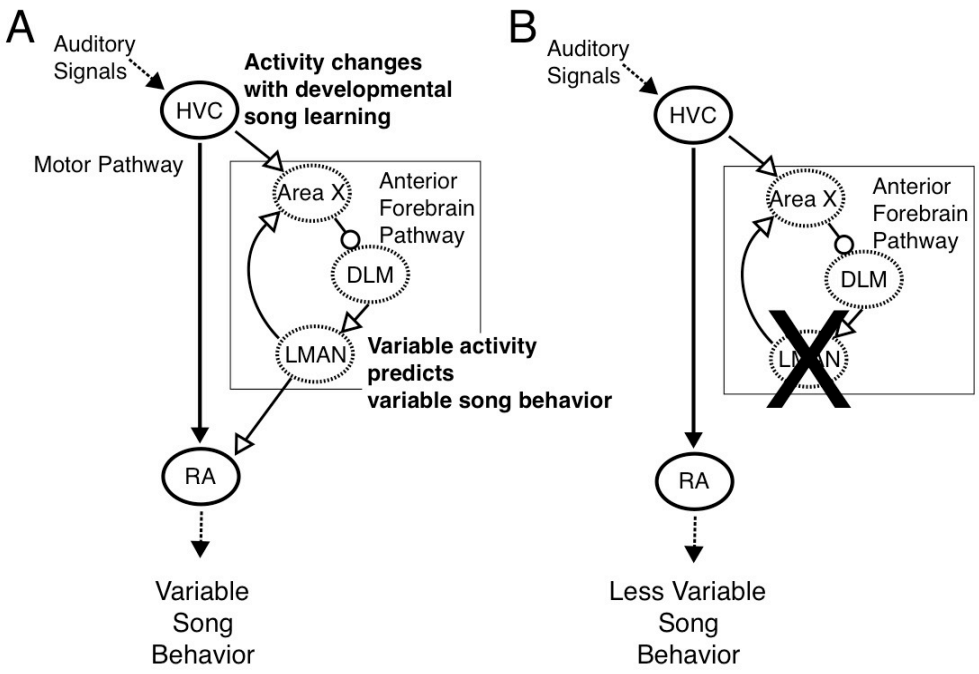

Figure 1. Recent data have suggested that the neural circuitry that generates vocal fluctuations is located outside and parallel to the song motor pathway in the Anterior Forebrain Pathway (AFP).
(A) The AFP appears to generate song variability, which is necessary for trial-and-error learning (Kao et al., 2005; Olveczky et al., 2005; Kao and Brainard, 2006). Stimulation of LMAN induces behavioral variability, possibly by inducing variability in the motor pathway through synapses in RA (Kao et al., 2005). (B) Lesioning or gross inactivation of LMAN decreases song variability (Kao et al., 2005; Olveczky et al., 2005; Kao and Brainard, 2006). How is song stabilized during development and shaped by auditory signals? Auditory signals selective for the learned song are passed from HVC to both the motor pathway and the AFP. Accumulating data indicate that activity in song nucleus HVC changes during the sensorimotor sensitive period of vocal learning (Volman, 1993; Nick and Konishi, 2005b, a; Crandall et al., 2007b; Crandall et al., 2007a), suggesting that HVC is involved in auditory-guided plasticity. The current study examines how HVC activity relates to behavior during singing on multiple timescales. Abbreviations, clockwise from top: HVC: this acronym is the proper name; Area X: this is the proper name; DLM: dorsolateral part of the medial thalamus (inhibited by Area X projections); LMAN: lateral magnocellular nucleus; RA: robust nucleus of the arcopallium.

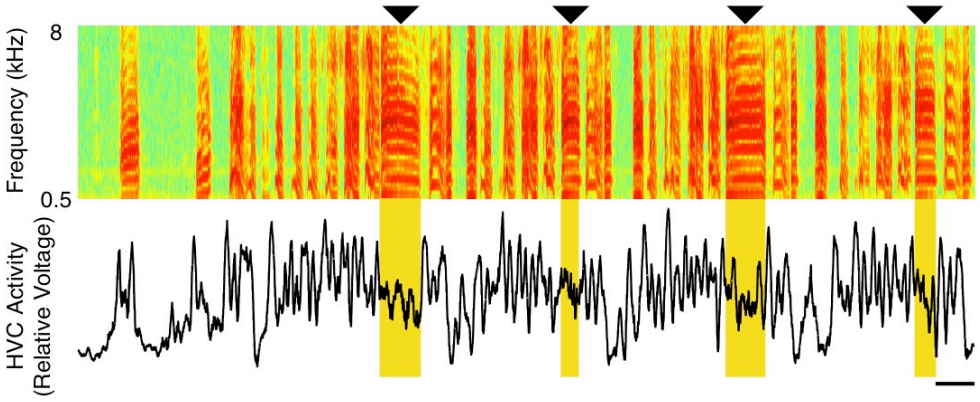

Figure 2. HVC activity oscillates with lower power during song syllables that are plastic. Data from a relatively mature animal illustrate differences in activity between relatively stable and more plastic syllables. The yellow boxes and black arrowheads indicate a syllable that varied in duration within single songs. Note the dampening of HVC oscillations during the plastic syllable relative to other activity within the same motif. HVC activity was rectified and smoothed. Scale bar: 200 msec. Representative data are from bird Blue-81, age 99 days.

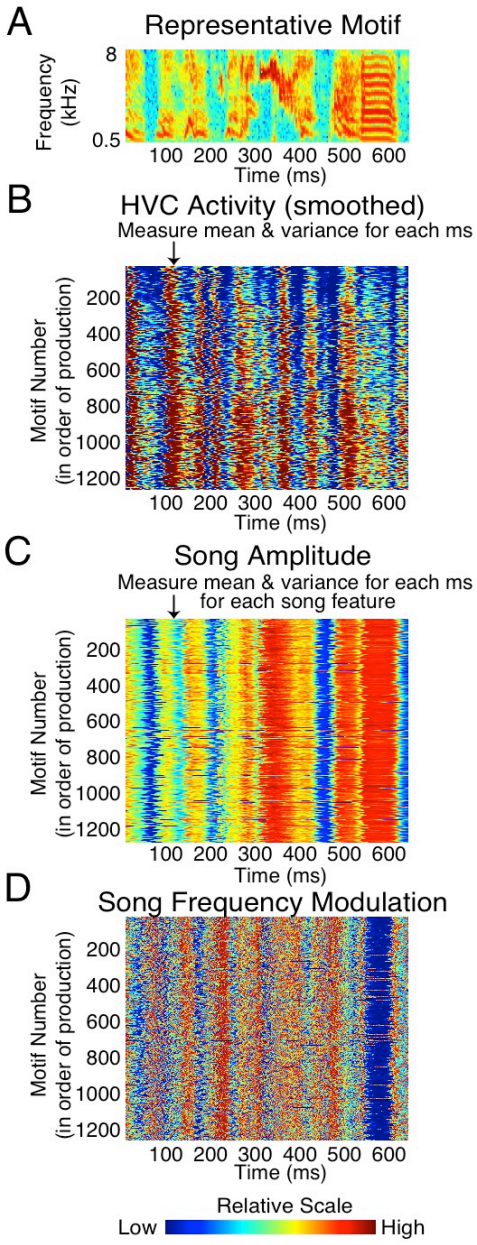

Figure 3. Example data from a single day show alignment of bursts of HVC neural activity, song amplitude, and song frequency modulation.
(A) A representative motif for comparison with cross-day data. (B) HVC activity bursts at reliable times during the motif. The arrow indicates that the mean of neural activity was calculated for each millisecond across motifs. (C) Song amplitude varies across each motif, but is relatively stable across motifs at a given millisecond. The arrow indicates that the mean and variance of song features were calculated for each millisecond across motifs. (D) Song frequency modulation is also relatively stable across motifs at a given millisecond. Example data are from bird Blue 70, age 90 days.

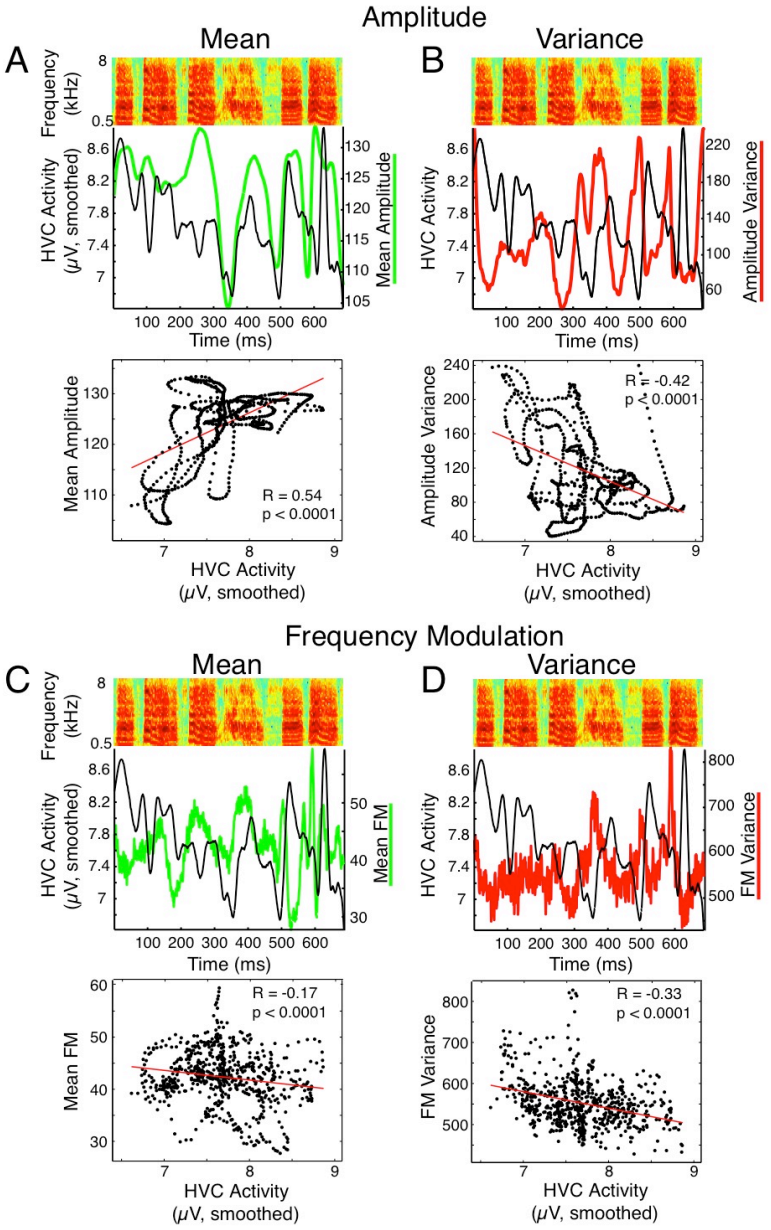

Figure 4. Neural activity correlates with some song features, but not others.
(A) Mean HVC activity correlates with mean song amplitude. (Top) The same representative sonogram is shown at the top of each feature plot for temporal orientation purposes only. (Middle) Mean amplitude (green) is plotted on the same axis as mean smoothed HVC activity to demonstrate temporal correlations. Note that when HVC activity is high, so typically is the mean amplitude. (Bottom) Linear regression reveals a direct relationship between HVC activity and song amplitude. (B) Amplitude variance is negatively correlated with HVC activity. (Middle) Amplitude variance (red) appears to peak when HVC activity is low, and vice-versa. (Bottom) HVC activity inversely correlates with amplitude variance, as shown by linear regression. (C) Mean frequency modulation does not appear to be correlated with HVC activity. (D) In contrast, the variance of frequency modulation peaks when HVC activity is low, and vice-versa (Middle). (Bottom) This leads to a small inverse correlation. Example data are from bird Red 136, age 65 days.

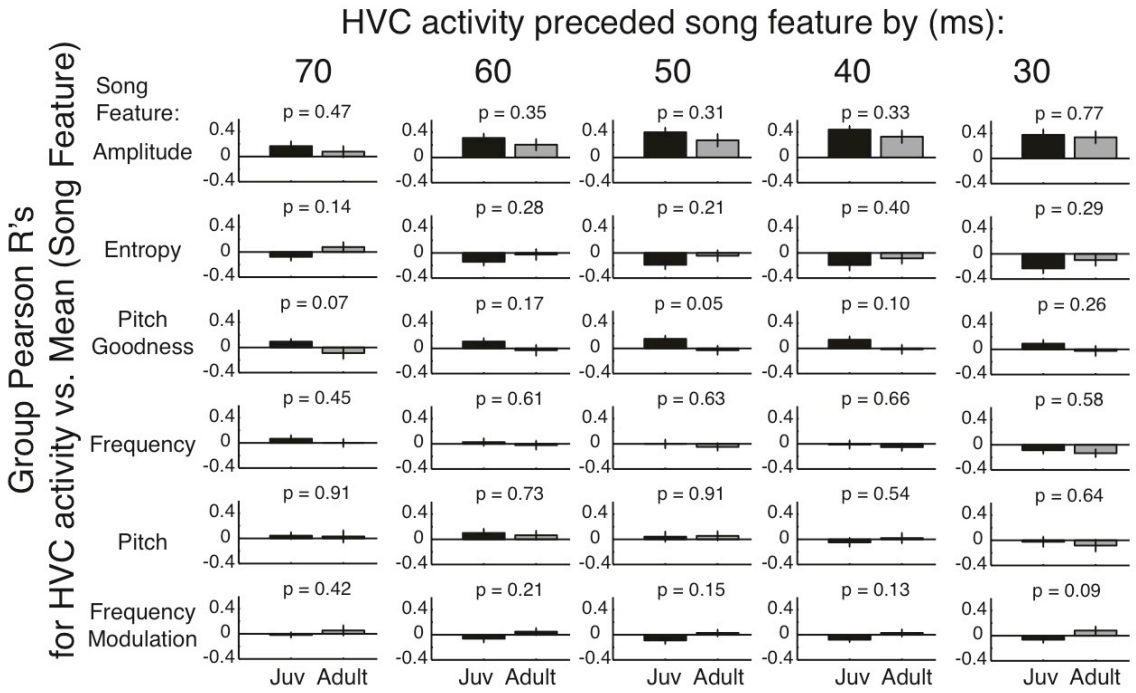

Figure 5. HVC activity directly predicts mean amplitude, but little else.
Bar plots of juvenile (black; N = 10 finches) and adult (gray; N = 8 finches) mean Pearson R's reveal no developmental modulation of HVC-song feature relationships. Each row contains the temporal data from a single song feature. Each column contains the feature for a given HVC-song lag. With a Bonferroni correction for multiple comparisons, significance is defined as $p < 0.01$.

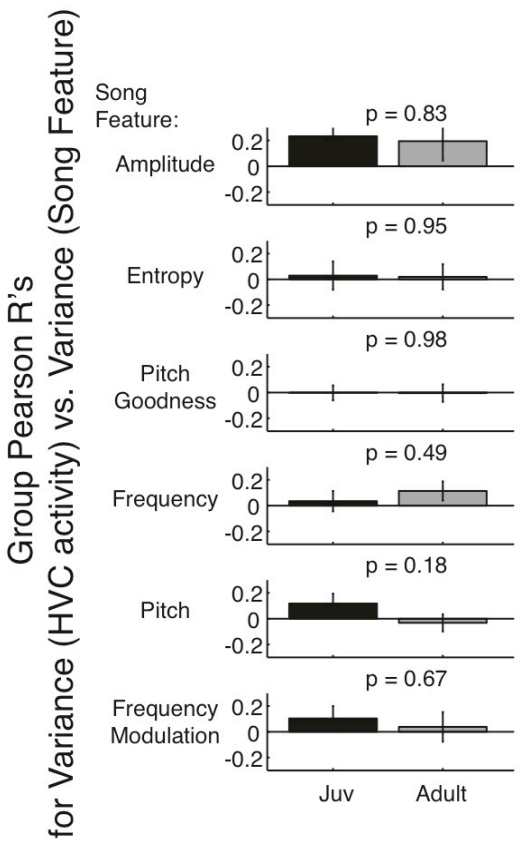

Figure 6. Variance in HVC activity directly predicts variance in amplitude, but little else. Bar plots of juvenile (black; N = 10) and adult (gray; N = 8) mean Pearson R's reveal no significant developmental modulation of variance of HVC - variance of song feature relationships. Each row contains the temporal data from a single song feature, with the compare HVC activity preceding the song feature by 50 ms.

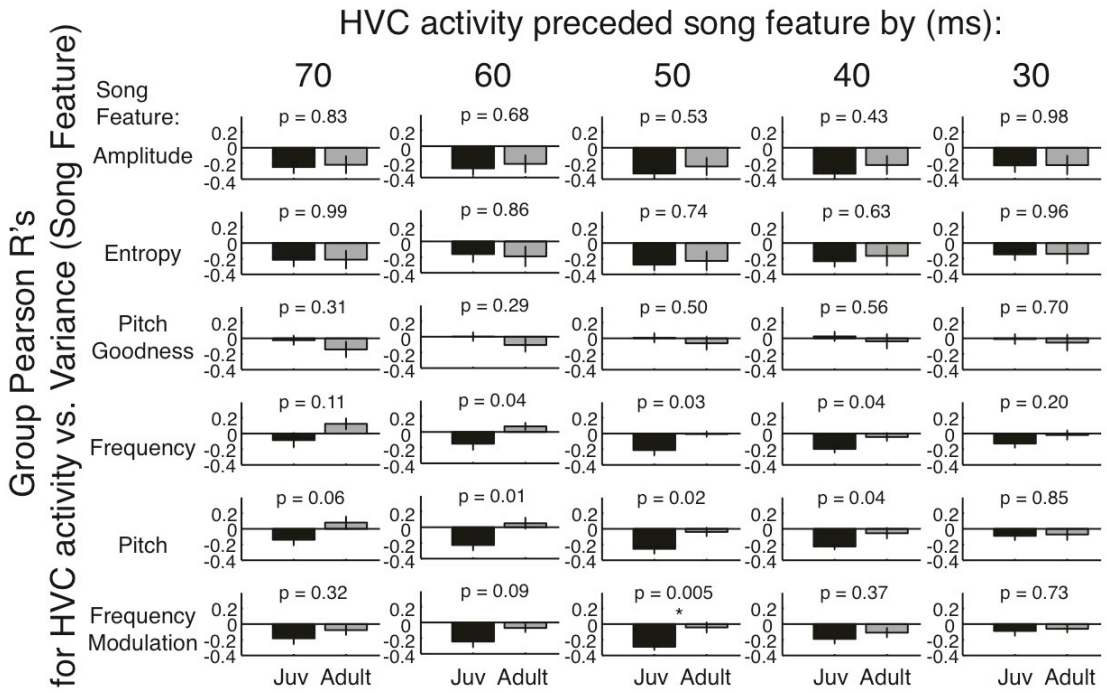

Figure 7. HVC inversely predicts song feature variance.
In juveniles (black; N = 10), all features examined except for pitch goodness were inversely predicted by mean HVC activity. This relationship did not change with development for amplitude and entropy. In contrast, the relationship of neural activity and frequency, pitch, and frequency modulation trended to be stronger in juveniles than in adults (gray; N = 8). This difference was significant for frequency modulation. As in Figure 5, each row contains the temporal data from a single song feature. Each column contains the feature for a given HVC-song lag. With a Bonferroni correction for multiple comparisons, significance is defined as $p < 0.01$.

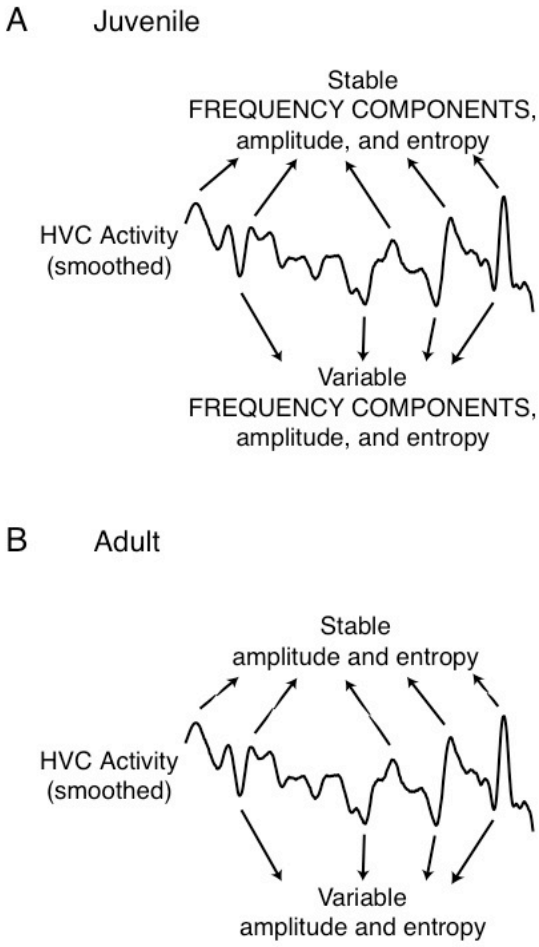

Figure 8. HVC activity peaks relate to stability in frequency components, amplitude, and entropy in juveniles, whereas activity troughs predict plasticity in these song features. In contrast, HVC activity in adults does not predict stability or plasticity in frequency components, but does relate to more stable amplitude and entropy.

Chapter 3

Daily and developmental modulation of "premotor" activity in the birdsong system

Day NF, Kinnischtzke AK, Adam M, Nick TA (2008)

Human speech and birdsong are shaped during a sensorimotor sensitive period in which auditory feedback guides vocal learning. To study brain activity as song learning occurred, we recorded longitudinally from developing zebra finches during the sensorimotor phase. Learned sequences of vocalizations (motifs) were examined along with contemporaneous neural population activity in the song nucleus HVC, which is necessary for the production of learned song (Nottebohm et al., 1976; Simpson and Vicario, 1990). During singing, HVC activity levels increased as the day progressed and decreased after a night of sleep in juveniles and adults. In contrast, the pattern of HVC activity changed on a daily basis only in juveniles: activity bursts became more pronounced during the day. The HVC of adults was significantly burstier than that of juveniles. HVC bursting was relevant to song behavior, since the degree of burstiness inversely correlated with the variance of song features in juveniles. The song of juveniles degrades overnight (Deregnaucourt et al., 2005). Consistent with a relationship between HVC activity and song plasticity (Day et al., 2008), HVC burstiness degraded overnight in young juveniles and the amount of overnight degradation declined with developmental song learning. Nocturnal changes in HVC activity strongly and inversely correlated with the next day's change, suggesting that sleep-dependent degradation of HVC activity may facilitate or enable subsequent diurnal changes. Collectively, these data show that HVC activity levels exhibit daily cycles in adults and juveniles, whereas HVC burstiness and song stereotypy change daily only in juveniles. In addition, the data indicate that HVC

burstiness increases with development and inversely correlates with song variability, which is necessary for trial and error vocal learning.

INTRODUCTION

Complex sequential behaviors such as speech require dynamic guidance from sensory feedback and temporal coordination of multiple muscles. Our understanding of how neural networks control these sensorimotor behaviors is improving. The birdsong system has been used to elucidate neural mechanisms of a complex motor skill (e.g., (McCasland and Konishi, 1981; Scharff and Nottebohm, 1991; Perkel, 2004; Prather et al., 2008). Birdsong and human speech are both learned vocalizations that are acquired during a sensitive period of development and controlled by a series of specialized forebrain nuclei (Marler, 1970; Nottebohm et al., 1976; Doupe and Kuhl, 1999). Songbirds and humans learn their vocalizations by first memorizing species-typical sounds from a tutor(s) during a sensory phase and then by matching their vocalizations to this memory using auditory feedback during a sensorimotor phase (Konishi, 1965). Birdsong consists of continuous sounds known as ‘syllables’ that are separated by silent periods. Syllables are arranged in a stereotyped sequence known as a ‘motif’, which is repeated to form songs. Song learning in the zebra finch can be observed motif-by-motif, syllable-by-syllable, as the bird slowly sculpts his song towards its mature form (Deregnaucourt et al., 2005).

The neural song system is a series of anatomically-distinct clusters of neurons (nuclei) in the thalamus, basal ganglia, and pallium (cortex) that are dedicated to the production and plasticity of song (Nottebohm et al., 1976; Bottjer

et al., 1984). HVC (this acronym is the proper name) (Jarvis et al., 2005) is a pallial song nucleus that controls song behavior (Nottebohm et al., 1976; Vu et al., 1994; Aronov et al., 2008). HVC lies at the interface of auditory and motor networks and transmits auditory signals to all downstream song nuclei, including the Anterior Forebrain Pathway (AFP), which has roles in plasticity and the induction of song variability (Doupe and Konishi, 1991; Kao et al., 2005; Olveczky et al., 2005).

Lesioning studies have confirmed that HVC is required for the production of learned song (Nottebohm et al., 1976; Simpson and Vicario, 1990; Aronov et al., 2008), but HVC's role in song plasticity, if any, has remained unclear. Data suggest that the AFP serves to destabilize song behavior (Kao et al., 2005; Olveczky et al., 2005; Thompson et al., 2007). Recently, our lab has shown that HVC 'premotor' activity during singing correlates with song stability, millisecond by millisecond (Day et al., 2008). This suggests that HVC and the AFP function antagonistically during song plasticity. In addition, HVC activity during singing changes with development (Crandall et al., 2007a) and HVC activity during sleep is positively correlated with overnight song stability in juveniles (Crandall et al., 2007b). HVC receives or generates neural signals that are selective for tutor song (Nick and Konishi, 2005a) and the bird's own song (Volman, 1993; Nick and Konishi, 2005b) depending on behavioral state (Nick and Konishi, 2005a). Collectively, these data indicate that HVC activity changes dynamically during song learning, responds selectively to tutor songs, and may stabilize the developing song.

Previously, Deregnaucourt and colleagues showed that song changes rapidly in the morning, stabilizes later in the day and degrades over a night of sleep (Deregnaucourt et al., 2005). The daily and nightly changes in behavior were dramatic, with the largest vocal changes in newly trained finches approaching 15% for some song syllables (Deregnaucourt et al., 2005). In addition, neurophysiological studies have implicated sleep in song learning (Dave and Margoliash, 2000; Crandall et al., 2007b; Shank and Margoliash, 2009). Based on these findings and recent data that show a correlation between HVC activity and song stability (Day et al., 2008), we hypothesized that premotor bursts in the juvenile HVC impede song plasticity and vary in predictable daily/nightly patterns. According to this hypothesis, HVC bursts will be weakest in the morning when song is most plastic and will become stronger later in the day as the song stabilizes. Here, we investigated daily and overnight changes in the HVC bursting activity that occurred during singing. We report that HVC 'premotor' bursting activity strengthened each day and degraded each night. These results are consistent with the hypotheses that HVC bursting activity stabilizes song and that daily changes in song behavior (Deregnaucourt et al., 2005) are driven by daily changes in HVC bursting activity.

METHODS

Subjects

41 juvenile (age 61 - 90 days; in the late sensorimotor stage of song development) and 22 adult (> 100 days) male zebra finches (*Taeniopygia guttata*) were subject to surgical implantation with chronic population recording

electrodes. Of the juveniles, 17 had high quality neural recordings (premotor RMS signal:noise > 2), but only 10 sang at least 100 motifs in at last 1 day and were used for this study. Of the adults, 5 had high quality neural recordings and sang ≥100 motifs in one day. Three of the finches implanted as juveniles (Blue-70, Blue-15, and Blue-81) were also recorded after day 90 (≥100 motifs/day) and thus included in the adult group for those days. All song recordings were made in the absence of a female (undirected). Undirected song is thought to reflect song practice (Jarvis et al., 1998). All juveniles and 3 of the adult finches were reared by their parents until day 45 in our facility on a 14:10 light cycle. Two adult birds were obtained from a commercial supplier. Although experience and not age is probably the strongest predictor of song system maturity, age is correlated with experience and presents a more quantifiable parameter. None of the finches used in these experiments were ever exposed to auditory playback. The finches were allowed to hear their own vocalizations. The University of Minnesota Institutional Animal Care and Use Committee approved all procedures.

Chronic physiological recording

Basic methods have been previously described (Crandall et al., 2007b). For population recordings, finches were implanted with a set of recording electrodes: 1 or 2 50-μm nichrome-formvar electrodes in HVC or a control brain area adjacent to HVC (not plated, 1.1 – 1.8 MΩ, AM Systems, Carlsborg, WA), a 50-μm nichrome-formvar electrode adjacent to HVC for use as a reference electrode, and 75-μm silver ground and electroencephalogram (EEG) electrodes.

The headstage and recording environment have been previously described (Schmidt and Konishi, 1998).

All data were acquired with custom-written (Datafleet, Minneapolis, MN) LabView software (National Instruments, Austin, TX) at a sampling frequency of 44.1 kHz. During recording, a lightweight operational amplifier was attached to the bird and connected to a mercury commutator via a flexible cable. HVC neural activity was amplified 1000 times and filtered 300 – 10,000 Hz. Song was monitored with a microphone (Earthworks, Milford, NH), high-pass in-line filtered at 100-Hz (Shure, Chicago, IL), and recorded. Localization of electrodes to HVC was confirmed with premotor activity in all cases and cresyl-violet histology in 7 of 15 finches.

Song Behavior Analysis

All data were analyzed with custom-written Matlab functions. Initial song analysis consisted of the sorting of sound data and exclusion of movement noise. Sound data were further sorted according to temporal properties. Preliminary songs were defined as sounds lasting ≥ 500 msec with time gaps of no more than 20 msec.

To specifically study activity during learned song behavior, a canonical motif was identified by a skilled observer from the oldest day available from each finch and used to extract motifs and corresponding neural activity throughout the recording period. Multiple motif forms could have been selected depending on the developmental stage. However, we were most interested in how HVC activity changed once the overall vocal pattern had been established and, thus, selected

the most mature motif available. Since perfusion and histology were performed as soon as the electrode recording declined, some subjects may not have achieved the final mature motif.

We focused our analysis on behaviorally relevant vocalizations (instead of, for example, movements) by band-pass filtering our vocal data (1 – 8 kHz). Amplitude envelopes of the canonical motif and all preliminary songs were constructed by filtering the rectified sound recording with a Savitzky-Golay smoothing filter (4^{th} order polynomial fit; 20-msec frame size). Then, amplitude envelopes of the canonical motif and preliminary songs were cross-correlated (Crandall et al., 2007a). Sharp peaks in the cross-correlation revealed the onset of a motif that matched the canonical motif. Behavioral song motifs and corresponding neural activity were then excised and saved for further analysis.

Song was quantified based on 6 features: amplitude, Weiner entropy, frequency, pitch, frequency modulation, and pitch goodness (a measure of sound periodicity). These features were calculated from the raw motif in 9.3-msec bins with sliding 1-ms steps with a subprogram of Sound Analysis Pro (Tchernichovski et al., 2000), ported to Matlab by S. Saar. The variance of vocal features was calculated across 20 aligned motifs millisecond by millisecond relative to time within the motif. The mean variance across all milliseconds in the motif provided a measure of song variance that was calculated separately for the first and last 20 motifs.

Multi-Unit Physiology Analysis and comparisons to song features

Data for analysis was limited to days during which a bird produced at least 100 identified motifs. HVC activity during motifs was digitally band-pass filtered 300 – 6000 Hz. To examine levels of population spiking activity during the motif, the root mean square (RMS) was calculated across the entire motif.

In preliminary studies, it appeared that the level of population burstiness was changing throughout the day in juveniles. Therefore, we developed a method to quantify the level of burstiness. Because of our requirement that the RMS of premotor activity be at least double the RMS of non-singing 'noise' activity, the bulk of the multi-unit data presented in this manuscript meets or exceeds a 3:1 signal:noise (Kao et al., 2008). This may not be obvious on the long time scales of songs and motifs (Fig. 1 A, B). However, increasing the temporal resolution during non-singing periods reveals large units that peak at >3 times the noise (Fig. 1C1). Even higher resolution examination reveals single units that fire alone or in population bursts (Fig. 1C2, asterisk). This quality of data has been subjected to thresholding and detailed temporal analysis of population activity (Crandall et al., 2007a). During periods of high HVC population activity, such as during singing, the amount of activity is so great that spiking events of multiple units are consistently simultaneous or near-simultaneous (Fig. 1D1-2). Smaller events can sum to exceed the threshold. Events that might normally be above threshold can be lost because they overlap temporally with other events of opposite polarity (Fig. 1D2, asterisk). In addition, multiple synchronous or near-synchronous events register as a single spike or no

spike at all. If synchrony of activity changes during development and/or the day, thresholding methods applied to multi-unit recordings will fail to detect it. Because of these concerns, we sought to analyze our data with a technique that is better at quantifying multi-unit activity. We used a Savitzky-Golay filter which smoothes data in the temporal domain (4^{th} order polynomial fit; 50-msec frame size). To measure burstiness, the peak:valley % was computed by first creating an HVC activity envelope by rectifying and smoothing the mean HVC activity amplitude. Then, the mean of the millisecond time windows that contained the maximum 25% of amplitude measurements was divided by that of the minimum 25%. This quotient was then decremented by 1 and multiplied by 100 to obtain the peak:valley %.

Statistical analysis and data presentation

All bars represent means and all error bars represent standard errors of the mean. Paired t-tests were used to compare data from the same finch. Unpaired t-tests were used to compare mean data across days from juveniles and adults. Significance was defined as $\alpha = 0.05$.

RESULTS

To investigate the role of the song nucleus HVC in song learning, we measured neural sensorimotor activity during singing across days, over nights, and through the end of the sensitive period for vocal plasticity. HVC population activity during stereotyped vocal sequences (motifs) was measured across multiple consecutive days. Zebra finches were maintained in the same recording chamber for many days before and during recording. Finches were only

disturbed for food and water replenishment. The electrodes were never experimentally adjusted. These efforts resulted in stable recordings over many days and, in a few cases, for several weeks. For all data shown, the finch had recovered from surgery for at least 6 days, been in the recording chamber for at least 3 days, and sang in the chamber at least one day prior. All HVC activity in this manuscript occurred during song motifs. These points are relevant to the daily changes in HVC activity that we report below.

HVC activity levels during singing increase daily and decrease overnight in juveniles and adults

From all available song data, we extracted and temporally aligned song motifs using established methods (Crandall et al., 2007a). The RMS of HVC population activity during song motifs increased over the course of the day in juveniles and adults (Fig. 2 and Supp. Video; example raw traces across days and development are shown in Supp. Fig. 1). HVC activity during a motif that was produced early in the day (Fig. 2A,C) was smaller amplitude than a motif produced later in the day (Fig. 2B,C and Supplementary Figure 2). We often noted obvious diurnal increases and nocturnal resets in HVC activity levels (Fig. 3 and Supp. Video). These changes in HVC activity occurred in the absence of fluctuations in recording chamber temperature (data not shown), which indicates the involvement of an intrinsic physiological process. We quantified the daily increase in HVC activity by examining 94 days of data in which each bird sang at least 100 motifs that were recognized by our motif finder program. We compared the HVC activity during the first 20 motifs with that during the last 20 motifs. HVC

activity significantly increased between the first and last motifs produced each day (Fig. 2, D,E; $p < 0.03$, paired t-test). The daily change was quite variable across animals and days (Supp. Fig. 3). The percent daily increase (Fig. 2F) was not different in juveniles compared to adults. To examine the overnight change in HVC activity, we used pairs of consecutive days during which at least 100 motifs were produced. The last 20 motifs from the first day were compared with the first 20 motifs from the next day. The overnight decrease in HVC activity level was not significantly different in juveniles and adults (Fig. 2G). Collectively, these data indicate that HVC activity levels during singing increase through the day and decrease overnight.

Simultaneous control recordings of a non-song related brain area immediately adjacent to HVC and within 200 μm of the HVC recording electrodes were achieved in four juveniles. In contrast to the singing-related HVC activity, which increased each day, control activity during singing decreased (HVC: +3.25 ± 6.19 RMS μV; Control: -5.83 ± 6.12 RMS μV; N = 4). The daily change in activity in HVC versus the control brain region was significantly different ($p = 0.03$, paired t-test).

During the day, the pattern of HVC activity becomes burstier in juveniles

Diurnal and nocturnal changes in HVC activity levels were similar in adults and juveniles (Figure 2). We asked whether the changes in activity were evenly distributed across the motif. We found that peaks of activity increased more than valleys during the day in juveniles, but not adults (Fig. 4). Peaks are upward deflections of the rectified and smoothed voltage trace (as in Fig. 4C), whereas

valleys are downward deflections. During the day, the juvenile HVC typically concentrated more power into specific time windows that already had more power (peaks) and less power in other windows (valleys). That is, HVC became more ‘bursty’. The term ‘burst’ refers to intermittent bouts of accelerated spiking activity, whereas the term ‘bursty’ refers to the tendency to exhibit bursts of activity. The examples in Fig. 4 A-B show sonograms of the first and last motifs of a given day for a juvenile (A) and an adult (B). Note the relative variability of the juvenile motifs (Fig. 4A) compared to those of the adult (Fig. 4B), as previously reported (Immelmann, 1969; Deregnaucourt et al., 2005). Comparison of mean smoothed and rectified HVC activity during the first and last 20 motifs reveals that peaks increased more than valleys in the juvenile (Fig. 4C), but not the adult (Fig. 4D). Millisecond-by-millisecond, juvenile HVC activity during the first 20 motifs correlated with the change in HVC activity during the same day, indicating that peaks preferentially increased (Fig. 4E). Further analysis of the data shown in Fig. 2 provides another example of increased juvenile burstiness during the day (Supp. Fig. 1). In the adult example, the relationship between HVC activity and the change in HVC activity that day was negative (Fig. 4F). These data show that HVC burstiness increases during the day in juveniles, but not adults.

To quantify the increase in burstiness, we calculated the percent of amplitude difference between peaks and valleys of rectified, smoothed HVC activity (such as that shown in Fig. 4C-D). As with RMS calculations, data for analysis was limited to days in which at least 100 motifs were produced. In

juveniles, peak:valley % significantly increased between the first 20 and the last 20 motifs (Fig. 5A; example RMS and peak:valley % data for 10 days and 3 finches are shown in Supp. Fig. 4). In adults, peak:valley % trended to decrease or not change over the day, and was not significant (Fig. 5B). Comparison of the mean diurnal change in peak:valley % by animal revealed that the daily change in burstiness was significantly greater in juveniles than adults (Fig. 5C). In contrast to diurnal changes, HVC burstiness during singing slightly decreased between the last 20 motifs on one day to the first 20 motifs on the next day (Fig. 5D). This overnight change was rather variable and not significantly different between juveniles and adults (Fig. 5D). Collectively, these data indicate that HVC burstiness increases during a day of singing in juveniles, but not adults.

HVC burstiness increases with development

Data above show that juvenile HVC burstiness increases diurnally and decreases to a lesser extent nocturnally. This suggests that diurnal increases in HVC burstiness may accumulate in development. To test this hypothesis, we compared the HVC peak:valley % in juveniles and adults. Comparing day-by-day, the juvenile HVC is less bursty than that of the adult (Fig. 6A; For this panel, each day is considered a separate data point, so individual finches may contribute more than one data point). Collapsing data by taking the mean peak:valley % of all days for each animal revealed the same trend as the day-by-day data, but was not significant (Fig. 6B; For this panel, each finch was only allowed to contribute one data point).

The cumulative developmental increase in HVC burstiness could be observed in single juveniles. In Fig. 6C, the peak:valley % for all recorded motifs from a single finch from days 82 – 99 are indicated by gray dots. The red line indicates the median peak:valley % for each day. Note how the red line becomes more positive, indicating that HVC becomes burstier, as the animal aged. We noted very little change in burstiness on days when this finch sang relatively little (days 82-84), but we did not observe this correlation in every finch (data not shown). Combining all juvenile data, we found a significant positive correlation between age and the HVC burstiness during the first 20 motifs (Fig. 6D). Interestingly, the correlation between age and the burstiness of last 20 motifs was less correlated (Fig. 6E). Subtraction of the burstiness values for the first 20 motifs from the last 20 motifs provides a daily change metric. Since the burstiness of the first 20 motifs increased with age and this relationship was degraded during the last 20 motifs, we predicted that the relationship of the daily change in burstiness to age would be negative to account for this difference. Indeed, the degree of daily change in burstiness decreased with age in juveniles (Fig. 6F). These data reveal a developmental change in HVC bursting activity that accrues daily during song learning. As the animal ages, the degree of daily change decreases and burstiness stabilizes.

HVC burstiness is inversely correlated with song variance in developing finches

How might HVC activity impact behavior? HVC activity is correlated with behavioral stability 50-ms into the future (Day et al., 2008). Higher amplitude bursts appear to finely limit song variability in discrete time windows.

Correspondingly, increased HVC burstiness may enable fine song stability control in multiple time windows, locking in additional temporal fragments of the song as sensorimotor development proceeds. If so, increased HVC burstiness should predict decreased overall song variability. To calculate overall motif variability, we temporally aligned the first 20 motifs of each day and, for each millisecond of the aligned motifs, took the variance across motifs of 6 song features: entropy, amplitude, frequency modulation, frequency, pitch, and pitch goodness (periodicity). We then took the mean variance of each feature across all milliseconds of the motif. We found that the variance of all 6 song features was inversely correlated with HVC burstiness (Fig. 7, Supp. Fig. 5). Fig. 7 shows one of the features, FM variance. The other 5 features can be seen in Supp. Fig. 5. On a day when HVC had relatively little bursting activity during singing (lower panels of A,C,E), the behavior was variable (upper panels of A,C,E). Several spectrotemporal segments that were particularly variable are highlighted in red and yellow. Later in development, HVC was more bursty (lower panels of B,D,F), and the behavior was less variable (upper panels of B,D,F). This correlation was significant for data from all juveniles (Fig. 7G), but not adults (Fig. 7G inset). The burstiness-song variance relationship was also noted across days in individual finches (data from each finch shown in Fig. 7G are represented by a different colored symbol in Supp. Fig. 6).

The overnight change in burstiness is inversely correlated with the change in burstiness the next day

Previous work has shown that juvenile finches have less nocturnal HVC activity and that nocturnal HVC activity appears to stabilize song behavior (Crandall et al., 2007b). In addition, the total degree of overnight destabilization of song in juveniles positively correlates with how well they ultimately copy their tutor's song (Deregnaucourt et al., 2005). Collectively, these studies suggest that nocturnal changes in HVC activity may have a role in song learning. These data, combined with the data presented above, led us to hypothesize that HVC burstiness should degrade more overnight early in song learning, relative to later in development. This should enable destabilization of song, if HVC burstiness stabilizes behavior (Fig. 7). Consistent with this hypothesis, we found that the degree of overnight decrement in HVC burstiness became smaller with development (Fig. 8A). We next asked how the overnight decrement in HVC burstiness related to daily changes in song: did daily changes in burstiness correlate more with the change in burstiness the night before or the night after? We found that the overnight change in HVC burstiness strongly and inversely correlated with the change in burstiness the next day (Fig. 8B). The day's change in burstiness also correlated with the next night's change, but not as strongly (Fig. 8C). Combined with the data in Fig. 7, these data suggest that nocturnal degradation of HVC activity enables variability in song behavior the next day and subsequent reforming of a new HVC activity pattern.

DISCUSSION

This study investigated the role of the song control nucleus HVC in vocal learning. It provides the first evidence that HVC activity levels change on a daily cycle. In juveniles and adults, HVC activity levels increased during the day and decreased at night. Burstiness increased each day in juveniles, but not in adults. These observed changes in burstiness are relevant to behavior: HVC activity levels inversely correlate with song variability, on the timescales of milliseconds (Day et al., 2008), days, and development (current study). In addition, burstiness degrades overnight in juveniles, which may underlie a daily cycle of song learning and stabilization during the day and destabilization at night.

Daily cycling of HVC activity

We have found that HVC activity increases daily. In juveniles, the oscillatory burst pattern of HVC activity typically becomes stronger during the day (Fig. 9A). In adults, overall HVC levels increase, but burst dynamics are relatively unchanged (Fig. 9B). Figure 9C presents a simple representation of our results in juveniles. In the morning, lower HVC activity levels and bursting correlate with plasticity in song behavior (upper right 3 sonograms). Later in the day, increased HVC activity correlates with increased song stereotypy, particularly during parts of the song that correspond to bursts in HVC activity (Day et al., 2008). As development proceeds, burstiness continues to increase, which may enable tight temporal control of song in the adult. The correlation between HVC burstiness and behavioral variance on the timescales of days and development could indirectly result from processes that coincidentally affect both (e.g. circadian

rhythms). However, another study found the same inverse relationship between HVC bursts and song variance on a millisecond timescale (Day et al., 2008), which suggests that daily and developmental processes alone cannot explain the predictive capacity of HVC activity on song variability. In addition, neural activity recorded outside of the song system does not show the same pattern of increased activity during the day in juveniles as it does in HVC (see Results).

Why cycle premotor activity?

Avian and mammalian sleep share fundamental characteristics, such as slow wave activity (Jones et al., 2008; Low et al., 2008). The synaptic homeostasis hypothesis (Tononi and Cirelli, 2006) posits that sleep serves in a homeostatic fashion to downscale synaptic strengths that are enhanced during waking. Sleep-dependent downscaling may increase the signal-to-noise ratio by decreasing the probability that a neuron will fire in response to a given input. The model proposes that slow-wave sleep increases the energetic efficiency of the brain by universally downscaling all synaptic strengths without regard to previous experience-dependent change. Our data are consistent with this model. We observe: (1) HVC activity potentiates during waking, which may reflect synaptic potentiation (current study); (2) Increased burst strength correlates with stability of song 50-ms into the future, suggesting underlying mechanisms of activity-dependent plasticity (Day et al., 2008); (3) HVC activity levels typically decrease after sleep regardless of developmental status or learning state, which may reflect nonspecific synaptic downscaling (current study). We have found that levels of HVC singing activity in adult finches increase each day and decrease

each night, but oscillatory bursting and song stability do not appear to cycle. In contrast, juveniles cycle all three parameters: HVC activity levels, burstiness, and song stereotypy. These data are consistent with a synaptic homeostasis model in which, throughout the day, synapses are selectively strengthened during specific song time windows (during bursts), perhaps due to an auditory-driven instructive signal (Nick and Konishi, 2005a). According to this model, relative differences in strengths of adult synapses are already large, as evidenced by strong bursts during the first 20 motifs, relative to juveniles. Thus, further synaptic strengthening has little effect on the burst pattern. In contrast, juveniles have relatively weak bursts, a subset of which become stronger as the day progresses. In this model, during sleep, both adults and juveniles would experience nonspecific synaptic decrement, which would be reflected in a decrease in activity levels. Decreases in activity levels need not be reflected in a decrease in measured burstiness and a change in burst pattern in animals with already strong HVC bursts (i.e., adults). Since HVC bursts predict song stability (Day et al., 2008), adults may exhibit increased song stereotypy because they have a strong and stable HVC burst pattern that is resistant to the effects of sleep-dependent synaptic downscaling.

In the context of the synaptic homeostasis hypothesis, the sleep ‘replay’ or 'rehearsal' of song-related neural patterns (Dave and Margoliash, 2000; Shank and Margoliash, 2009) represents a trace of synaptic enhancement that occurred during waking (Tononi and Cirelli, 2006). If the activity pattern that we observe during singing is a neural prediction or expectation that is strengthened after

each of thousands of motifs, then the synaptic trace pattern should be very strong in adults and weaker in juveniles. Consistent with this hypothesis, previous work has shown that putative sleep 'replay' activity is significantly less in juveniles as compared to adults (Crandall et al., 2007b).

Pressing questions

The current study has raised many new questions regarding birdsong learning. In terms of percentages, are the small changes in activity levels behaviorally significant? Since daily cycling of neural activity levels during a specific behavior has never been reported and never shown to correlate with behavioral variability (as we observe), no scale exists with which to judge the size of the effect. Is sleep required for the resetting of HVC activity, or does it result from a circadian rhythm? Developmental analysis of song behavior has revealed that sleep can reset the song entropy variance (Deregnaucourt et al., 2005; Shank and Margoliash, 2009), which suggests that sleep may be involved in the resetting of HVC activity that is closely tied to behavioral variance (current study, (Day et al., 2008). Does auditory feedback affect the oscillatory burst strength of HVC population activity? Auditory signals increase HVC population activity during singing (Sakata and Brainard, 2008), consistent with the hypothesis that an instructive signal strengthens HVC bursts (Nick and Konishi, 2005a). Does HVC entrain the activity of the AFP? Fast-spiking putative interneurons dominate multi-electrode single-unit recordings (Crandall et al., 2007a) and may become more synchronous with development (N. Day, S. Kerrigan, N. Aoki, D.Q. Nykamp, and T.A. Nick, unpublished observations). Thus,

the strong oscillatory bursting activity characteristic of the adult HVC (Crandall et al., 2007a) may be due to the synchronous firing of interneurons. Synchronous activity in HVC interneurons may entrain the activity of Area X-projecting HVC neurons and thus entrain the activity of the AFP. Alternatively, HVC interneurons may directly entrain the HVC neurons that project down a pathway directly involved in producing song (the neurons that project to the Robust Nucleus of the Arcopallium). What stabilizes the neural oscillation at the end of vocal learning and decreases song variability in adults? Other studies have found that perineuronal nets may close critical periods and stabilize developing networks (Sur et al., 1988; Pizzorusso et al., 2002). Perineuronal nets appear in the song system during the sensorimotor phase (T. Balmer, V. Carels, J. Frisch, and T.A. Nick, unpublished observations). Answering these questions will illuminate the role of HVC in vocal learning and, perhaps, reveal fundamental mechanisms of sensorimotor learning.

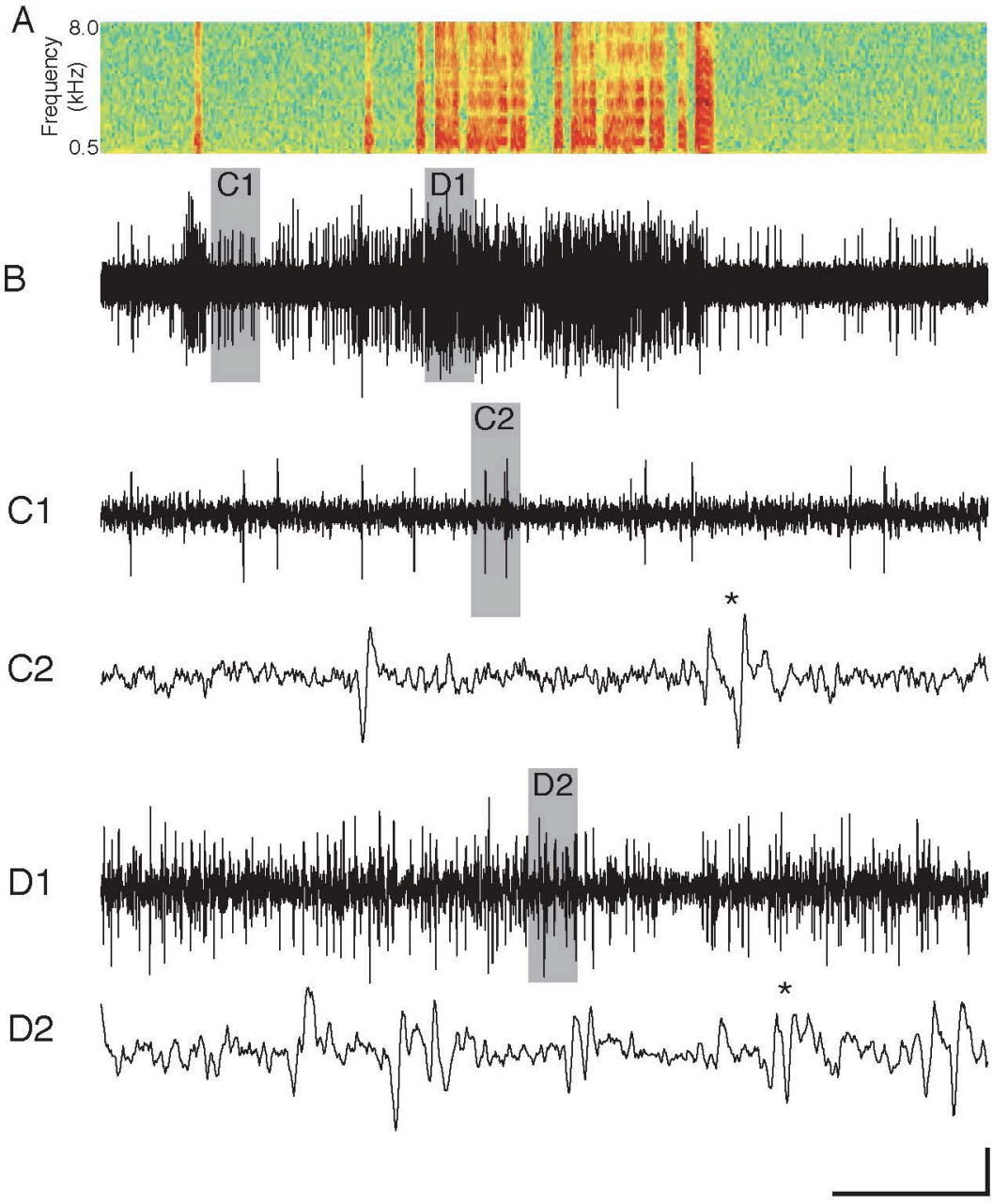

Figure 1. The complex nature of HVC multi-unit activity during singing renders thresholding methods inadequate.
(A) For reference, a sonogram of recorded vocalizations is temporally aligned with B. (B) HVC population activity typically increases during singing as compared to non-singing. In juveniles, there is often some activity immediately preceding and/or following song (Crandall et al., 2007a). Gray bars indicate time windows that are shown at higher resolution below. (C1,2) Higher temporal resolution of activity during non-singing reveals relatively distinct spiking events. (D1, D2) Higher resolution singing activity reveals near continuous overlap and interference of multiple units. All data are from finch Blue-46, age 66 days. Scale bar: 100 μV, A,B: 1 s; C1, D1: 55 ms; C2, D2: 3 ms.

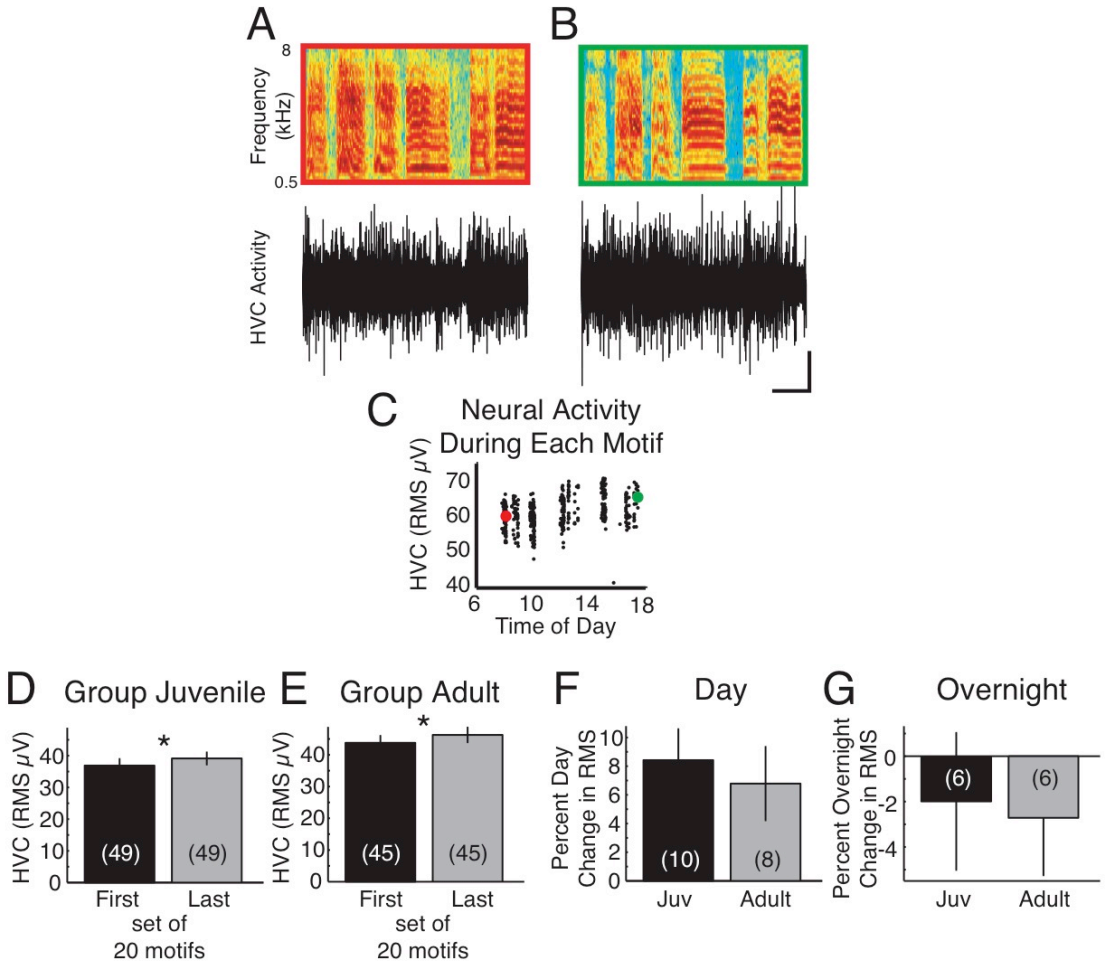

Figure 2. HVC activity levels increase each day and decrease overnight in juvenile and adult zebra finches.

A motif produced early in the day (A, red), was accompanied by less HVC activity than a motif produced later in the same day (B, green). In A and B, the top panel is a sonogram of a stereotyped learned motif. The colored outlines correspond with the colored points in C. The lower panel shows HVC population activity. Scale bar: 100 ms; 100 μV. (C) The RMS of HVC population activity during singing increases during the day. The RMS's during the motifs shown in A and B are indicated by their respective colors. Exemplar data in A-C are from bird Blue-81 age 87 days. (D-E) Comparing the first 20 motifs with the last 20 motifs of each day revealed that HVC activity significantly increases in juveniles (D) and adults (E; *$p < 0.03$, paired t-test; day Ns in parentheses). By finch, the percent change in singing activity levels over the day (F, $p = 0.63$) and overnight (G, $p = 0.86$; finch Ns in parentheses) was not different between juveniles and adults. In general, HVC activity during singing increased during the day (F) and decreased overnight (G).

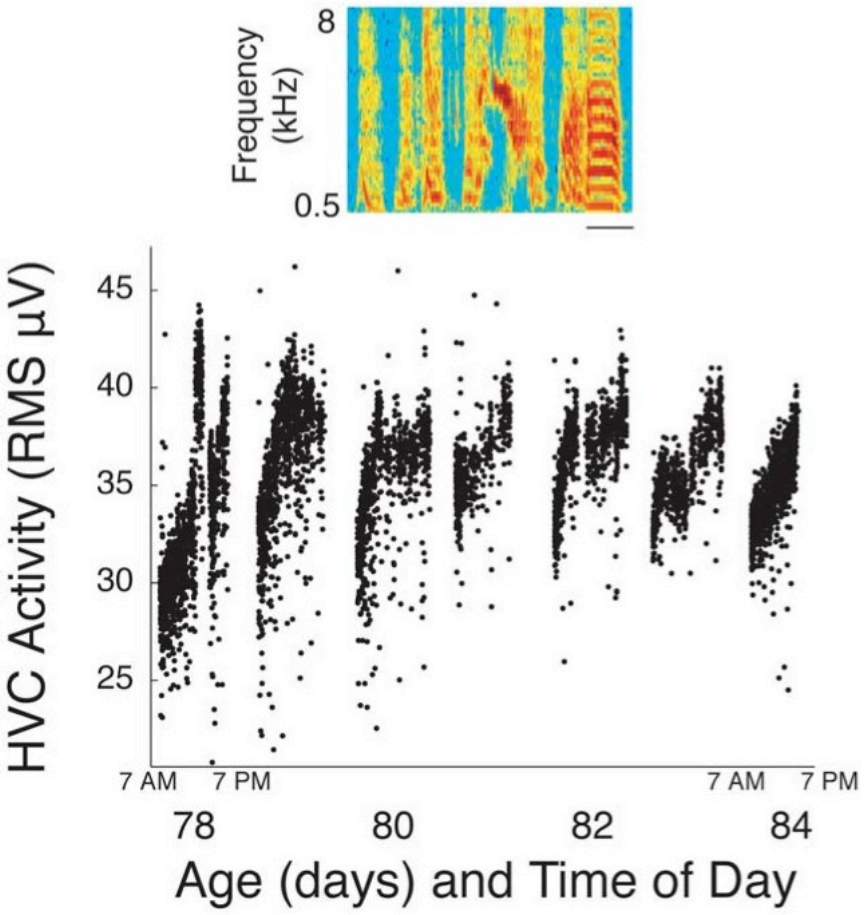

Figure 3. Apparent cycling of HVC activity during song motifs was noted in many longitudinal recordings.
Mean HVC activity during each motif for seven consecutive days is plotted versus the time of day. HVC activity increased until approximately noon each day. Occasionally activity decreased during the day, as on day 78. HVC activity in the morning trended to be lower than the evening before. The inset shows a sonogram of the canonical motif from bird Blue-70 that was used to extract all other motifs. All data in this figure are from Blue-70. Scale bar: 100 msec.

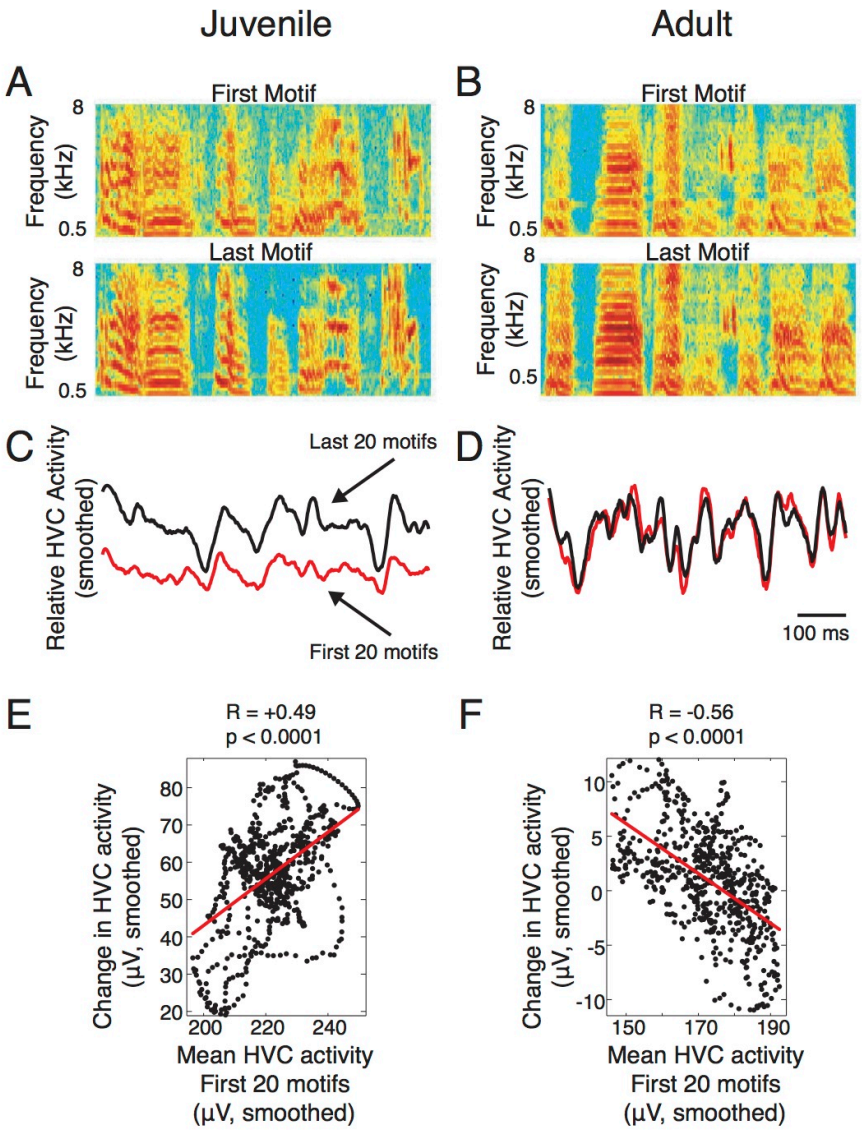

Figure 4. HVC singing activity becomes burstier each day in juveniles, but not adults. In juveniles (A,C,E), peaks in neural activity during motif production typically increased more than valleys, such that the burstiness increased. In adults (B,D,F), HVC bursting activity was generally stable or declined during the day. (A) Sonograms of the first and last song motif reveal that the juvenile song (A) became more structured and complex during the day, whereas the adult song (B) was relatively stable. (C,D) HVC neural activity also changed more in the juvenile (C) than in the adult (D). (C) In the juvenile, comparison of the mean rectified and smoothed HVC activity of the first 20 motifs (red) to that of the last 20 motifs (black) reveals that the increase in neural activity during the day was focused in the peaks, with relatively little increase in activity during troughs. (D) An adult recording with an overall decrease in burstiness highlights the differences between adults and juveniles. (E,F) The change in HVC activity was directly proportional to the starting HVC activity (the first 20 motifs) during the same millisecond (relative to the motif) in the juvenile (E), and inversely proportional in the adult (F). Data are from Blue-57, age 58 days and Orange-331 age 100 days. Two other examples similar to C and D are shown in Fig. 8 A,B.

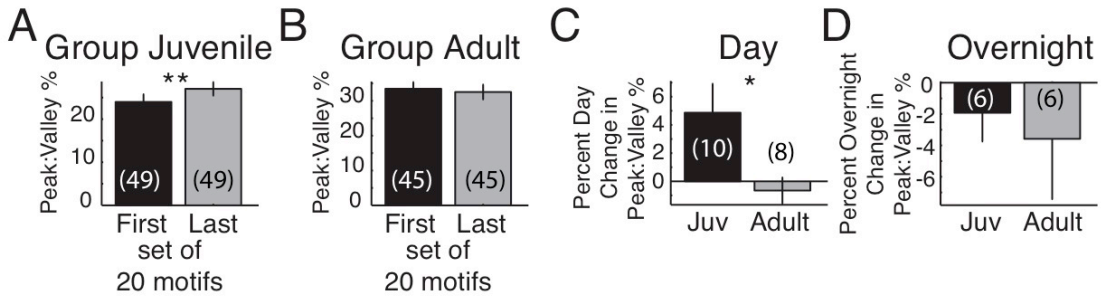

Figure 5. As measured by peak:valley comparison, HVC singing activity becomes burstier each day in juveniles, but not adults.
Juveniles (A; **p < 0.02, paired t-test), but not adults (B, p = 0.75), exhibit significant changes in HVC burstiness (peak:valley %) each day. (C) The percent daily change in juvenile burstiness was significantly greater in juveniles (*p < 0.04, unpaired t-test). (D) The percent overnight change in burstiness was not significantly different in juveniles versus adults (p = 0.77).

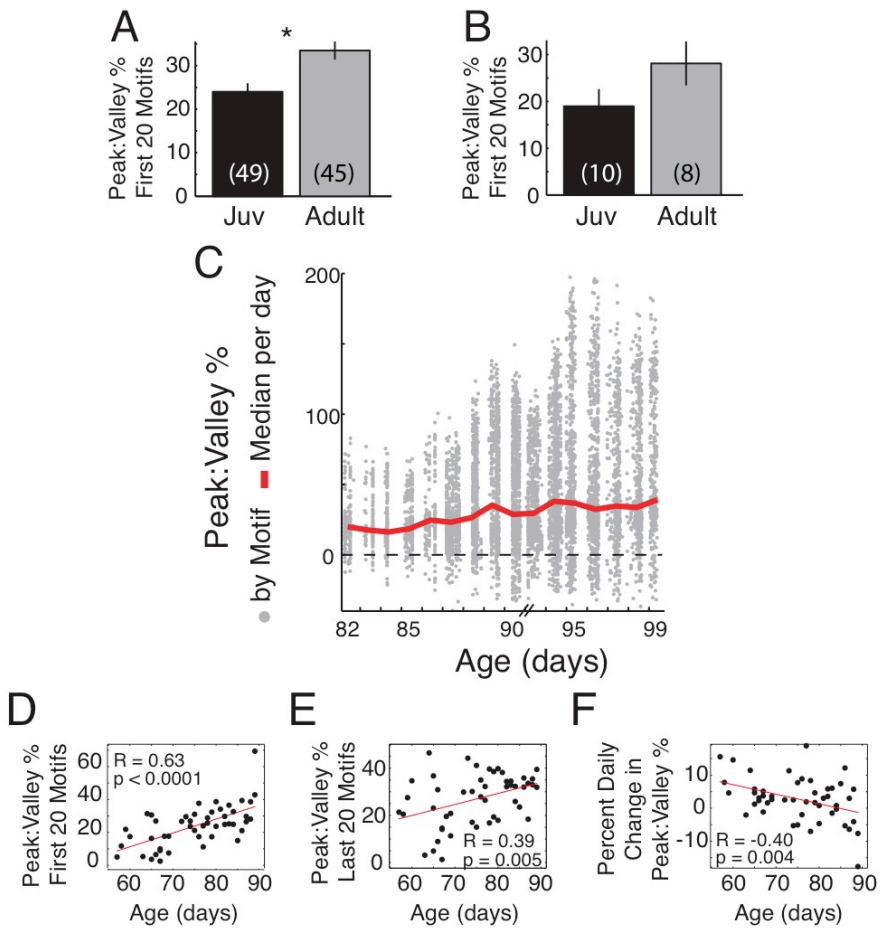

Figure 6. HVC burstiness increases with development, whereas the degree of diurnal change in burstiness decreases with development.
(A) HVC burstiness, as measured by peak:valley % across days, was greater in adults than juveniles (*$p < 0.0005$, unpaired t-test). (B) Analysis of data by bird yielded the same trend as the daily analysis, but the difference was not significant ($p = 0.12$). (C) The increase in HVC burstiness was observed day-by-day in longitudinal recordings of juveniles. The peak:valley % for each motif is shown in gray dots, the median per day is indicated by the line. (D) Burstiness increased with age in juveniles and was most clearly observed in the first 20 motifs of each day. (E) HVC burstiness during the last 20 motifs of each day also correlated with age, but the relationship appeared more complex. (F) The percent daily change in burstiness decreased with age in juveniles.

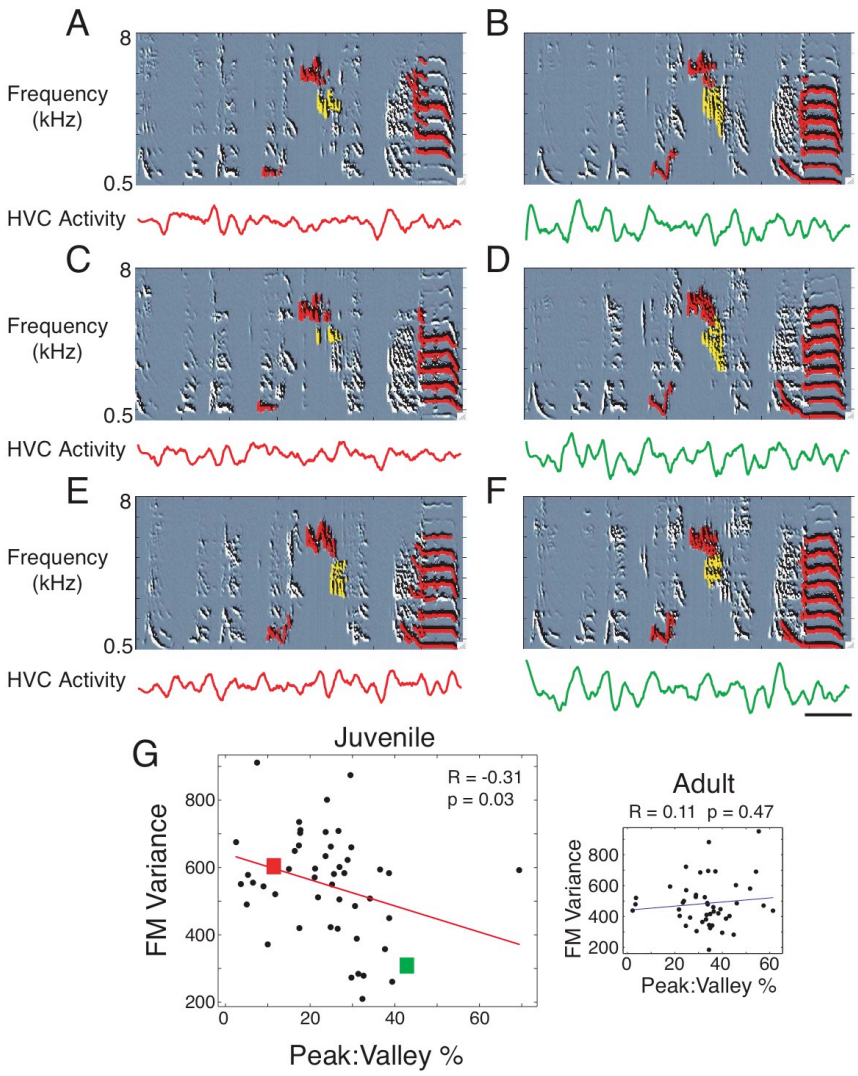

Figure 7. HVC burstiness inversely correlates with behavioral variability.
A day during which behavior was relatively more variable and HVC activity was less bursty is shown in the left column (A,C,E; bird Blue-70 age 77 days), compared to a day during which behavior was more stable and HVC activity was more bursty (B,D.F; bird Blue-70 age 89 days). The top panel of each example shows spectral derivatives, which are similar to sonograms, with parts of the song spectrotemporal field labeled with red and yellow for clarity. These song segments were relatively variable in the left column compared to the right. For example, note the leftmost red mark in each panel. Note how this sound is variable in A,C compared to E. Note how this sound is relatively simple compared to the corresponding and stable sound in B,D, and F. The bottom panel of each example shows rectified and smoothed HVC activity. Note the shallow peaks and valleys in the left column compared to the right. (G) Day-by-day comparison of HVC burstiness (peak:valley %) and FM variance reveals a weak but significant correlation in juveniles. Data are from 10 birds, with multiple values reflecting multiple days from the same finch (Supp. Fig. 6). Exclusion of the rightmost outlier results in R = -0.39, p = 0.006. Inset: Adult HVC burstiness and song variance are not well correlated.

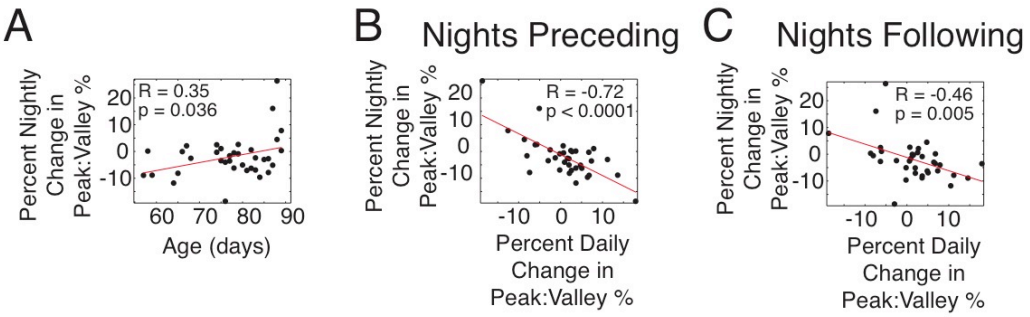

Figure 8. Nocturnal decrements in burstiness decrease with development and inversely correlate with the next day's change in burstiness.
(A) Overnight decrements in HVC burstiness decreased with age in juveniles. (B) The overnight change in burstiness strongly and inversely correlated with the degree of change in burstiness the next day. (C) The change in burstiness during the day correlated with the overnight change in bursting the following night.

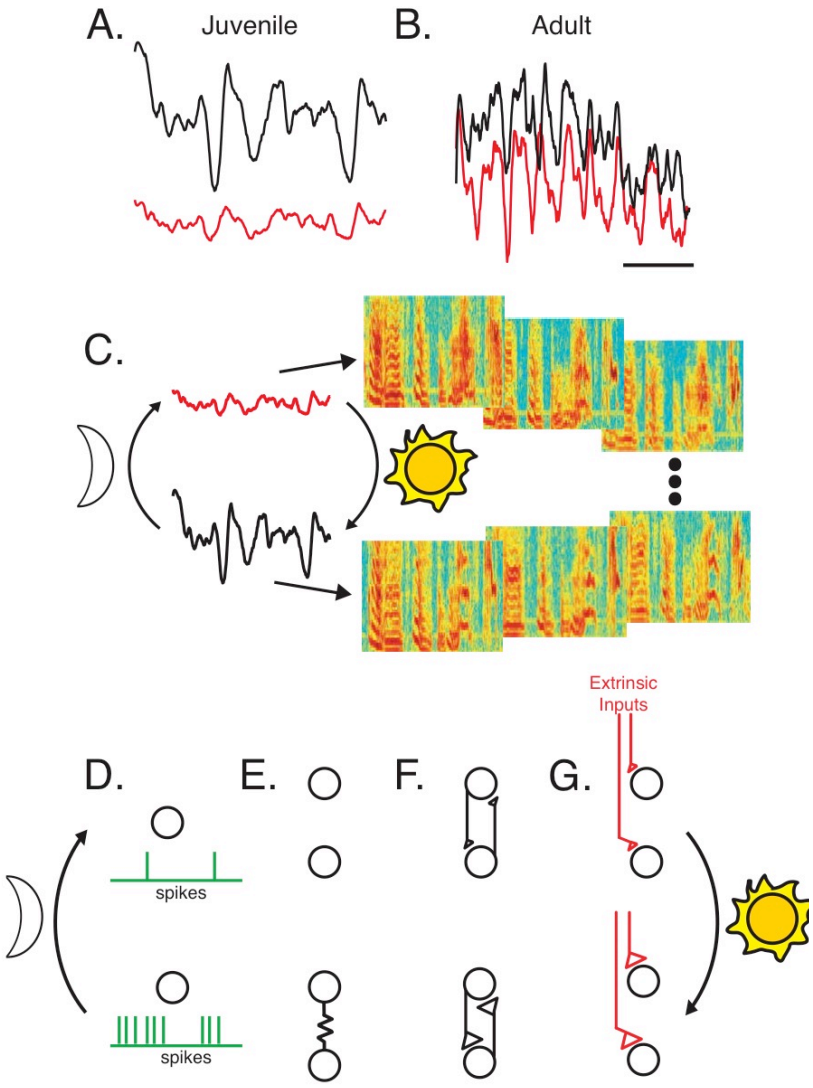

Figure 9. A model of daily cycling of motor control during song learning.
Nightly decrement of HVC activity levels and bursting may allow song variability and subsequent rearrangement and/or strengthening of HVC activity pattern via activity dependent plasticity. In turn, activity-dependent plasticity may result in daily increment in HVC activity levels and bursting. (A) Mean juvenile HVC activity for the first 20 motifs (red) was greater than that of the last 20 (black) motifs on the same day. (B) Mean adult HVC activity for the first 20 also increased relative to the last 20 motifs on the same day. HVC activity levels cycled in both juveniles (A) and adults (B). In contrast, the relative burstiness increased during the day in juveniles (A), but was relatively stable in adults (B). For A and B, y-axis: relative voltage; x-axis: time relative to the motif; scale bar: 200 msec. (C) We propose that increasing HVC activity increases the entrainment of projection neurons and stabilizes behavior. In the morning, juvenile HVC population activity is low amplitude with low oscillatory power (red). Lack of correlated interneuron bursting in juveniles may allow variability in the firing of projection neurons and consequent variability in song behavior (the 3 motif sonograms in the upper right). Later in the day, HVC interneuron population activity is higher amplitude (black) and concentrated in more compact bursts. The hypothesis predicts that putative interneuron bursts entrain the activity of projection neurons and thereby decrease behavioral variability (the 3 motif sonograms in the lower right.

Chapter 4

Identification of single neurons in a forebrain network

Day NF, Kerrigan SJ, Aoki N, Nick TA (in press) *J Neurophys*

Behaviors are generated from complex interactions among networks of neurons. Single unit ensemble recording has been used to identify multiple neurons in functioning networks. These recordings have provided insight into interactions among neurons in local and distributed circuits. Recorded units in these ensembles have been classed based on waveform type, firing pattern, or physical location. To specifically identify individual projection neurons in a cortical network, we have paired tetrode recording with antidromic stimulation. We have developed techniques that enable antidromic identification of single units and study of functional interactions between these neurons and other circuit elements. These methods have been developed in the zebra finch, and should be applicable, with potential modifications that we discuss here, to any neural circuit with defined subpopulations based on projection target. This methodology will enable elucidation of the functional roles of single identified neurons in complex vertebrate circuits.

INTRODUCTION

Neural circuits have been probed using multi-unit, serial single-unit, and single-unit ensemble (e.g., tetrode or multi-electrode) recordings. In the investigation of circuit dynamics, single unit ensemble recordings such as tetrode recordings have effectively isolated the simultaneous spike trains of multiple single units. These powerful recordings enable analysis of spike time relationships and the functional connectivity among neurons.

Tetrode recordings have been used extensively in the forebrain to study functional ensembles of single units (McNaughton et al., 1983; Gray et al., 1995; Hargreaves et al., 2005; Johnson and Redish, 2007). Analysis of tetrode records relies on spike waveform characteristics to enable sorting of single-unit activities. In ensemble recordings, cellular identity is inferred by spike waveforms, firing patterns, and physical location. However, in many systems, identification using these parameters is not feasible due to the relative homogeneity of spike trains and waveforms in functionally heterogeneous neuronal subtypes. In these instances, methods that have been used to identify single neurons in the population have included dye fills (Steinberg and Schmidt, 1970; Hill and Oliver, 1993; Dutar et al., 1998; Mooney and Prather, 2005) and antidromic stimulation (Lipski, 1981; Swadlow, 1998; Hahnloser et al., 2002; Lim and Anderson, 2007; Prather et al., 2008). In some of these cases, network connectivity has been determined using paired intracellular recordings (Mooney and Prather, 2005; Yoshimura and Callaway, 2005; Debanne et al., 2008) or inferred using post-hoc alignment of serially recorded neurons with stereotyped behavior or stimuli

(Richmond et al., 1990; Kozhevnikov and Fee, 2007). Intracellular recording in vocalizing and sleeping animals has provided insight into the membrane properties of antidromically-identified neurons that are active during specific behaviors (Long et al., 2010).

Understanding the neural control of behavior cannot be achieved without an understanding of neural networks (Harris-Warrick and Marder, 1991). In invertebrate systems, study of neural circuits has been facilitated by simultaneous recording of multiple neurons with known identities (e.g.(Selverston et al., 1976; Stent et al., 1978; Church and Lloyd, 1994). To identify neurons in a vertebrate forebrain circuit, we combined two powerful techniques: tetrode recording and antidromic stimulation. Living neurons with specific projection patterns can be identified by antidromically stimulating their axons (Ranck, 1975). Previous studies have used combined multi-electrode single unit recording and antidromic stimulation in order to examine the relationship of the activity of single identified neurons to Local Field Potentials and behavior (Soteropoulos and Baker, 2006; Witham and Baker, 2007). Combining single unit ensemble recording with antidromic identification enables the study of identified neurons within the broader circuit, the measurement of functional interactions among neurons in real time, and the enhancement of neuron classification, particularly interneurons, based on functional interactions.

To functionally characterize cell-cell interactions in a forebrain region that contains heterogeneous neuronal subtypes, we recorded ensembles of single units with a four tetrode array in HVC (this acronym is the proper name). HVC is

a cortical nucleus that is critically involved in the production of learned song (Nottebohm et al., 1976; Simpson and Vicario, 1990; Vu et al., 1994; Aronov et al., 2008). HVC contains a functionally heterogeneous population of neuronal subtypes based on projection target: interneurons and two populations of pyramidal-like neurons with mutually-exclusive projection targets (Dutar et al., 1998; Mooney, 2000; Wild et al., 2005). A population of cortico-cortical neurons (HVC_{RA}) projects to the Robust Nucleus of the Arcopallium (RA), a song motor control area. Another population of cortico-basal ganglia neurons (HVC_{X}) projects to Area X, the song basal ganglia. We have achieved antidromic identification of both of these subtypes in neuronal ensembles of up to 21 units. This has enabled examination of the spike trains of identified projection neurons in the context of local forebrain circuits and how these spike trains relate to those of other neurons in the ensemble. Determining the interactions among neurons in a circuit is a critical step in understanding the mechanisms of behavior. Below, we describe the approach, pitfalls, and available solutions that are inherent in this new combined method. Proof-of-concept is provided using conditional probabilities to assess spike train relationships of projection neurons with other neurons that were recorded at the same time. We report the novel finding that both projection neuron subtypes are co-active with multiple other HVC neurons in population recordings. This finding would be impossible with serial single unit recordings.

METHODS

Animals and surgery

Tetrode recordings were obtained from 42 male zebra finches (*Taeniopygia guttata*) that originated from our breeding colony or an outside supplier. Birds were housed under a 14:10-hr light:dark cycle and were given food and water *ad libitum*. The Institutional Animal Care and Use Committee at the University of Minnesota approved all procedures.

Prior to surgery, all animals were deprived of food and water for a minimum of 1 hour before an initial intramuscular injection of 20% urethane (60-70 µl). Two additional subdoses (not exceeding 30 µl/dose) were given at 30-45 minute intervals. The bird was placed in a stereotaxic apparatus (Herb Adams Engineering) and lidocaine (1%, Xylocaine) was injected under the scalp. After resection of the scalp, craniotomies were made over the following right hemisphere brain regions using established coordinates from the bifurcation of the midsagittal sinus at a 20˚ head angle from the horizontal: HVC (approximately 2.5 mm lateral), Area X (1.4-1.6 mm lateral, 3.9-4.1 mm anterior) and RA (1.8-2.2 mm lateral, 1.8-2.0 mm posterior).

Custom-made bipolar stimulating electrodes were constructed from 200 µm Teflon-coated tungsten wires (A-M Systems, Sequim, WA) that were stripped ~2 mm at the tips and epoxied together approximately 200-500 µm apart (100 kΩ impedance). These stimulating electrodes were cemented into place with dental acrylic within Area X at a depth of 3.9-4.5 mm (Figure 1A). A reference electrode (125 µm bare silver wire) was cemented in place between the dura mater and the

brain approximately 3.5 mm anterior and 4 mm lateral. A headpost was also cemented to the bird's skull. After this preparatory surgery, the animal was moved into a sound-attenuating chamber (Industrial Acoustics Company, New York) on an air table (TMC, Peabody, MA). Once the animal was secured by the headpost, a bipolar stimulating electrode (FHC, Bowdoin, ME) (300-500 kΩ impedance) was inserted 0.9-1.4 mm at a 30˚ angle into RA under the control of a microdrive (Siskiyou, Grants Pass, OR).

Recording and electrical stimulation

Ensembles of single units in HVC were recorded extracellularly in 10-15 minute recording sessions with a 4-tetrode array (A4x1; Neuronexus Technologies, Ann Arbor, MI) attached to a 16-channel headstage preamplifier (10x) that fed to a Model 3600 16-channel AC amplifier (A-M Systems). Tetrodes were linearly distributed within HVC parallel to the midsagittal sinus in the rostral-caudal plane with a spacing of 150 μm (Figure 1B). Tetrodes (0.5-2.0 MΩ impedance) with a recording site area of 312 μm^2 were used. Signals were amplified (1000x), filtered (300 – 10,000 Hz), and acquired at 22.05 kHz with custom written Matlab (Mathworks, Natick, MA) software. Activity in HVC was collected on 15-channels; the 4^{th} recording site on one tetrode was not recorded to enable collection of chamber sound events on our analog-to-digital PCI card that was limited to 16 channels (PCI 6251; National Instruments, Austin, TX).

Electrical stimulation was delivered by a stimulus isolation unit triggered by a Master 8 (AMPI, Jerusalem, Israel) to the efferent targets of HVC: Area X and RA. Only one target was stimulated at a time. Single, monophasic pulses of

200 μs duration were delivered at a rate of 0.5 Hz. Stimulation intensity (20 - 400 μA) was gradually increased until reliable spikes were observed in HVC.

In a subset of recordings, collisions of spontaneous and antidromic spikes were obtained to further test the antidromicity of the stimulation and to rule out the possibility of intervening synapses. For spike collisions, a two-window discriminator (FHC) was used to identify specific spontaneous spikes and selectively trigger stimulation within 1.5-ms.

For the study shown in Figure 8, auditory stimuli were played back through a tweeter speaker (Focal, France). Song playback order was randomly determined within each trial. Each auditory playback session consisted of 15 trials, with an 8 second interstimulus interval. Auditory stimuli included Bird's Own Song (BOS), reverse BOS (REV), conspecific, and silence.

Spike Sorting

Multiple spikes on the same tetrode were sorted using automatic clustering followed by manual checking of each cluster. Following each recording, spikes were identified using custom software if they crossed a relatively low, predetermined threshold (mean + 4 SD; calculated independently on each channel for each recording session). 22-point (~1-ms) spike waveforms and their corresponding timestamp were used for clustering. Our conservative threshold included numerous 'noise' events, which were subsequently sorted out, to eliminate the possibility of excluding part of a cluster. Features of thresholded waveforms (for each channel: Energy, Derivative of Energy, and First Principal Component) were then calculated (MClust, A.D. Redish) and subjected to

unsupervised clustering with a Gaussian mixture model with unconstrained covariance matrices (KlustaKwik; K. Harris, Rutgers, http://klustakwik.sourceforge.net) to obtain a maximum of 30 initial “preclusters” using a classification expectation-maximization (CEM) algorithm. KlustaKwik allows for a variable number of clusters, penalized by the Akaike Information Criterion (AIC). The initial preclusters identified by KlustaKwik were manually checked and occasionally combined or split, using MClust 3.5 (A.D. Redish; http://redishlab.neuroscience.umn.edu/MClust/MClust.html) in the Matlab environment. Clustering was performed either on a Dell PC or blade server with a 64-bit processor and Windows XP.

Four criteria were used to determine cluster quality to ensure that sorted spikes accurately reflected single units. Clusters that did not pass all criteria were discarded. The four criteria for cluster quality were: (1) L-ratio score < 0.1; (2) Isolation Distance >16; (3) less than 1% violations of a 1-ms refractory period; and (4) visual separation of the cluster from “noise” and other clusters on at least 2 (of 12) dimensions. The L-ratio is a measure of the compactness of a cluster. Isolation distance quantifies how well a cluster separates from other clusters (Harris et al., 2001; Schmitzer-Torbert et al., 2005; Jackson et al., 2006). For spike waveform display (Figures 5C, 6B, 7E-F) and analysis, a matrix of 64-point waveforms was created with the same indices as the 22-point waveform matrix that was used for clustering.

Identification of clustered antidromic single units

All units used for further analysis, including those identified by antidromic stimulation, met the criteria described above. A subset of spontaneous spikes typically co-clustered with antidromic spikes. In collision experiments, spontaneous spikes that triggered spike collisions also co-clustered with antidromic spikes. The co-clustering of spontaneous spikes with antidromic and/or collided spikes enables the identification of spontaneous events. Spike times for events occurring after stimulation were used to identify fixed latency spikes and to calculate latency time and latency variability relative to the stimulus. Antidromic latency was defined as the mean time from stimulation to the peak of the action potential for at least 10 trials. Latency variability was defined as the standard deviation of the latencies. All spikes that were collected during and 100 ms after stimulation were excluded from analyses of waveform properties and network interactions. Based on our finding that antidromic units identified with spike collisions had latency variabilities of up to 119 μs (see Results, Figure 5), the latency variability cut-off for identification of unit as a projection unit was set at <125 μs.

Auditory analyses

Post-hoc alignment of song stimuli and corresponding neural activity enable comparison of single neuron auditory responses across multiple trials. Dot rasters were created with a 1-ms binning window. Peristimulus time histograms (PSTHs) were constructed by smoothing the summed dot raster from all trials using a moving average with a 5-ms binning window.

Analyses of functional interactions and statistics

All units used for quantitative analysis were from recordings of spontaneous activity in adult finches (> 130 days post hatch). Conditional probabilities were calculated to assess functional connectivity between pairs of units in the circuit. The entire recording between stimulation epochs was used for conditional probability calculations. We examined spike train relationships by calculating the probability of projection neurons firing pair-wise with all other neurons: the conditional probability of the projection neuron firing ± 5 ms, given that the other unit had fired. We shuffled the interspike intervals of one of the spike trains to control for overall firing rate (Nadasdy et al., 1999). All conditional probability calculations were performed on both the recorded and shuffled data sets. The Wilcoxon signed-rank test was used to compare recorded and shuffled conditional probabilities with significance defined as $p < 0.05$. Except where noted, all measures of central tendency are shown as median with interquartile range.

RESULTS

Using combined antidromic stimulation and tetrode recording, we identified single projection neurons within ensembles of single units in the zebra finch HVC song nucleus. Figure 1 schematically diagrams the placement our recording tetrode array and two stimulating electrodes in the brain nuclei of the song system. 41 adult zebra finches were successfully implanted with stimulating and recording electrodes. Of these, 35 animals had antidromic units that fired reliably with low latency variability during the recording. Eleven animals had

antidromic units that met our four conservative criteria for cluster quality. Identified units from two of these animals were discarded because their latency variability was greater than 125 μs and collision data were unavailable. Ultimately, 12 antidromic units (HVC_X= 5; HVC_{RA}= 7) from 9 animals were included in our final quantitative analyses.

Tetrode recordings in the zebra finch

Figures 2A and 3A show spontaneous activity on four channels of a single tetrode from different adult animals, including the activity of an identified HVC_X or HVC_{RA} neuron, respectively. Because the relative distance from the neuron varies across the sites of a tetrode, spike waveforms of individual neurons differ across each of the recording channels of the tetrode. Sorting techniques exploit these differences to identify spikes of single units. The raster plot (Figure 2B) shows the relative spike times of 3 single units recorded by the 4-tetrode array during a 30-ms period, including an HVC_X. The time frame is expanded in Figure 2C (3 sec) to show the simultaneous activity of these 3 units plus an additional 12 that were recorded and clustered in the same session. Similar plots of the activity of an HVC_{RA} unit in the context of its local circuit are shown in Figure 3. Figure 4 shows that spike sorting of tetrode data captures every spike in the characteristic high-frequency burst of an HVC_{RA} projection unit previously reported using single wire recordings (Hahnloser et al., 2002) The firing rate of spikes within HVC_{RA} bursts using our methods was 248 ± 121 Hz, which is consistent with previously published results (Hahnloser et al., 2002; Long et al., 2010). Across all 9 adult animals included in this study, we recorded 134 single

units over 12 10-15 minute recording sessions. The number of simultaneously recorded units ranged between 5 and 21.

Antidromic stimulation is used to identify single units in an ensemble

In order to identify specific cell types, we drove antidromic spikes in HVC with stimulation of Area X or RA and recorded the resulting activity with a 4-tetrode array. The stimulus parameters were similar to those used in single unit studies (Hahnloser et al., 2002; Hahnloser et al., 2006): 0.5 Hz stimulation between 20 and 400 μA. Figure 5A shows action potentials in an HVC neuron stimulated with an electrode in RA. Six sets of simultaneously acquired and temporally aligned traces from the 4 recording channels of one of the 4 tetrodes are overlaid. This HVC_{RA} neuron had an antidromic latency of 8.09 ms stimulated with 110 μA of current. The latency variability of the antidromic spike was 119 μs. This was higher than that previously reported (94 μs; (Hahnloser et al., 2006). The range of antidromic latencies for putative HVC_X and HVC_{RA} neurons agreed with previously reported data (Hahnloser et al., 2006): The latency to first spike of HVC_X neurons was 3.4 - 9.9 ms (Mean ± SD: 5.1 ± 2.4 ms), whereas that of HVC_{RA} neurons was 3.5 - 8.1ms (5.4 ± 1.7 ms). The latency variability of HVC_X neurons used in further analyses was 0.078 ± 0.035 ms (Mean ± SD), whereas that of HVC_{RA} neurons was 0.072 ± 0.027 ms. Antidromically identified projection units were included in later analyses if the latency variability of a given unit was less than 125 μs.

To definitively determine that the spikes triggered in single neurons were antidromically stimulated, we performed collision tests on a subset of units that

had a fixed latency. Collision tests are the best method for confirming the direct stimulation of a neuron's axon, as opposed to synaptic activation of that neuron through axonal stimulation of a cell that is presynaptic to that neuron (Lipski, 1981). "Orthodromic" refers to the physiological conduction of an action potential from cell body to synaptic terminal, whereas "antidromic" refers to the reverse conduction of an action potential from axon to cell body. The cell body of a projection neuron, which lies in HVC, can be invaded antidromically after a spontaneous spike with a minimal delay of *T*, which is defined as:

$$T = R + 2T_1,$$

where *R* is the absolute refractory period and T_1 is the antidromic latency. If a stimulus is applied during the critical delay (*W*),

$$W = T_1 + R,$$

no antidromic spike will be observed in the cell body due to collision of the orthodromic and antidromic spikes within the axon.

For these experiments, a two-window discriminator identified spontaneous spikes that were used to trigger stimulation. In pilot experiments, it was determined that a single trigger window was insufficient to isolate the spikes of single neurons in an ensemble recording due to triggering by multiple units. The stimulation occurred within 1.5 ms of the spontaneous spike, and resulted in a collision of the orthodromic and antidromic spikes (Figure 5B).

Solutions to the problem of multiple overlapping spikes triggered by the antidromic stimulation

A problem inherent in the combination of tetrode recording and antidromic stimulation is that of overlapping waveforms. A barrage of near-simultaneous spikes can be triggered by stimulation. The waveforms can then overlap and preclude clustering based on the features of waveforms. To overcome this problem, site areas and spacing for the tetrode configuration, as well as the duration of waveforms used for feature calculation, must be carefully selected and optimized for each brain region. Larger site areas that record many units may be ideal for monitoring large numbers of neurons, but may be unsuitable for identifying a subset of single units using antidromic stimulation due to co-activation of many neurons and subsequent occlusion of waveforms used in clustering. In addition, the lowest stimulation current that triggers an antidromic spike in at least one high signal:noise unit should be used. Attempting to increase the number of antidromically-identified units by increasing stimulation strength can be detrimental by causing near-simultaneous activation of multiple units and subsequent failure of spike feature clusters to meet quality criteria. If, even with these steps, two units are consistently stimulated at latencies that yield overlapping waveforms, the tetrode array could be moved slightly to eliminate one of the units from the recording. Alternatively, spontaneous spikes that drive collisions may be used to identify each unit, as described below.

Clustering of antidromic units

We determined that antidromically identified units could be successfully isolated from other simultaneously recorded units (Figure 6). Antidromic stimulation was delivered at the beginning and end of each recording session. Antidromic spikes and spontaneous spikes that were used to trigger collisions were clustered with all other spikes from the recording, as described above. All antidromic units were held to the same cluster quality criteria as other units (L-ratio, Isolation Distance, ISI Violations, visual separation). Note the separation of a cluster that was identified as an HVC_{RA} from both noise and other clusters in Figure 6A-B.

A solution to the potential problem of waveform differences in spontaneous and antidromically-stimulated spikes

Comparison of overlaid waveforms (Figure 6C) demonstrates that antidromic and spontaneous waveforms were very similar. However, there were slight differences in waveform, such as the initial segment spikes (arrowheads in the left panel of Figure 6C) characteristic of antidromic waveforms (Lipski, 1981). The appearance of this voltage fluctuation that is typical of intracellular recordings in the median of our extracellularly-recorded events demonstrates the reliability and fidelity of this method. Because of these differences in antidromic and spontaneous waveforms, two visually-separated subclusters often appeared in a subset of dimensions (data not shown). It is possible that, in some systems, antidromic spikes may differ sufficiently from orthodromic spikes to produce two separate clusters in several dimensions, preventing identification of the

spontaneous and other orthodromic spikes of the projection neuron as those of a projection neuron. However, we found that spontaneous spikes that triggered spike collisions reliably clustered with those obtained in the absence of stimulation based on our four criteria (Figure 6C-D). The co-clustering of spontaneous spikes that resulted in collisions with other orthodromic events enables antidromic identification while avoiding the complications of both the overlap of near-simultaneous antidromically-stimulated waveforms and any potential differences between orthodromic and antidromically-driven waveforms.

Simultaneous recording of multiple projection neurons

Tetrode recording with antidromic identification enables simultaneous recording of multiple identified projection neurons. Figure 7 shows the activity of three HVC_X neurons together with other neurons in a population burst. Two of these projection neurons were recorded on the same tetrode, and were identified as different units based on their locations in feature space and different fixed latencies. Each of the identified neurons passed our strict cluster criteria. Simultaneous recordings of multiple projection neurons will facilitate understanding of the functional interactions among the projection neurons, their regulation by the local network (e.g., Do the same interneurons appear to functionally inhibit multiple projection neurons?), and the output of the network.

Auditory playback

Neurons in HVC respond preferentially to playback of Bird's Own Song (BOS) and have stereotyped responses to BOS and other auditory stimuli (Margoliash, 1983; Margoliash and Fortune, 1992). Combining tetrode recording

with antidromic stimulation enables simultaneous recording of identified projection neurons and their local circuits. Figure 8 shows the patterns of activity of two single units to playbacks of different song stimuli (A1-D1). One unidentified unit responds more to Bird's Own Song (Figure 8 A2-A3), than to other songs (B, C, D 2-3). An HVC_X fires precisely across repeated playbacks of BOS, as observed in previous single unit studies (Prather et al., 2008).

Evaluating spike time relationships among identified HVC neurons

Combining antidromic stimulation with tetrode recording enables analysis of spike time relationships among projection neurons and their local circuits. A defining characteristic of song system activity is its bursts of population activity that involve multiple neurons (Schmidt and Konishi, 1998; Crandall et al., 2007a; Day et al., 2009; Shank and Margoliash, 2009). However, the types of HVC neurons that are co-active in the bursts have not been determined. Toward this end, we have calculated conditional probabilities between pairs of neuronal spike trains. We determined the probability that a projection neuron fired, given that another neuron in HVC had fired. The conditional probability was obtained using a ± 5 ms window set by the spike time of any neuron in the recording. This allowed us to examine the probability of a projection neuron firing with other neurons. Conditional probabilities of pairs of real spike trains were compared to conditional probabilities calculated from the same data, but with one spike train shuffled by randomly reordering the interspike intervals (Nadasdy et al., 1999). We found that both types of HVC principal neurons were active with the population under anesthesia (Figure 9). Example raster plots in Figure 9A-B

show HVC_X and HVC_{RA} neurons that were active during population bursts. Compared to shuffled spike times, both HVC_X and HVC_{RA} units were significantly more likely to fire when other units were active (N: HVC_X = 86 pairs; HVC_{RA} = 43 pairs; $p < 1.5 \times 10^{-6}$, $p < 2.1 \times 10^{-7}$ respectively; Figure 9C-D).

DISCUSSION

We have combined two electrophysiological techniques to identify individual neurons in ensemble recordings. This new method of combined tetrode recording and antidromic identification enables the study of identified neurons in the context of a functioning forebrain network. Knowledge of the network, in turn, can provide information, such as functionally inhibitory interactions, that enables classification of additional neurons. Although the study of identified neurons in a functioning circuit has been possible for decades in invertebrate preparations (Frazier et al., 1967; Selverston et al., 1976), vertebrate work has focused on the physiological study of either single identified neurons (Hahnloser et al., 2002; Herfst and Brecht, 2008; Long et al., 2010) or large neuronal ensembles (Nicolelis et al., 1997; Johnson and Redish, 2007). Reliable and repeatable identification of neuronal subtypes using a combination of electrophysiological techniques will facilitate and hasten understanding of vertebrate circuits and their regulation.

One particular advantage of ensemble recordings is the large number of units that can be simultaneously monitored. This approach allows for analysis of hundreds of neuron-neuron interactions using analytical methods such as conditional probability or coherence. However, one limitation of tetrode

recordings in brain areas with functionally heterogeneous subtypes has been the definitive identification of these subtypes and subsequent attribution of patterns of activity and functional connectivity. Identification of specific cells in the circuit, when possible, can more accurately inform our understanding of neuronal interactions. In many systems, cellular identification technologies limit the possible number of recorded pairs (e.g., dye fills) or the ability to detect real-time interactions (e.g., antidromic stimulation combined with serial single-unit recording). Functionally inhibitory interactions, in particular, are impossible to detect with serial single-unit recording.

There are potential pitfalls and limitations of this technique. Antidromic stimulation of multiple neurons or single neurons with local synaptic connections can result in a barrage of near-simultaneous spiking activity. Coincident, overlapping spiking events occlude waveforms that are used for spike clustering. We addressed this significant problem in several ways: (1) the spacing and site area of the tetrodes was optimized to record a relatively small number of neurons; (2) the stimulation magnitude was always decreased to the minimum required for the activation of at least one antidromic unit in the entire recording; (3) the waveform duration used for feature calculation was optimized; and (4) spike collisions were developed as an alternative method to identify units, since the spontaneous spike that triggers the collision typically occurs in relative isolation and thus provides a less occluded waveform. The first two of these optimizations required a compromise, because the number of all possible units and antidromic units, respectively, had to be decreased in order to achieve

unoccluded waveforms. For any system, application of this technique will require a compromise, since the number of units in the recording will likely need to be decreased to enable antidromic identification of a small subset of those units. Because the stimulation is minimal, some units in the recording that project to the target will not be identified as such. This is also the case with antidromic identification in serial single-unit recordings (Ranck, 1975; Lipski, 1981).

Another potential pitfall of using antidromic stimulation to identify single units in an ensemble is the inherent difference between spontaneous and antidromically-driven waveforms. This is best exemplified by the initial segment spike that appears in antidromically-driven, but not spontaneous, waveforms (Figure 6C;(Lipski, 1981). We found that, in the system under study (the birdsong nucleus HVC), spontaneous and antidromic waveforms still tended to co-cluster in most dimensions that we analyzed and met our conservative criteria for cluster quality. To ensure that the antidromic and spontaneous waveforms did indeed represent the activity of the same unit, we examined spike collisions: Spontaneous spikes that triggered collisions possessed waveforms that were identical to spontaneous waveforms that were used for the analysis of circuit activity.

A problem inherent in antidromic stimulation in general, whether used in combination with serial single-unit or multiple-unit ensemble recordings, is that the stimulation itself may alter circuit activity. We sought to minimize this problem by stimulating only at the beginning and end of each recording session, with continuous recording throughout initial stimulation, circuit monitoring in the

absence of stimulation, and re-stimulation. Our stimulation parameters matched those used in previous serial single-unit studies, with stimulation magnitudes consistently on the lower end of the range reported (Hahnloser et al., 2002).

Even with these optimizations, there are neural systems where antidromic identification of units in neural ensembles may not be useful for identifying cells as a particular projection neuron subtype. For example, in brain areas where all neurons project to the same target, such as the granular layer of the dentate gyrus, antidromic stimulation would not provide additional information regarding cellular identity. However, in these cases, this technique may provide valuable information by confirming the identity of a specific projection neuron at the beginning and end of the recording. Additionally, in situations in which pharmacological manipulation may cause spontaneous spiking to cease, antidromic stimulation can be used to reconfirm that the cell is still alive and capable of spiking. In brain areas where cells are densely packed or tend to fire simultaneously in population bursts, combination of antidromic stimulation and tetrode recording may theoretically not be possible. However, we were able to identify multiple single units in HVC, which has relatively dense clusters of tightly-packed neurons that are characterized by their population bursts. This suggests that this technique can be applied to other brain areas that might initially seem intractable.

Combining antidromic stimulation with tetrode recording has allowed us to identify single projection units in a complex neuronal network during both spontaneous and auditory-evoked neural activity. This technique could be easily

adapted to recordings of neuron populations with multiple single wire electrodes instead of the compound multi-electrode (tetrode) recordings that we describe here. Assessing the functional connectivity of neural networks is paramount in understanding how circuits work. We have demonstrated the feasibility of the technique in a challenging brain area with dense clusters of neurons that fire in bursts. In the intact, anesthetized birdsong system, we found that both types of projection neurons (cortico-cortical and cortico-basal ganglia) participate in population bursts. Future experiments will utilize functional interactions with identified projection neurons to class other neuronal subtypes. Other potential applications of this technique are to enable the discovery of the topography of activity within and among neuronal subtypes across heterogeneous brain areas and to delve into circuit changes that occur in tandem with behavioral plasticity and the insertion of new neurons.

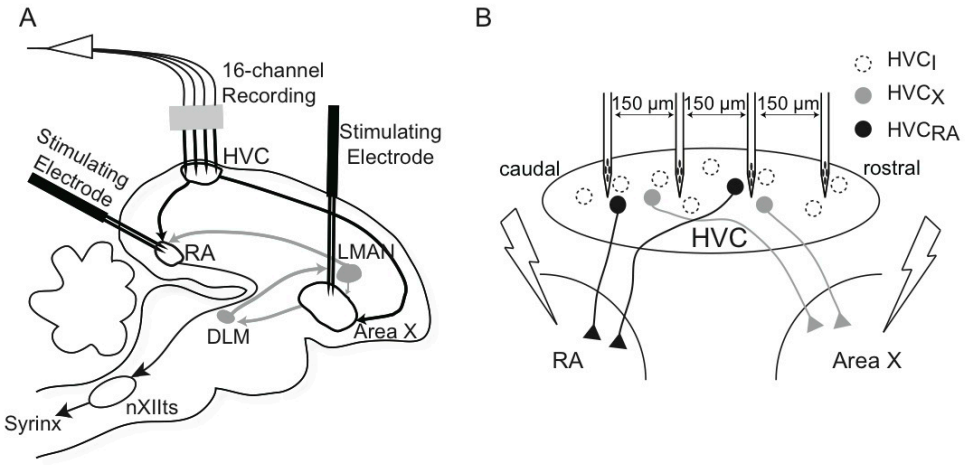

Figure 1. Schematic of the recording design used in this study.
A) A 16-channel, 4-tetrode recording array was placed in the cortical song nucleus HVC in the zebra finch brain. Bipolar stimulating electrodes were placed in its efferent projection targets, the song motor nucleus RA, and song basal ganglia, Area X. Arrows denote a subset of connections among song system nuclei. B) A simplified diagram of HVC shows the tetrode configuration. Tetrode shanks were placed 150 µm apart along the rostral-caudal axis. HVC neurons may project to either RA or Area X, but not both. The axons of a third group, interneurons, ramify solely within HVC. For this study, axons that terminated in Area X or RA were stimulated to antidromically identify single units in the context of a functioning forebrain network.

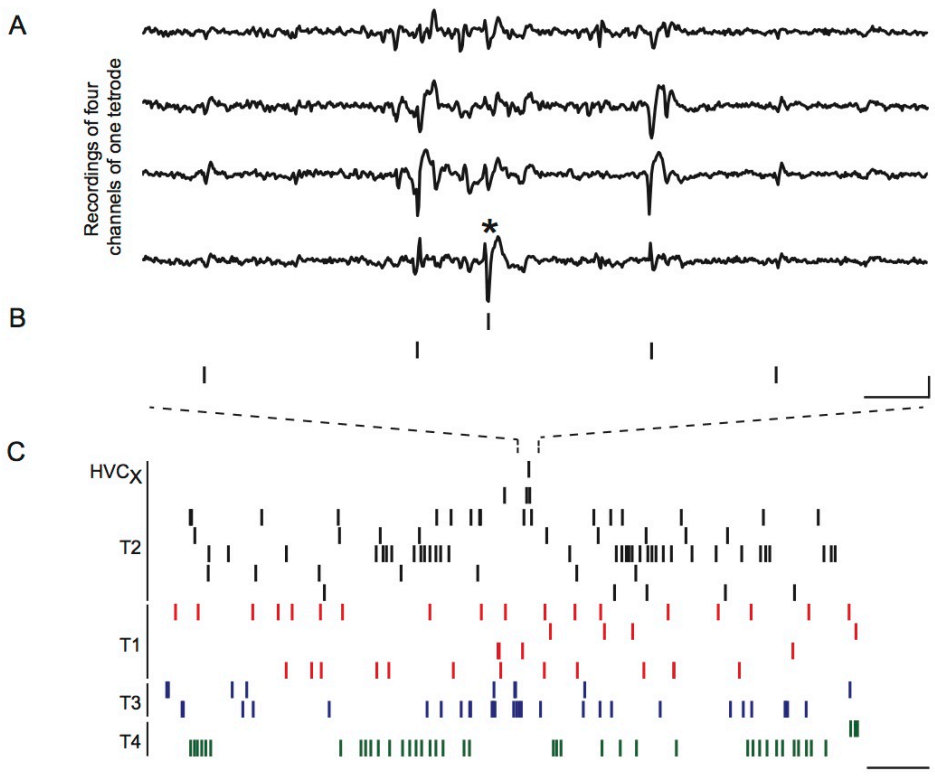

Figure 2. Ensemble recordings capture the simultaneous activity of an HVC_X with its local circuit neurons.
A) Raw HVC multi-electrode voltage traces of four recording sites from the same tetrode are temporally aligned. The asterisk indicates the action potential of an HVC_X. Voltage events have variable amplitudes across channels depending on the recording site location relative to the neuron. B) Raster marks indicate the activity of the HVC_X and two other neurons from the same tetrode that were active in the same time frame. Scale bar: 2.5 ms, 100 µV. C) Spikes of single neurons across all tetrodes of the recording are shown in a longer time window. The activity of the HVC_X unit (top) is labeled on the left. All clusters (n=15) from a given tetrode are indicated by the line at left and shared color. Scale bar: 125 ms. Data are from O-365, age 155 days.

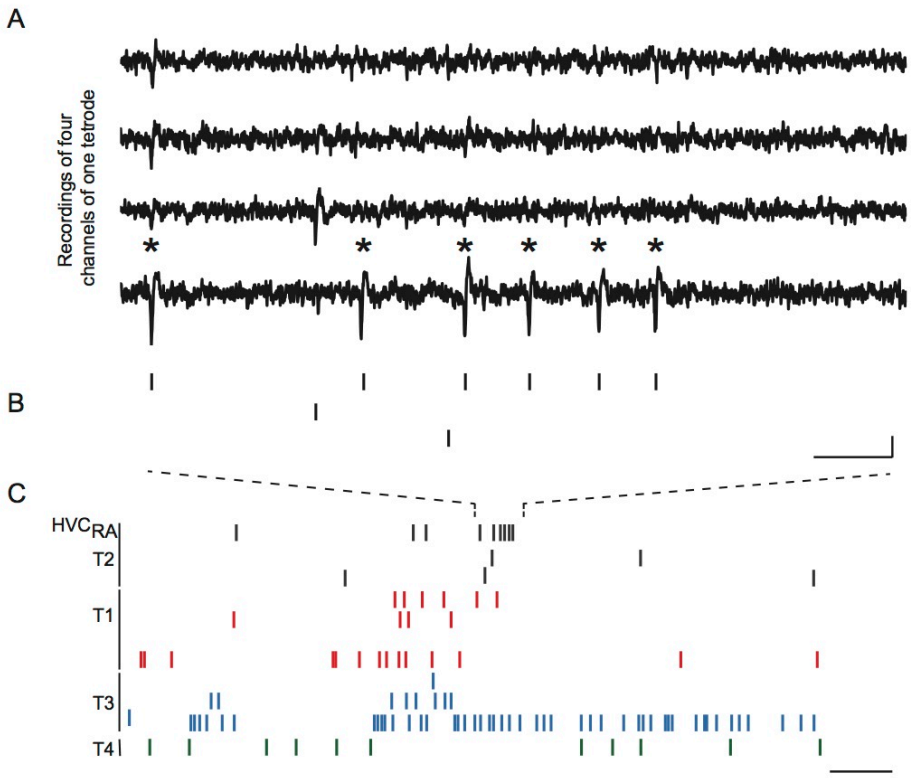

Figure 3. Ensemble recordings capture the simultaneous activity of an HVC$_{RA}$ with its local circuit neurons.
A) Raw HVC multi-electrode voltage traces of four recording sites from the same tetrode are temporally aligned. Asterisks indicate the action potentials of an HVC$_{RA}$. B) Raster marks indicate the activity of the HVC$_{RA}$ and two other neurons from the same tetrode that were active in the same time frame. Scale bar: 10 ms, 50 μV. C) Spikes of single neurons across all tetrodes of the recording are shown in a longer time window. The activity of the HVC$_{RA}$ unit (top) is labeled on the left. All clusters (n=15) from a given tetrode are indicated by the line at left and shared color. Scale bar: 125 ms. Data are from O-236, age 139 days.

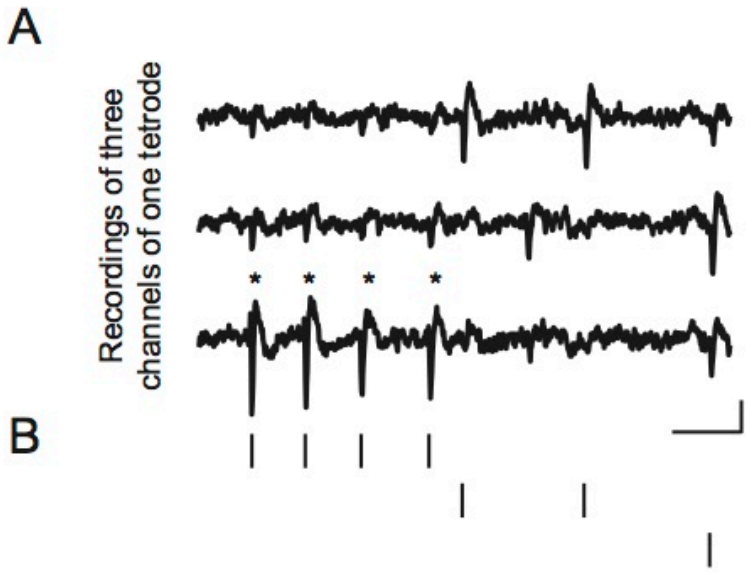

Figure 4. Single-unit bursts are captured in ensemble recordings and subsequent clustering analysis.
A) Raw HVC multi-electrode voltage traces of three recording sites from the same triode are temporally aligned. Asterisks indicate each action potential in an HVC_{RA} burst. B) Raster marks indicate the activity of the HVC_{RA} and two other neurons from the same tetrode that were active in the same time frame. Scale bar: 5 ms, 50 μV. Data are from R-757, age 270 days.

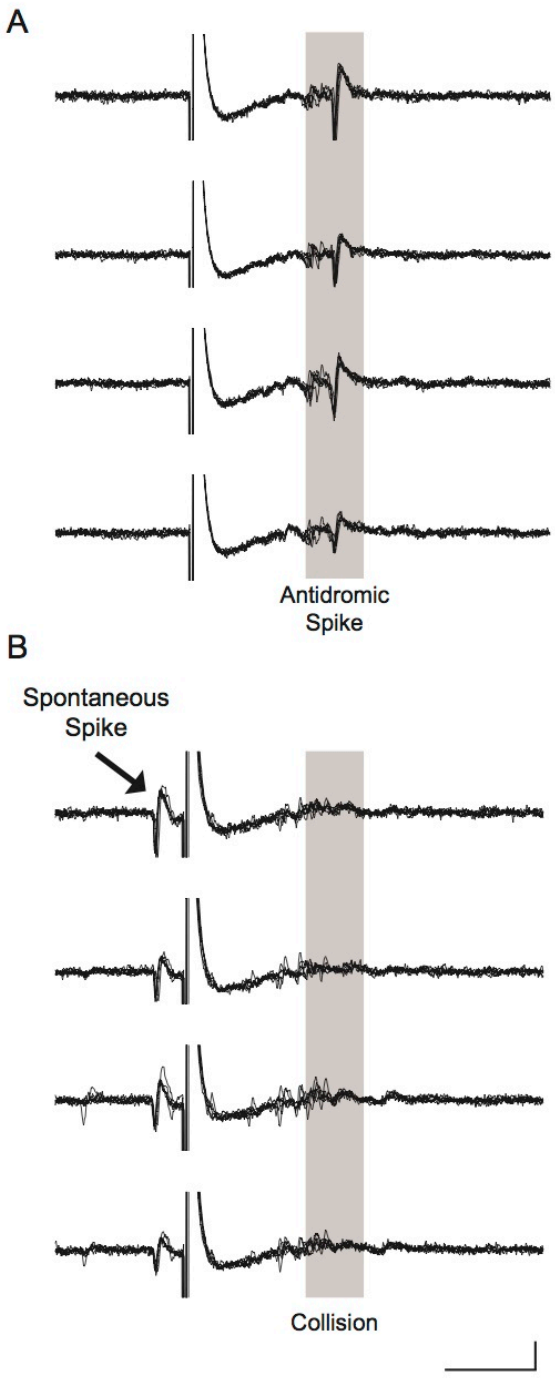

Figure 5. Antidromic identification of projection neurons can be achieved in ensemble recordings.
A) Stimulation of the premotor song nucleus RA (200 μs, 42 μA) reliably drove an HVC spike at a latency of 8.1 ms. Six traces are overlaid and temporally aligned for each of the four recording sites. B) Triggering the stimulus by a spontaneous spike resulted in orthodromic-antidromic collisions. Scale bar: 5 ms, 500 μV. Data are from bird R-161, age 150 days.

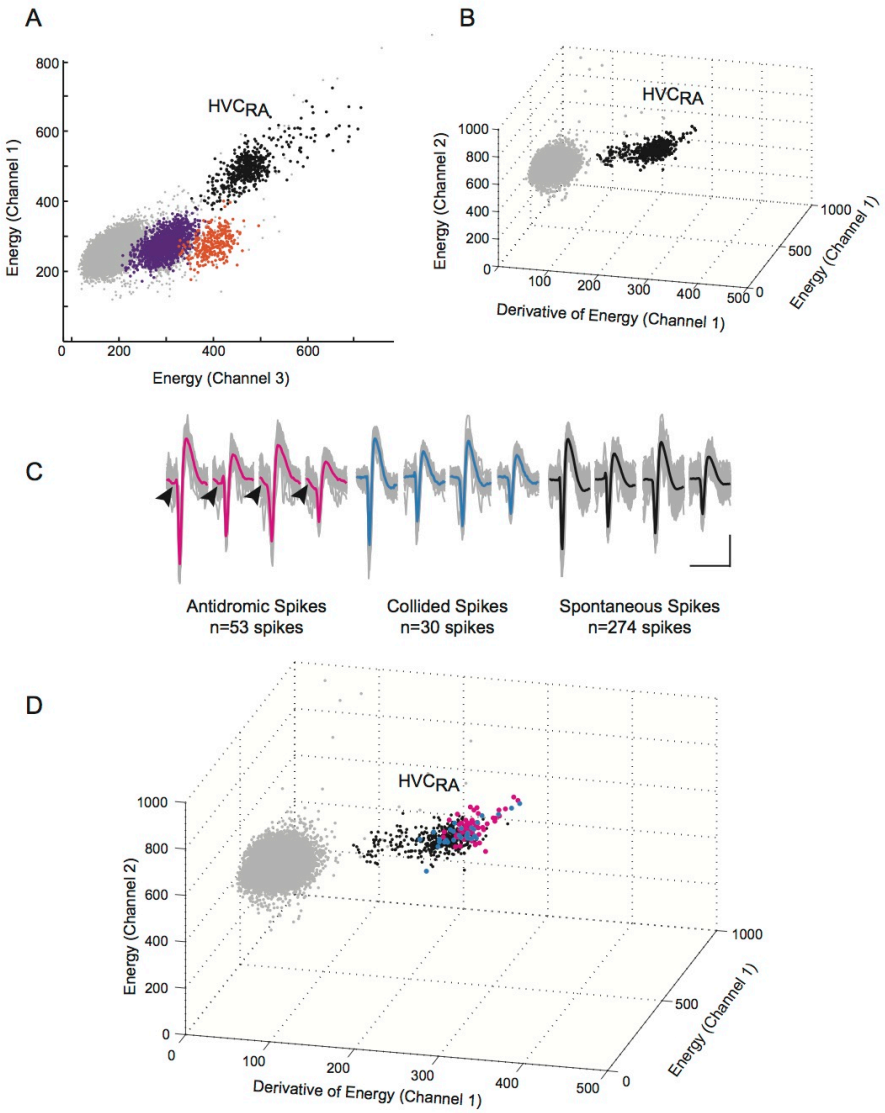

Figure 6. The spikes of antidromically-identified units were identified by co-clustering spontaneous and stimulus-related waveforms.
A) A plot of 2 of the 12 parameters used to sort spikes shows the spike event cluster of an RA-projecting unit (black) and 2 other units (purple, orange) that were identified. Light gray dots consist of spikes and noise that were not clustered in the dimensions shown. The black cluster contains spontaneous as well as antidromic spikes that were evoked by stimulation of RA. L-ratio: 0.001; Isolation Distance: 120.9. B) A 3D plot shows 3 additional waveform features that were used to identify the RA-projecting unit. Note that in this view, the orange and purple clusters are no longer visually separated, but the HVC_{RA} (black) cluster remains well isolated. C) Waveforms are segregated according to their relationship to antidromic stimulation: antidromically driven (magenta), spontaneous collided (blue), and spontaneous during the stimulation-off recording period (black). Note the similarity of all waveforms. The median voltage is overlaid in the color corresponding to the graph in D. Arrowheads (left panel) indicate the presence of the initial segment spike in antidromically-stimulated waveforms. Scale bar: 2 ms, 40 μV. D) The antidromic, collided, and spontaneous spikes cluster together based on clustering criteria and visual inspection. Data are from bird R-161, age 150 days.

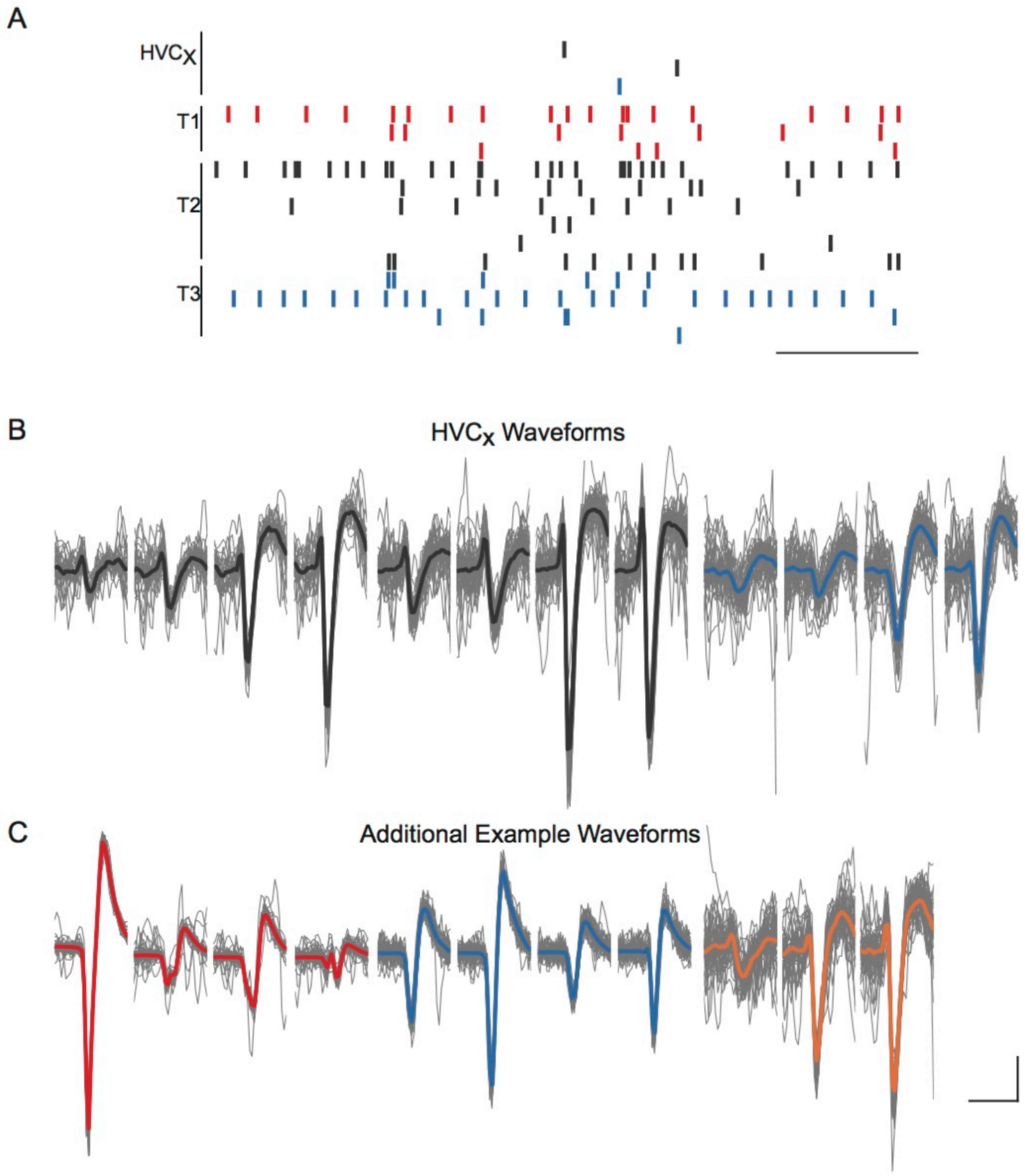

Figure 7. Multiple simultaneously active projection neurons can be studied using combined tetrode recording and antidromic identification.
Raster marks indicate the activity of all units that were active during a 1.2 sec time window. 3 HVC_X neurons recorded on two tetrodes were active in a population burst. Antidromic latencies for each projection neuron were (from top to bottom): 9.47 ms, 10.85 ms, 6.68 ms. Single units from the same tetrode are indicated by the same color. Scale bar: 250 ms. B) Overlaid waveforms (gray) and the median waveform (color) of 50 randomly selected spikes of simultaneously active HVC_X units shown in (A) and three other example waveforms of units active in the same session. Single units from the same tetrode are indicated by the same color. Tetrode 1: Red; 2: Black; 3: Blue; 4: Orange. Scale bar: 150 μV (all but C, Left), 250 μV (C, Left); 1 ms. Data are from O-37, age 49 days.

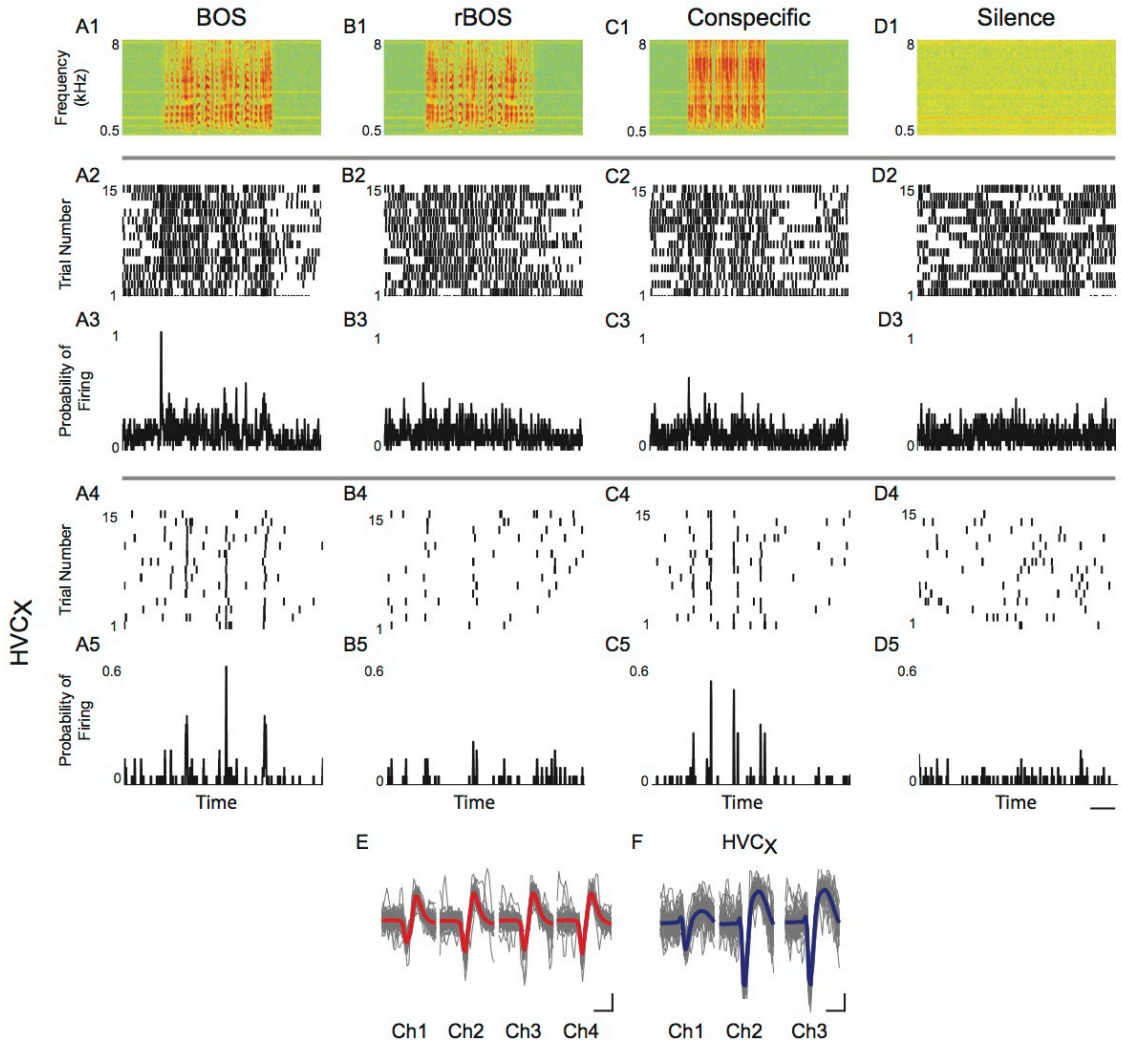

Figure 8. Auditory-evoked activity in simultaneously-recorded single units.
A1-D1) Spectrograms of auditory stimuli. A2-D2, A4-D4) Aligned raster plots of individual units for 15 trials of auditory playback show responses to an HVC_X (bottom) and a unit in its local circuit (top). Note the precision of firing in the HVC_X. A3-D3, A5-D5). Peristimulus time histograms (PSTHs) show the moving averages with a 5-ms binning window. Scale bar: 1 sec. E, F). Waveforms of each unit. 50 randomly selected spikes are overlaid in gray. The median waveform is plotted in red (E) or blue (F; HVC_X). Scale bar: 20 µV, 0.5 ms. Data are from R-493, age 166 days.

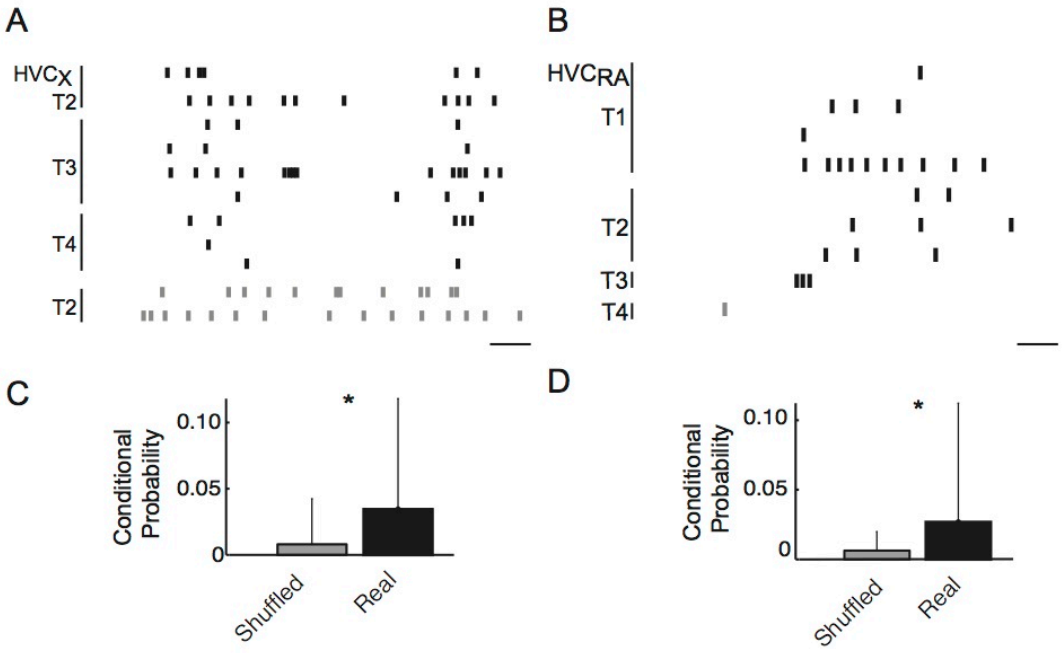

Figure 9. Both types of cortical projection neurons are active during HVC population bursts.
A, B) The rasters show the temporally-aligned spike times of all units from four tetrodes that were simultaneously active within a population burst (black) or outside of the bursts (gray). HVC_X and HVC_{RA} units spiked within population bursts. A, B are from different animals. Scale bar: 10 ms. C, D) Group data show the conditional probabilities of a projection unit firing given that any other unit has fired ± 5 ms. HVC_X (C) and HVC_{RA} (D) recorded spikes were more likely to occur within ± 5 ms of another unit firing than shuffled controls (age > 130 days; N: HVC_X = 86 pairs; HVC_{RA}= 43 pairs; $p < 0.005$ for both).

Chapter 5

Network analysis of single unit ensembles during song learning

INTRODUCTION

Vocal learning in oscine songbirds is an excellent model for human speech acquisition (Marler, 1970; Doupe and Kuhl, 1999) and, thus, sensorimotor integration and learning. Humans and songbirds learn their vocalizations during two developmental sensitive periods for sensory and sensorimotor learning (Thorpe, 1958; Konishi, 1965; Marler, 1970; Marler and Peters, 1977). During the sensorimotor phase, animals match their vocalizations to a memory of a tutor song, but the neural mechanisms underlying song crystallization (mature song resilient to further modification) are not well understood.

The neural pathways for song learning during the sensorimotor phase are well described (Nottebohm and Arnold, 1976; McCasland and Konishi, 1981; Bottjer et al., 1984). The song control nucleus HVC receives auditory input and sends efferents to the two pathways critical for song production and learning. The motor pathway consists of HVC and one of its efferent targets, RA (the Robust Nucleus of the Arcopallium), an avian analog of motor cortex. The motor pathway is critical for song production and temporal patterning of the song, but it is not considered as critical for song learning as the Anterior Forebrain Pathway (AFP).

The second set of neurons in HVC project to Area X, a basal ganglia homologue, which is the input to the AFP. Area X is one of the three song nuclei in the AFP, a well-characterized basal ganglia thalamo-cortical loop, which is critical for song learning. Area X sends inhibitory projections to the medial

dorsolateral nucleus of the thalamus (DLM), which, in turn, sends excitatory efferents to the lateral magnocellular nucleus (LMAN; cortical). LMAN and HVC share the same output nucleus, RA, which projects to motor neurons in the brainstem to drive vocalizations. Given that activity in LMAN is modulated by social interactions in adults; LMAN shows more precise firing when animals sing directed song (to a female, or other audience) when compared to activity during undirected song. Therefore, adult songs are composites produced by the integration of LMAN and HVC activity within RA (Thompson & Johnson, 2011).

The AFP is required for song learning; lesions to Area X prevent juvenile songs from progressing towards a match to a tutor model, leaving them in a perpetually variable state (Sohrabji et al., 1990; Scharff and Nottebohm, 1991). Conversely, song crystallizes when LMAN is lesioned, even if song is still in an immature state (Bottjer et al., 1984; Kao and Brainard, 2006). These data implicate LMAN, and the AFP, in the generation of variability that is necessary for trial-and-error learning.

Early in the sensorimotor phase, initial vocalizations (referred to as subsong or babbling and characterized by highly variable, unpatterned sounds) are primarily generated by LMAN, without input from HVC (Aronov et al., 2008). However, by the conclusion of sensorimotor learning, patterning of song is controlled by the motor pathway. HVC input to RA is weak during subsong; the projections are present but synapses are sparse (Mooney, 1992; Foster and Bottjer, 1998). However, at the time of song crystallization, anatomical changes in both HVC and LMAN result in a ratio of HVC:LMAN synapses on RA neurons

of ~2:1 (Herrmann and Arnold, 1991).

How is variability in the AFP restricted in adults? Does input from HVC modulate activity in the AFP? The role of the motor pathway in song learning is difficult to determine, in large part because lesions to HVC prevent vocalization (Nottebohm et al., 1976). Longitudinal studies using multi-unit recordings have explored the role of HVC in song learning. Activity in HVC is developmentally regulated; in juveniles, HVC is characterized by bursts of population activity that begin before and outlast the vocalization. In adults, HVC activity is more tightly correlated with song production. The change in HVC burst duration is correlated with song maturation (Crandall et al., 2007a). In addition, bursts in HVC are correlated with spectral and temporal stability during singing, and decrements in HVC activity are correlated with plasticity in syllable structure (Day et al., 2008; Chapter 2). This change in HVC activity may restrict plasticity during sensorimotor learning (Day et al., 2008). Collectively, these data point to HVC as a node of control of sensorimotor development in the song system.

The microcircuitry of the adult HVC has been described (Mooney and Prather, 2005), but many details remain unresolved. For example, interneurons in HVC are poorly understood, and their interactions with each other and with the projection neurons. The roles of HVC output neurons (HVC_X and HVC_{RA}) during singing and auditory playback in adults have been better characterized (see Chapter 1; (Mooney, 2000; Hahnloser et al., 2002; Mooney and Prather, 2005; Kozhevnikov and Fee, 2007), but their activity has been inferred using serial, paired recordings. The neural control of song production cannot be fully

understood until an understanding of how networks of neurons in song system nuclei interact with each other to produce a complex, stereotyped behavior. In addition, understanding how relationships among neurons in HVC change during learning has important implications for development of neural networks and critical period plasticity.

To monitor developmental changes in ensembles of cells in HVC and to capture dozens of pairs of simultaneously recorded neurons of different types, we recorded HVC activity in juvenile and adult birds with an extracellular 4-tetrode array. We paired tetrode recording with antidromic stimulation to identify individual projection neurons (Day et al., in press). We previously found that both types of projection neurons are active in bursts in adults (Day et al., in press). Here we ask how the overall population of single units and how smaller subpopulations of cells change with development. We characterized cells in juvenile and adults based on their functional interactions with other cells in the network. One set of cells was asynchronous and did not fire in population bursts with other units. Coherence measurements demonstrated that these cells were functionally inhibitory. Inhibitory interneurons are involved in network oscillations and synchrony, detecting sensory input, experience-dependent refinement of local circuits (Benes and Berretta, 2001; Belmonte et al., 2004; Gonzalez-Burgos and Lewis, 2008; LeBlanc and Fagiolini, 2011). We saw differences in the waveforms, timing, and bursting properties of these groups in juveniles and adults. In addition, we observed that the identified population of cells that project to the AFP pathway change with development. These changes may reflect an

important role that basal ganglia-projecting neurons play in regulating activity in a basal ganglia-thalamo-cortical loop, and/or transmitting an efference copy for a forward model of motor learning and control.

METHODS

Fifty-two (age 45-61 days; in the middle of the sensorimotor stage of song development) and 26 adult (>120 days) male zebra finches (*Taeniopygia guttata*) were subjected to surgical and recording methods described by Day et al. (2011; Chapter 4). Briefly, birds were anesthetized with intramuscular injections of urethane. Craniotomies over right hemisphere brain regions (HVC, Area X, and RA) were made using established coordinates; a headpost and a reference electrode were cemented in place.

Following the preparatory surgery, the animal was moved into a sound-attenuating chamber (Industrial Acoustics Company, New York) on an air table. Once the animal was secured by the headpost, a bipolar stimulating electrode (FHC, Bowdoin, ME) (300-500 kΩ impedance) was inserted 0.9-1.4 mm at a 30˚ angle into RA under the control of a microdrive (Siskiyou, Grants Pass, OR). A bipolar stimulating electrode (FHC, Bowdoin, ME; or custom-made using 200μm telflon-coated tungsten) was inserted into Area X at a depth of 3600-4600 μm.

Recording and electrical stimulation

Ensembles of single units in HVC were recorded using a 4-tetrode array (A4x1; Neuronexus Technologies, Ann Arbor, MI) linearly distributed within HVC. A 16-channel headstage preamplifier (10x) fed signals into a 16-channel AC amplifier (Model 3600, A-M Systems, Sequim, WA) and were amplified an

additional 1000x. Signals were filtered (300-10,000 Hz) and acquired at 22.05 kHz using the custom-written Matlab (Mathworks, Natick, MA) software. In a subset of animals, one or two channels were filtered 1-10,000 Hz to collect local field potentials.

To identify projection neurons to RA or Area X using antidromic stimulation, single, monophasic electrical pulses of 200 μs duration were delivered at a rate of 0.5 Hz. Stimulation intensity (20 - 400 μA) was gradually increased until reliable spikes were observed in HVC. Collisions were also obtained in a subset of sessions to verify their efferent targets.

Spike Sorting

Multiple spikes on the same tetrode were sorted using automatic clustering followed by manual checking of each cluster. Following each recording, spikes were identified using custom software if they crossed a relatively low, predetermined threshold (mean + 4 SD; calculated independently on each channel for each recording session). 22-point (~1-ms) spike waveforms and their corresponding timestamp were used for clustering. Features of thresholded waveforms (for each channel: Energy, Derivative of Energy, and First Principal Component) were then calculated (MClust, A.D. Redish) and subjected to unsupervised clustering using KlustaKwik (K. Harris, Rutgers, http://klustakwik.sourceforge.net). The initial preclusters identified by KlustaKwik were manually checked and occasionally combined or split, using MClust 3.5 (A.D. Redish; http://redishlab.neuroscience.umn.edu/MClust/MClust.html) in the

Matlab environment. Clustering was performed either on a Dell PC or blade server with a 64-bit processor and Windows XP.

Four criteria were used to determine cluster quality to ensure that sorted spikes accurately reflected single units. Clusters that did not pass all criteria were discarded. The four criteria for cluster quality were: (1) L-ratio score < 0.1; (2) Isolation Distance >16; (3) less than 1% violations of a 1-ms refractory period; and (4) visual separation of the cluster from "noise" and other clusters on at least 2 (of 12) dimensions.

Identification of clustered antidromic single units

All units used for further analysis, including those identified by antidromic stimulation, met the criteria described above. Antidromic, collided, and spontaneous spikes coclustered. Spike times for events occurring after stimulation were used to identify fixed latency spikes and to calculate latency time and latency variability relative to the stimulus. Antidromic latency was defined as the mean time from stimulation to the peak of the action potential for at least 10 trials. Latency variability was defined as the standard deviation of the latencies. The latency variability cut-off for identification of unit as a projection unit was set at <125 μs unless collision were available.

Calculation of spike parameters

To analyze changes in the population of HVC neurons during development, we quantified properties of spike waveforms (e.g. spike width, amplitude, asymmetry), spike timing (e.g. interspike interval distribution, instantaneous spike rate, overall firing rate, 95%ile ISI), and bursting properties

(e.g. burst duration, burst rate). Spike width was measured at 25% peak amplitude (Rauske et al., 2003). Burst duration was determined for each unit by calculating the length of each burst with varying maximum ISI cutoffs – 10 ms, 20 ms, and 50 ms. Each burst contained at least 3 spikes. The burst rate was determined by dividing the number of bursts determined in each maximum ISI category by the trial length.

Analyses of functional interactions and statistics

Functional connectivity was assessed using coherence to measure correlated spontaneous activity between each set of two cells in each recording (Rosenberg et al., 1989; Kimpo et al., 2003). The coherence was calculated by normalizing the cross-correlation of two spike trains by the autocorrelation of both spike trains. We used a 5 ms binning window to observe interactions between units up to delays of ±500 ms. Coherence is useful to evaluate spike time relationships, particularly in a 'bursty' nucleus such as HVC, because it corrects for overall firing rate and for bursting activity more than the cross-correlation alone. The sampling error was estimated using the jackknife resampling technique (Thomson and Chave, 1991). The jackknife is a non-parametric measure of variance and a reliable method for assessing confidence intervals. The jackknife is calculated by successively deleting each spike time in the spike train and recomputing the coherence. The jackknife is the variance of estimate of the cross-correlation. Pairs of cells are considered significantly correlated if the coherence value exceeds three times the standard deviation, which corresponds to a 99% confidence level. Figure 1A plots the coherence of a single unit (HVC_X;

T3C8) against 10 additional single units in the recording. The coherence is plotted in black, and time = 0 is the time when the other unit fired. Positive coherence values indicate that the activity of two cells is correlated (functionally excitatory); negative interactions suggest that the other cell functionally inhibits the HVC_X.

Unidentified individual units were classified based on their functional interactions with other units in the ensemble recorded simultaneously. Pairs of cells were considered "coactive" if they were significantly positively correlated at time = 0 using the coherence measure (Figure 1A). Anticorrelated units were identified as having more significantly negative interactions with other units in the recording than significantly positive interactions (Figure 1B).

The Wilcoxon rank sum test was used to compare juvenile and adult spike parameters. Significance was defined as $P < 0.05$.

RESULTS

Ensembles of single units were obtained in juvenile and adult zebra finches. Fifty-two adult and 26 juvenile zebra finches were implanted with stimulating electrodes and a recording tetrode. Of these, 42 adults and 18 juveniles had antidromic units that fired reliably during the recording. Sixteen adults and 10 juveniles had antidromic units that met our four criteria for cluster quality. Antidromic units from 5 adults and 8 juveniles were discarded because their latency variability was >125 μs, and collision data were unavailable. Ultimately, 20 antidromic units in the adult (HVC_X: 13; HVC_{RA}: 7) and 17 units in the juvenile (HVC_X: 11; HVC_{RA}: 6) were used in our final analyzes.

The population of single units in HVC changes with development

Activity in HVC is characterized by its bursts of population activity that involve multiple neurons (Schmidt and Konishi, 1998; Crandall et al., 2007a; Day et al., 2009; Shank and Margoliash, 2009). We analyzed 213 individual single units in the adult and 219 single units in the juvenile to assess overall changes during development in a heterogeneous population of cells.

Changes in bursting occur with development (Day et al., 2009). Qualitative comparisons of population activity showed that population bursts in HVC were longer in adults and there was a change in the spiking activity of HVC_X units (Figure 2). The example shown in Figure 2 summarizes the changes that we observed in HVC in juvenile and adult animals in different subpopulations of cells. To determine how the overall population of cells in HVC changed with development, we overall firing rate, and bursting properties (e.g. burst duration, burst rate) for each unit.We found that burst duration (maximum interspike interval =20 ms) increases with development (Figure 3C).

Coactive neurons were defined as those neurons that had more significantly positive coherences with other neurons in the same recording than significantly negative at t = 0-5 ms. We found that neurons that were coactive with the population increased the duration of their bursts with development (Max (ISI) within the burst = 20 ms; Juv (113 units): 10 ms, IQR: 8-14 ms; Ad (118): 12 ms, 10-14 ms; Rank Sum, $p < 0.0029$).

HVC_X units change with development

To determine if specific populations of cells changed their burst durations with development, we compared the burst duration (maximum ISI = 20 ms) of HVC_X and HVC_{RA} in juveniles and adults. HVC_X cortico-basal ganglia projection neurons increased their burst durations with development (Juv (11): 6 ms, 5-8 ms; Ad (13): 9 ms, 8-12 ms; $p < 0.007$). The burst duration of RA-projecting cortico-cortical neurons was not different in juveniles versus adults (Juv (6): 10 ms, 8-15 ms; Ad (7): 9 ms, 9-11 ms; Rank Sum, $p = 1$).

'Anticorrelated' units in juveniles have prolonged bursts

Figure 4A-B shows the activity of a subpopulation of cells in juveniles and adults that we characterized as being 'anticorrelated' relative to overall population activity in HVC. We observed that some units tended not to fire within population bursts, and appeared to fire independently of the rest of the circuit. To class these cells, we identified them based on their functional interactions with other units based on coherence measurements (Figure 1B). Anticorrelated units were defined as having more significant negative interactions at time = 0 (the time at which the other cell fired) than positive interactions. These coherence calculations indicate that these cells are functionally inhibitory. We have found that the percent of recorded neurons that are functionally inhibited (as defined by significant negative coherence at t = 0-5 ms) by single anticorrelated neurons increases with development (Juvenile (33 anticorrelated units): 35%, IQR: 21-46%; Adult (22): 48%, 33-59%).

We quantified the burst duration of anticorrelated units in juveniles and adults. In contrast to the findings in single units that tended to fire with the population, the anticorrelated neurons did not increase their burst durations with development, and, in fact, decreased their burst durations if measure with a 50-ms maximum ISI (Figure 4F, N: Juv = 33, Ad = 25). These neurons changed their activity patterns from a regularly-firing beating style to a burst-pause phenotype. This is best exemplified by the 95th percentile ISI. This is a conservative estimate of the maximum ISI and will be lower in neurons that cannot shut up and tend to fire regularly in a beating pattern. Juvenile anticorrelated neurons had a significantly lower 95th percentile ISI (Fig. 4C). The overall firing rate is greater in juveniles than in adults (Figure 4D), but overall firing rates vary considerably in juveniles (4F-G). The maximum firing rate of anticorrelated cells in adults is ~15 Hz; 19 of 25 anticorrelated units in the adult have overall firing rates less than 5 Hz. The remaining 6 units have median firing rate of 12 Hz. In juveniles, 3 of 33 of the anticorrelated units fire with a median firing rate of 41 Hz, and 19 of 33 have overall firing rates greater than 5 Hz.

DISCUSSION

This study investigated changes among single units in the song control nucleus HVC. These data provide evidence that there are overall developmental changes in the HVC network. In addition, we report changes in HVC_X units that provide input to the AFP, which is critical for song learning. This finding further implicates HVC as brain region that is not only critical for song production, but also for song learning.

Network population activity can be analyzed using ensembles of single units

Changes in neural activity during development have primarily been studied at using serial single unit recordings or longitudinal multiunit recordings (Nick and Konishi, 2005b; Crandall et al., 2007a; Day et al., 2008, 2009). Analyzing large ensembles of single units from tetrode recordings from animals during sensorimotor learning or in adult animals provides unique insight into how populations of cells are changing during development. Previous work using longitudinal multiunit recordings during singing have already shown that HVC premotor population bursts, which are dominated by interneuron activity (Rauske et al., 2003; Crandall et al., 2007a), are developmentally regulated (Crandall et al., 2007a; Day et al., 2009) and are correlated with behavioral stability, millisecond-by-millisecond (Day et al., 2008). Our data show that ensembles of single units show developmental changes that reflect the increased burstiness observed in adult animals. Though developmental changes can be inferred using serial single unit recordings, there is great power in analyzing developmental changes at the level of the network.

Cortico-basal ganglia communication changes with development

We have previously found that both types of HVC projection neurons are coactive in bursts with multiple other neurons under anesthesia (Day et al., in press), Chapter 4). Overall, we have found that the burst duration of all single units increases with development; HVC_X units display similar patterns of activity. These data suggest that HVC_X units may be involved in neural changes that are important for stabilizing song.

The role of HVC in learning is difficult to ascertain; lesioning HVC eliminates all subsequent vocalizations (Nottebohm and Arnold, 1976). Therefore, evaluating HVC's role in learning must be determined by investigating its output to the motor and anterior forebrain pathways. How Area X and its downstream nuclei influence vocalization is ongoing research in many birdsong laboratories.

However, one important question is: How is behavioral plasticity limited in the AFP? Our data provide direct evidence that HVC may be controlling the AFP by modulating the output of basal ganglia-projecting neurons, supporting a hypothesis from Day et al. (2008) (Chapter 2). These data suggest that the signal to the AFP changes with song development. Increased activity to the AFP results in greater inhibition to LMAN and may decrease a "variability" signal that is conveyed to RA, the common output of the motor pathway and the AFP.

The input to RA is primarily from LMAN in juveniles, and from HVC in adults (Herrmann and Arnold, 1991; Aronov et al., 2008). This change in input to RA could result from multiple mechanisms. One hypothesis is that HVC could drive RA more strongly in adults. However, HVC activity and its prediction of variance in song (Day et al. 2008) suggested that HVC may be entraining the AFP via HVC_X projections. Data from this study reinforces that hypothesis by demonstrating that bursts to the AFP are greater in adults than in juveniles

The Anterior Forebrain Pathway has long been known for its role in learning during the sensorimotor phase in zebra finches, because lesions to the AFP stall vocal development. Therefore, understanding the signal it receives is

critical in resolving the neural mechanisms that drive learning. One signal that has been studied in detail is the auditory signal transmitted during singing and listening. HVC_X neurons are the sole source of auditory input to the AFP. These cells fire precisely to auditory stimuli, and their activity is shaped by interneurons (Mooney & Prather, 2005). Given that HVC_X are active during both singing and listening to BOS, it has been suggested that they may transmit an efference copy, a predictive signal of motor output (Prather et al., 2008, Troyer & Doupe, 2000a). Our data, which is collected in the absence both singing and listening, show that there are changes in spontaneous discharge of HVC_X neurons, which will be interesting to explore in greater detail in the future.

Anticorrelated units may regulate critical periods

In addition to identifying projection neurons to both efferent targets of HVC, we also classified a population of cells that are 'anticorrelated' with the projection neurons and other population neurons. This group of putative interneurons functionally inhibited large percentages of other neurons juveniles and adults. These putative inhibitory interneurons are of special interest, because their burst properties are altered throughout the sensorimotor phase of vocal learning and they have high firing rates. In addition, GABAergic interneurons detect changes in sensory input (Fagiolini et al., 2004), regulate excitability of glutamatergic pyramidal neurons (Rudolph 2007), refine local cortical circuits (Hensch 1998) and influence cortical development (Wang & Kriegstein, Zheng & Knutsen 1999) and synchronize brain regions (Galaretta & Hestrin 2001; Gonzalez-Burgos & Lewis 2008, Tamas 2000). Many

neurodevelopmental disorders are though to derive from alterations in cortical inhibitory tone, including autism, Tourette's syndrome, and schizophrenia (Hensch et al., 1998; Zheng and Knudsen, 1999; Tamas et al., 2000; Galarreta and Hestrin, 2001; Fagiolini et al., 2004; Di Cristo, 2007; Rudolph et al., 2007; Gonzalez-Burgos and Lewis, 2008; Wang and Kriegstein, 2009).

Prolonged bursting is a characteristic of developing sensory systems during a sensitive period (Hensch, 2005). Prolonged discharge is thought to reflect an immature state of neural circuitry; increased inhibitory interneuron activity may indicate the onset of experience-dependent plasticity. Facilitation of GABA neurotransmission decreases the incidence of prolonged bursts in mature sensory systems (Fagiolini and Hensch, 2000). A population of units that had prolonged bursting and fired at very high rates during singing was observed in the awake, behaving zebra finch (Crandall et al., 2007a). These units had both pre- and post-motor activity in juveniles, but not in adults, and responded to auditory stimulation. Although we are unable to observe their spiking properties in awake animals, anticorrelated cells observed in our recordings share similar properties to these putative fast-spiking, inhibitory interneurons; particularly a small group of cells that fire at extremely high rates (Figure 4G). Reponses to auditory feedback in anesthetized birds may illuminate the role of these cells in gating sensory information in HVC. Since these cells are functionally inhibitory, the decrease in burst duration and spike rate is consistent with the developmental increase in burst duration of the HVC coactive population and may be causative. It is possible that the effect of the anticorrelated neurons on

the population may increase with development due to increased synaptic efficacy and/or correlated firing among anticorrelated neurons, either of which could be enhanced by perineuronal nets (Hockfield and McKay, 1983; Zaremba et al., 1989; Dityatev et al., 2007).

This chapter sheds light on the neural changes that accompany sensorimotor learning in a critical period. Critical periods are characterized by prolonged bursting, expression of the calcium binding protein parvalbumin that is characteristic of fast-spiking neurons, the presence of extracellular matrix structures perineuronal nets (PNNs) (Hockfield and McKay, 1983; Sur et al., 1988), and changes in the excitatory-inhibitory balance (Hensch, 2005). Perineuronal nets are thought to stabilize sensory circuits and affect neuronal excitability (Hockfield and McKay, 1983; Zaremba et al., 1989; Dityatev et al., 2007). Maximal perineuronal net expression occurs in all seven song nuclei by the time song crystallizes and PNNs in the song nucleus HVC predict song maturity (Balmer et al., 2009). Although anticorrelated units share similar characteristics with other fast-spiking interneurons in developing sensory systems, it will be critical to determine whether these cells are A) parvalbumin-positive and/or B) surrounded by PNNs.

Next steps

Combining tetrode recordings with antidromic stimulation is a powerful approach to studying circuit development in a sensorimotor system. Using this technique we are able to monitor multiple populations of neurons within HVC and the spike time relationships among them. These studies uncover changes in

different populations of cells that would be impossible to study using other techniques. Implementing coherence to determine functional connectivity is necessary to identify subpopulations of cells that have similar effects on other neurons.

In addition to providing insight into developmental changes, these data also raise a number of interesting questions: What intrinsic or extrinsic changes in HVC_X account for the changes we observe? As the synaptic partners of HVC_X neurons, how do interneurons or HVC_{RA} cells shape their activity? How does auditory playback change the activity of these different types of neurons? Finally, are the fast-spiking, prolonged bursting putative interneurons that alter their activity during development surrounded by perineuronal nets? These questions, and others, will elucidate neural mechanisms of vocal learning during a critical period, as well as provide insight into cortical development and learning in general.

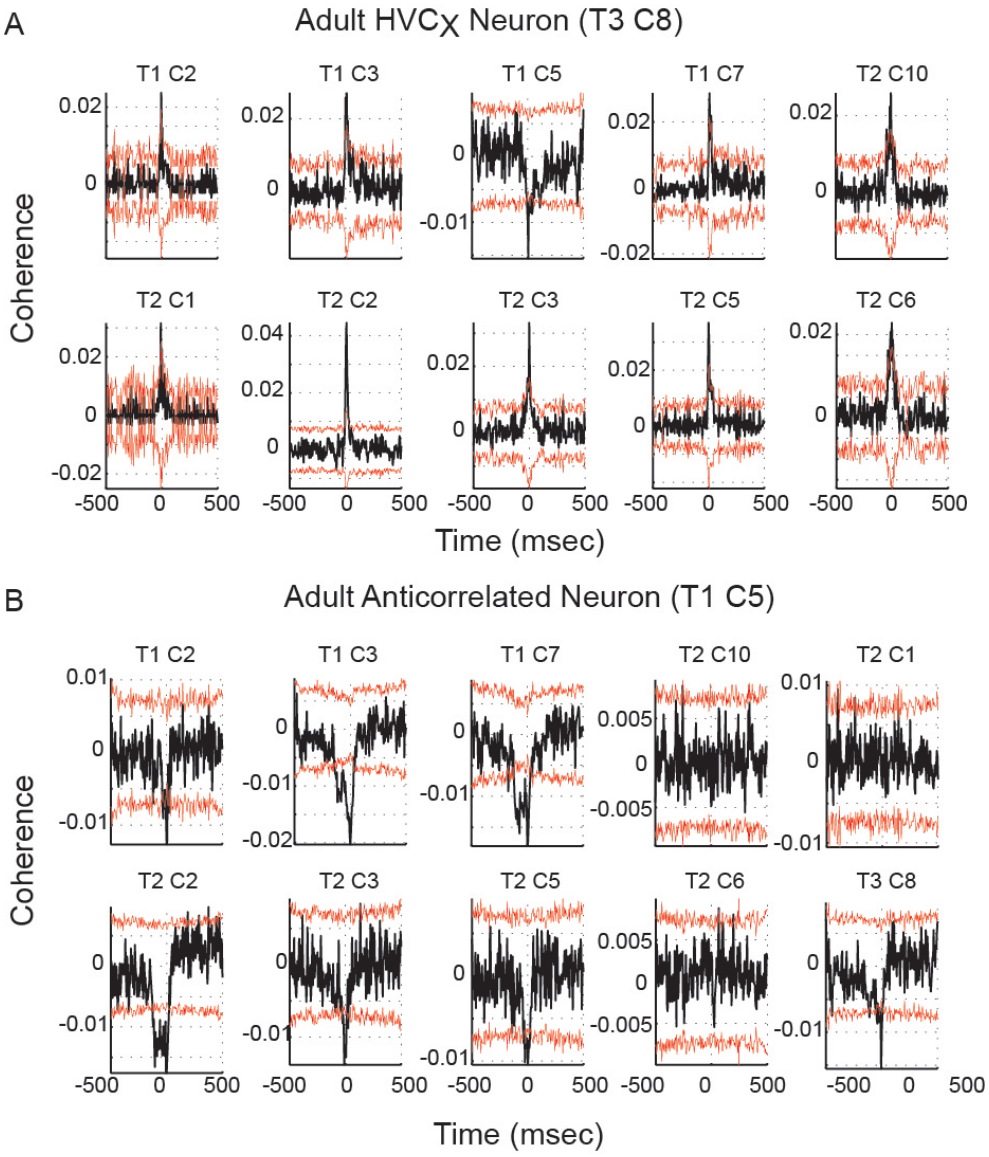

Figure 1. Coherence identified functionally coactive units and functionally anticorrelated units.

Coherence plots show the correlated activity between units in HVC. Coherence was calculated in 5 ms binning windows and a jackknife estimated the variance. A) Units were identified as 'coactive' if the activity (black) at time = 0 ms significantly positive jackknife (red). Activity is plotted as the activity of the indicated unit (T3C8) relative to the spike of the other unit. The adult HVC_X unit is coactive with other units in HVC. B) Example coherence plots of an 'anticorrelated' unit in an adult. Activity is plotted as the activity of the indicated unit (T1C5) relative to the spike of the other unit. Anticorrelated units were identified as having more significantly negative interactions with other units in the recording than significantly positive interactions. Data are from Orange-491; age 264. 10 representative of 36 available coherence plots are displayed.

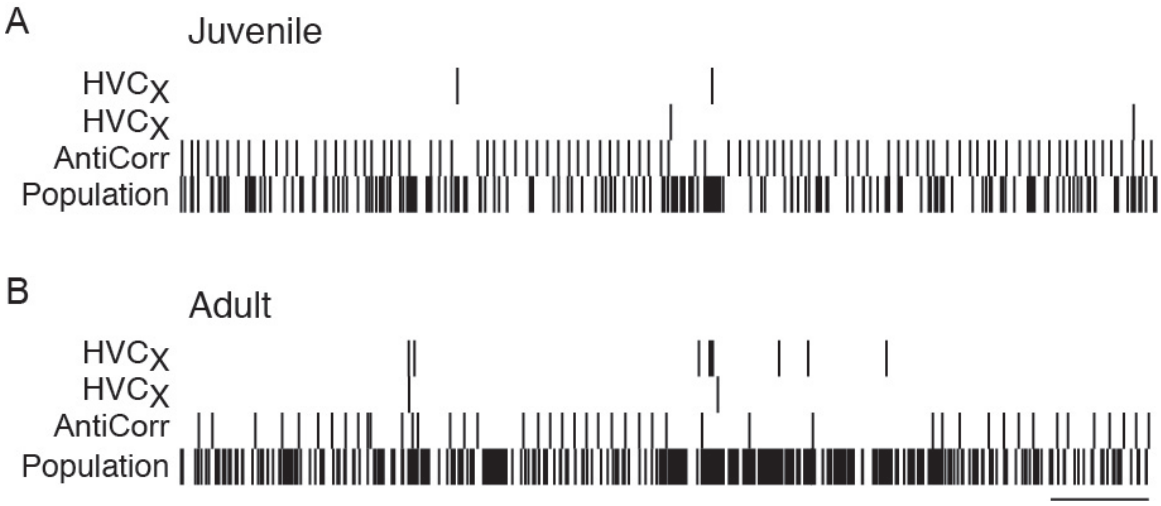

Figure 2. Representative spontaneous activity in juvenile and adult zebra finches.
Raster plots show the activity of a subset of identified or characterized units and their spiking events relative to the overall (summed multiple unit) population activity. A) Spontaneous activity in the juvenile has sparsely firing HVC_X units, and a high firing rate unit that is anti-correlated with population activity. B) Activity in the adult HVC: an HVC_X unit shows bursting activity in an adult, and a unit anti-correlated with the population activity. Scale bar is 500 ms. Data from juvenile O-37, age 49 days and adult O-491, age 264 days.

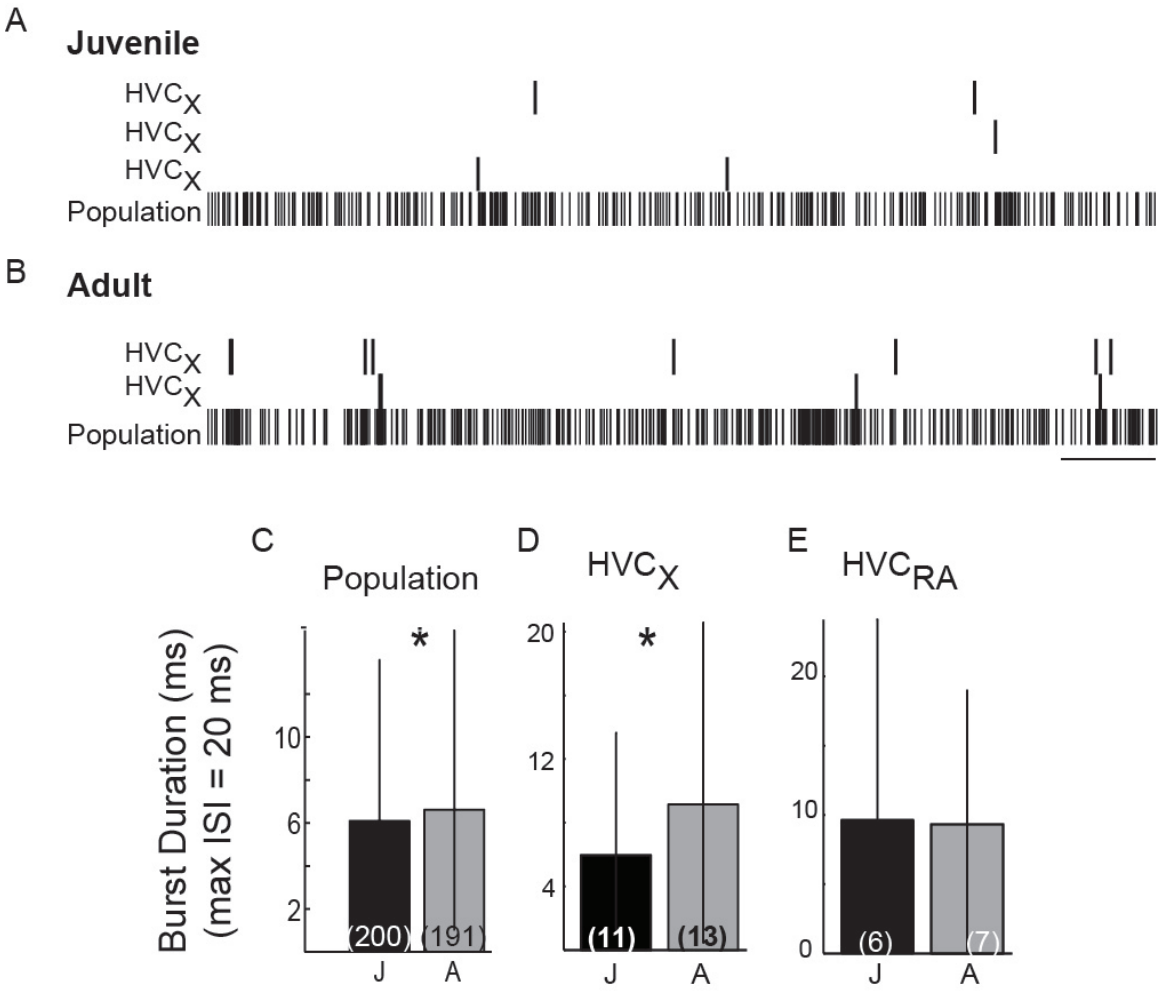

Figure 3. HVC$_X$ units change their bursting activity with development.
A – B) Raster plots show that multiple simultaneously recorded HVC$_X$ units have different firing patterns in juveniles and adults. C - E) Burst duration increases in the coactive population and in HVC$_X$ units during development. Burst duration of HVC$_{RA}$ units does not change. The burst duration in HVC$_{RA}$ does not change during development. Data are from juvenile O-37, age 49 days and adult O-491, age 264 days.

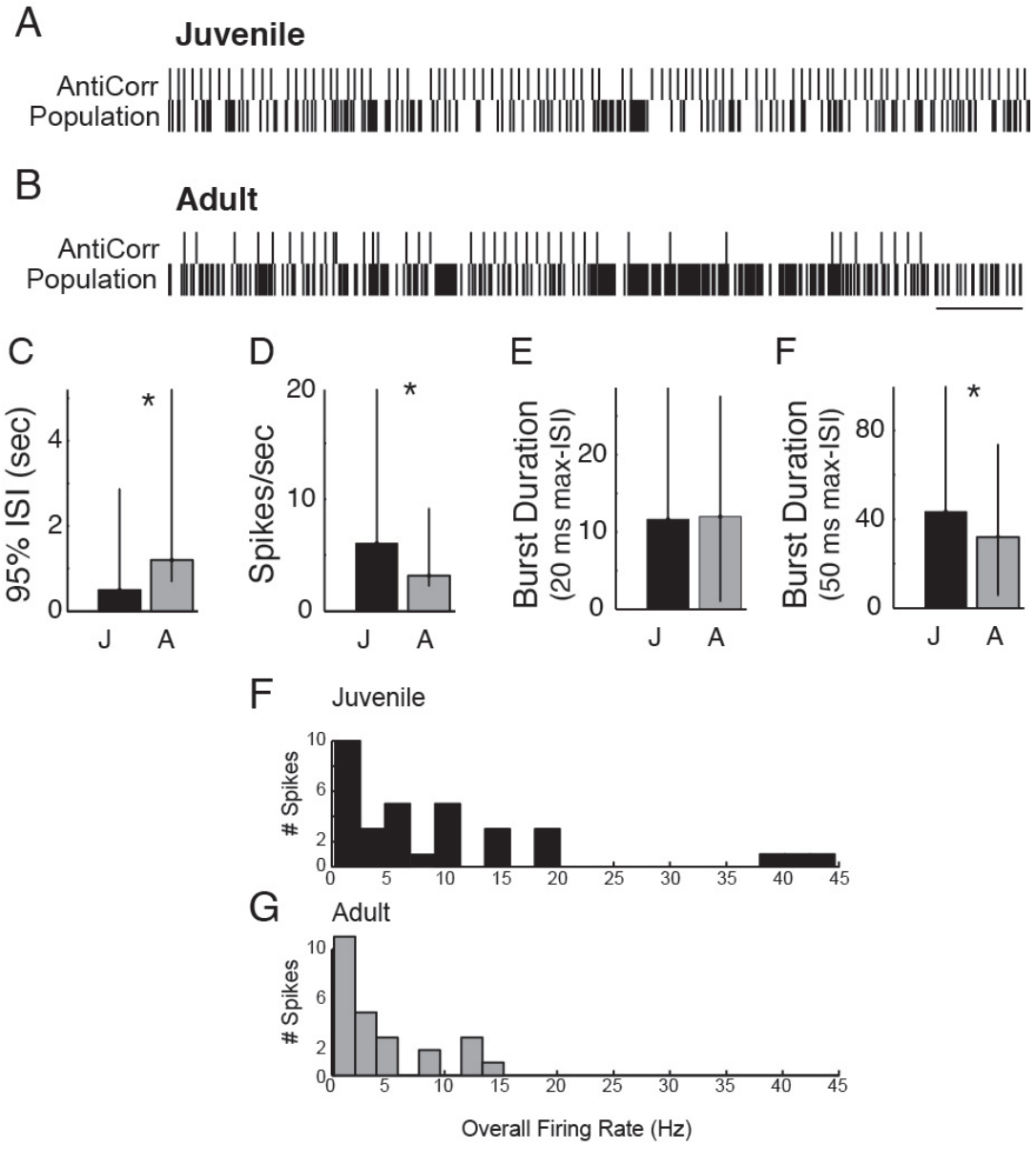

Figure 4. HVC neurons whose spike trains were anticorrelated with the population, and thus appeared to be inhibitory, exhibited distinct and significant changes with development.

A – B) Anticorrelated units in juvenile and adult finches do not spike with the rest of the population. C) The 95th percentile ISI increases with development, indicating that there are longer pauses between bouts of activity. D) The firing rate of these anticorrelated neurons decreases with development. (C) The duration of bursts with a 20-ms maximum ISI, which increases with development in the coactive population and in HVC_X neurons, does not change in anticorrelated neurons. E) Consistent with an increase in spike rate and decrease in 95th percentile ISI, the burst duration with a 50-ms maximum ISI decreases with development in this putatively inhibitory class of neurons.F-G) Histograms show that there is a subpopulation of anticorrelated units that fire at very high rates. Data are from juvenile O-37, age 49 days and adult O-491, age 264 days.

Chapter 6

Summary and Conclusions

Throughout life animals must learn and adapt to their environment to ensure survival. Neural plasticity enables animals to appropriately respond to dynamically changing environments. However, once a desired behavior is achieved, the brain must restrict neural activity to retain this stable behavior. Critical periods are specific time windows in which the nervous system is receptive to a specific experience and are excellent windows in time to study the characteristics of neural plasticity and behavioral development.

Birdsong is an excellent model for advancing our knowledge of speech acquisition that occurs during childhood in part because vocalizations are learned during a well-characterized critical period. This dissertation presented changes in neural activity that occur during a critical period for vocal learning in HVC, a sensorimotor brain region in the zebra finch. These changes occur over a period of approximately three months as a young bird slowly shapes his song vocalizations to match those of his tutor using auditory feedback. HVC is critical for song production, and receives both sensory input and sends a motor signal to control song. Thus, this brain area is positioned to play a key role during sensorimotor learning.

Summary of experiments

The foundation of this dissertation research comes from chronic recordings in awake, behaving (singing) birds. Previous studies have shown that, during singing, HVC population activity in juveniles precedes and outlasts a vocalization (Crandall et al., 2007b); in adults, HVC activity is very tightly

correlated to the song (McCasland and Konishi, 1981). By measuring the correlation in the activity in HVC to the song millisecond-by-millisecond, we were able to systematically assess how the activity in HVC changes during vocal development in tandem with song learning. Chapter 2 proposed a novel hypothesis that HVC might be involved in song learning by selectively stabilizing song behavior milliseconds at a time (Day et al., 2008). We found that bursts (time periods in which cells are exceptionally active) in HVC activity predict song stability on-line during singing, whereas decrements in HVC activity predict song variability.

But why does it matter that weaker activity in HVC leads to higher variability in song? Behavioral experiments demonstrated that juvenile zebra finches sing less mature songs in the morning than they did the night before (Deregnaucourt et al., 2005). This finding, in tandem with previous results that HVC activity regulates song stability/variability (Chapter 2), led to the hypothesis that the difference in song stability from evening to morning to evening may be due to changes in HVC. Data presented in Chapter 3 confirmed that HVC activity is weaker (less oscillatory power) in the morning and greater in the evening. However, we also observed that the pattern of HVC activity changes, such that there is increased ‘burstiness’ (greater oscillatory power) later in the day compared to the morning. This may reflect the increasing stability of small units of song over the course of the day. This increase in burstiness is only observed in juveniles. Adults show little to no

change in burstiness during the day or overnight. These results further implicated HVC as a brain region that may have a significant role in controlling song learning.

Following the observation that the pattern of HVC activity is changing, we combined two well-established electrophysiology techniques to study changes in populations of HVC neurons during sensorimotor learning. Antidromic stimulation identified single units with known projection targets within ensembles of neurons in an intact forebrain region of the song system (Chapter 4). As the output cells of HVC, the modulation of projection neuron activity during development is critical for our understanding of the role of HVC in song learning. How do the signals to the motor pathway differ from those to the anterior forebrain pathway? Is the activity of those cells shaped by other populations of cells in HVC? Chapter 5 detailed changes in two groups of cells – HVC_X (input to the AFP) and a group of fast-firing putative interneurons. These changes are of significant interest because no studies have implicated that HVC_X neurons provide an instructive signal to the AFP, and because the 'anticorrelated cells' exhibit changes that are known to occur at the closure of critical periods.

A model of top-down regulation of the AFP

The AFP is critical for song learning in the zebra finch (Bottjer et al., 1984; Kao et al., 2005; Olveczky et al., 2005; Aronov et al., 2008), but its input from HVC has not been thoroughly investigated. HVC_X neurons have properties

that have been described as a possible “auditory-vocal mirror neurons”, because they are active during singing and listening, and transmit a delayed corollary discharge (Prather et al. 2008). Additionally, given that HVC_X innervates a striatal structure that is important for song learning and perception (Scharff and Nottebohm, 1991; Scharff et al., 1998), it is not unreasonable to posit that control of AFP may be induced by a signal shaped within HVC.

Chapter 2 proposed that bursts in HVC activity might control song variability and trial-and-error learning by entraining the AFP. This hypothesis predicts that HVC_X neurons change during development. Less activity of HVC_X neurons may alter AFP activity. We posit that HVC_X neurons may entrain the AFP. If HVC_X neurons are less active, there will be less entrainment.

Data from Chapter 4 strengthen the argument that HVC_X neurons may play a critical role in limiting song variability, because we observed changes in the bursting properties of these cells that parallel changes in the population of cells as a whole. The increase in bursting in HVC_X neurons, but not in HVC_{RA} neurons does implicate the HVC-X pathway as a possible signal to restrict variability. These findings do not conflict with HVC and RA’s proposed roles in coding the temporal pattern and structure of song in adults (Vu et al., 1994; Long et al., 2011).

Behavioral variability in the song system may be restricted in RA from HVC projections or from LMAN projections, and/or both. LMAN drives initial vocalizations in the immature zebra finch (Aronov et al., 2008), but HVC_{RA}

neurons drive crystallized song (Thompson et al., 2011). Potentially, increased HVC activity could more strongly drive the HVC projection to RA and thereby reduce behavioral variability. We showed in Chapter 2 that the relationship between HVC activity levels and the variance of song was differentially regulated during development. These results suggested a more interesting model: Increased HVC activity could entrain the activity of the AFP via HVC's efferent connection to Area X.

Critical period regulation

We have found that populations of single neurons in HVC are changing during the sensitive period for sensorimotor learning. However, little is known about the regulation of sensorimotor activity during critical periods and the behaviors they underlie. There are two characteristics of the plastic song system: 1) Fast-spiking putative interneurons fire before, during, and after song behavior during sensorimotor learning but not later in development (Crandall et al., 2007b) and 2) the presence of perineuronal nets around a subset of PV+ inhibitory interneurons in HVC (and other song nuclei) correlates with song stability (Balmer et al., 2009).

Inhibitory neurons synchronize cortical networks and control the activity of excitatory neurons (Hasenstaub et al., 2005a; Cardin et al., 2009). Parvalbumin-positive interneurons, which are less than 8% of mammalian neocortical neurons (Xu et al., 2010), control cortical rhythms and, thus, circuit activity and function (Engel et al., 2001; Womelsdorf and Fries, 2007; Cardin et

al., 2009). In addition, changes in inhibition are critical for the closure of critical periods (Fagiolini and Hensch, 2000; Hensch, 2005). Data from Chapter 5 demonstrate that the inhibitory tone in HVC is changing with development (decreases in the burst duration of functionally identified inhibitory interneurons). Our data reflect changes solely in HVC, but do not preclude changes in other song control regions of the brain.

In HVC, our data indicate that functionally inhibitory neurons are changing during their firing patterns. One mechanism that may alter the activity of these neurons comes from the appearance of perineuronal nets at the end of the sensorimotor phase (Balmer et al., 2009). PNNs affect the electrical excitability of the neurons they surround (Hockfield and McKay, 1983; Zaremba et al., 1989; Dityatev et al., 2007), most prominently PV-positive fast-spiking neurons (Hartig et al., 1999). An addition, PNN expression is altered in animals that experienced sensory deprivation (Pizzorusso et al., 2002; Balmer et al. 2009). Developmental cellular changes in HVC are reflected in premotor activity bursts that decrease in duration (Crandall et al., 2007a) and become stronger (Day et al., 2009) during the sensorimotor phase. These stronger bursts appear to stabilize behavior (Day et al., 2008, 2009). We speculate that the change in prolonged bursting in anticorrelated cells may result from the PNNs restricting excitability of these cells in adulthood. This may be one of many neural mechanisms that restrict plasticity during development.

How perineuronal nets and, more broadly, the extracellular matrix stabilize neural circuits and behavior is not known. However, recent studies have suggested that effects of the ECM may be achieved at least in part through its effects on neuronal excitability, synaptic plasticity, dendritic function, and regulation of transmembrane proteins, including lateral stabilization of glutamatergic AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors and modification of the magnitude and kinetics of calcium influx through L-Type calcium channels (Dityatev and Schachner, 2003; Dityatev et al., 2007; Frischknecht et al., 2009; Kochlamazashvili et al., 2010; Dansie and Ethell, 2011).

One experiment that will directly test the influence of PNNs on the activity of HVC projection neurons and other neurons in the ensemble is applying ChABC to HVC to destroy the extracellular matrix using our tetrode setup. This approach will allow us to directly determine the effects of PNNs on the HVC circuit. We predict that the duration of population bursts, including the activity of Area X-projecting neurons, will decrease and that the activity of neurons that fire anticorrelated with the HVC population will change from a burst-pause to a more regular beating pattern (the reverse of normal development).

In awake, behaving juvenile zebra finches, one group of cells is activated by sensory stimulation (auditory playback). These same cells also show prolonged bursts (Crandall et al., 2007a). A second experiment using auditory

playback is also critical for our understanding of how single units interact in response to BOS or tutor song. Playback experiments may reveal 1) how auditory stimulation shapes the activity of the circuit and 2) the influence of PNNs on the circuit during auditory playback. We will directly compare data from before and after ChABC application. Auditory-evoked activity in HVC is greater in sleeping or anesthetized animals than in awake animals (Schmidt & Konishi, 1998), so changes observed during auditory playback will require additional scrutiny. Nonetheless, examining network activity in the presence of auditory stimulation may reveal functional connectivity in HVC that is necessary for proper song learning.

Atypical development of interneurons is implicated in several neurodevelopmental disorders, including autism (Di Cristo, 2007), suggesting that understanding interneurons and their roles in plasticity will illuminate human disease (LeBlanc and Fagiolini, 2011; Gogolla et al., 2009). Interneurons and PNNs appear to have roles in the regulation of sensory critical periods (Hensch, 2005) and vocal learning in the songbird (Crandall et al., 2007a; Balmer et al., 2009, Chapter 5). Understanding neural changes in this cortical song nucleus that occur during the critical period has the potential to unmask similar changes that may occur in human infants and children, and may motivate therapeutic interventions in the future.

Conclusion

The studies in this dissertation have demonstrated that the cortical song nucleus, HVC, undergoes developmental changes and may be important for song learning. Data presented in this thesis indicate that HVC is not solely a participant in the motor pathway, but may entrain activity in the Anterior Forebrain Pathway to help instruct song learning during trial-and-error learning. Chronic recordings are especially useful to evaluate how overall population activity changes during development (Crandall et al., 2007a,b). However, similar developmental changes can be observed in the anesthetized animals from ensemble recordings using tetrodes. Though these methods diverge significantly from the standard serial single unit recordings that a number of laboratories use to study the neural control of vocal learning and production, they contribute valuable insight into how song learning occurs using a circuit level analysis.

In nervous systems, inducing behavioral change in a dynamic environment is as necessary as it is to stabilize a particular behavior. My research has contributed to an improved understanding of how brain plasticity is regulated throughout the lifetime of an animal. Second, understanding how one brain area may influence the activity in another provides valuable insight about experience-dependent brain development. By improving our understanding of the neural mechanisms that underlie developmental changes in sensorimotor learning, we can better understand abnormalities in disease models.

Literature Cited

Adret P (1993) Operant-conditioning, song learning and imprinting to taped song in the zebra finch. Anim Behav 46:149-159.

Alvarez-Buylla A, Kirn JR, Nottebohm F (1990) Birth of projection neurons in adult avian brain may be related to perceptual or motor learning. Science 249:1444-1446.

Andalman AS, Fee MS (2009) A basal ganglia-forebrain circuit in the songbird biases motor output to avoid vocal errors. Proc Natl Acad Sci USA 106:12518-12523.

Aronov D, Andalman AS, Fee MS (2008) A specialized forebrain circuit for vocal babbling in the juvenile songbird. Science 320:630-634.

Ballentine JJ, Hyman J, Nowicki S (2004) Vocal performance influences female response to male bird song: an experimental test. Behav Ecol 15:163:168.

Balmer TS, Carels VM, Frisch JL, Nick TA (2009) Modulation of perineuronal nets and parvalbumin with developmental song learning. J Neurosci 29:12878-12885.

Belmonte MK, Cook Jr. EH, Anderson GM, Rubenstein JLR, Greenough WT, Beckel-mitchener A, Courchesne E, Boulanger LM, Powell SB, Levitt PR, Perry EK, Jiang YH, Delorey TM, Tierney E (2004) Autism as a disorder of neural information processing: directions for research and targets for therapy. Molecular Psychiatry 9:646-663.

Benes FM, Berretta S (2001) GABAergic interneurons: Implications for understanding schizophrenia and bipolar disorder. Neuropsychopharmacology 25:1-27.

Bolhuis JJ, Gahr M (2006) Neural mechanisms of birdsong memory. Nat Rev Neurosci 7:347-357.

Bottjer SW, Arnold AP (1984) The role of feedback from the vocal organ. I. Maintenance of stereotypical vocalizations by adult zebra finches. J Neurosci 4:2387-2396.

Bottjer SW, Miesner EA, Arnold AP (1984) Forebrain lesions disrupt development but not maintenance of song in passerine birds. Science 224:901-903.

Bottjer SW, Halsema KA, Brown SA, Miesner EA (1989) Axonal connections of a forebrain nucleus involved with vocal learning in zebra finches. J Comp Neurol 279:312-326.

Brainard MS (2004) Contributions of the anterior forebrain pathway to vocal plasticity. Ann N Y Acad Sci 1016:377-394.

Brainard MS, Doupe AJ (2000) Interruption of a basal ganglia-forebrain circuit prevents plasticity of learned vocalizations. Nature 404:762-766.

Brenowitz EA (1991) Altered perception of species-specific song by female birds after lesions of a forebrain nucleus. Science 251:303-305.

Buschman TJ, Miller EK (2007) Top-down versus bottom-up control of attention in the prefrontal and posterior parietal cortices. Science 315:1860-1862.

Buzsaki G, Chrobak JJ (1995) Temporal structure in spatially organized neuronal ensembles: a role for interneuronal networks. Curr Opin Neurobiol 5:504-510.

Cardin JA, Carlen M, Meletis K, Knoblich U, Zhang F, Deisseroth K, Tsai L-H, Moore CI (2009) Driving fast-spiking cells induces gamma rhythm and controls sensory responses. Nature 459:663-667.

Chi Z, Margoliash D (2001) Temporal precision and temporal drift in brain and behavior of zebra finch song. Neuron 32:899-910.

Church PJ, Lloyd PE (1994) Activity of multiple identified motor neurons recorded intracellularly during evoked feeding-like motor programs in Aplysia. Journal of Neurophysiology 72:1794-1809.

Cobb SR, Buhl EH, Halasy K, Paulsen O, Somogyi P (1995) Synchronization of neuronal activity in hippocampus by individual GABAergic interneurons. Nature 378:75-78.

Coleman MJ, Mooney R (2004) Synaptic transformations underlying highly selective auditory representations of learned birdsong. J Neurosci 24:7251-7265.

Crandall SR, Aoki N, Nick TA (2007a) Developmental modulation of the temporal relationship between brain and behavior. J Neurophysiol 97:806-816.
Crandall SR, Adam M, Kinnischtzke AK, Nick TA (2007b) HVC neural sleep activity increases with development and parallels nightly changes in song behavior. J Neurophysiol 98:232-240.
Dansie LE, Ethell IM (2011) Casting a net on dendritic spines: The extracellular matrix and its receptors. Dev Neurobiol 71:956-981.
Dave AS, Margoliash D (2000) Song replay during sleep and computational rules for sensorimotor vocal learning. Science 290:812-816.
Day NF, Kinnischtzke AK, Adam M, Nick TA (2008) Top-down regulation of plasticity in the birdsong system: "premotor" activity in the nucleus HVC predicts song variability better than it predicts song features. J Neurophysiol 100:2956-2965.
Day NF, Kinnischtzke AK, Adam M, Nick TA (2009) Daily and developmental modulation of "premotor" activity in the birdsong system. Dev Neurobiol 69:796-810.
Day NF, Kerrigan SJ, Aoki N, Nick TA (in press) Identification of single neurons in forebrain network. J Neurophysiol.
Debanne D, Boudkkazi S, Campanac E, Cudmore RH, Giraud P, Fronzaroli-Molinieres L, Carlier E, Caillard O (2008) Paired-recordings from synaptically coupled cortical and hippocampal neurons in acute and cultured brain slices. Nat Protoc 3:1559-1568.
Deregnaucourt S, Mitra PP, Feher O, Pytte C, Tchernichovski O (2005) How sleep affects the developmental learning of bird song. Nature 433:710-716.
Di Cristo G (2007) Development of cortical GABAergic circuits and its implications for neurodevelopmental disorders. Clin Genet 72:1-8.
Dityatev A, Schachner M (2003) Extracellular matrix molecules and synaptic plasticity. Nature Rev Neurosci 4:456-468.
Dityatev A, Bruckner G, Dityateva G, Grosche J, Kleene R, Schachner M (2007) Activity-dependent formation and functions of chondroitin sulfate-rich extracellular matrix of perineuronal nets. Dev Neurobiol 67:570-588.
Doupe AJ, Konishi M (1991) Song-selective auditory circuits in the vocal control system of the zebra finch. Proc Natl Acad Sci USA 88:11339-11343.
Doupe AJ, Solis MM (1997) Song- and order-selective neurons develop in the songbird anterior forebrain during vocal learning. J Neurobiol 33:694-709.
Doupe AJ, Kuhl PK (1999) Birdsong and human speech: common themes and mechanisms. Annu Rev Neurosci 22:567-631.
Doupe AJ, Perkel DJ, Reiner A, Stern EA (2005) Birdbrains could teach basal ganglia research a new song. Trends in neurosciences 28:353-363.
Draganoiu TI, Nagle L, Kreutzer M (2002) Directional female preference for an exaggerated male trait in canary (Serinus canaria) song. Proc Biol Sci 269:2525-2531.
Driver J, Frith C (2000) Shifting baselines in attention research. Nat Rev Neurosci 1:147-148.
Dutar P, Vu HM, Perkel DJ (1998) Multiple cell types distinguished by physiological, pharmacological, and anatomic properties in nucleus HVc of the adult zebra finch. J Neurophysiol 80:1828-1838.
Eales L (1985) Song learning in zebra finches: some effects of song model availability on what is learnt and when. Anim Behav 33:1293-1300.
Engel AK, Fries P, Singer W (2001) Dynamic predictions: oscillations and synchrony in top-down processing. Nat Rev Neurosci 2:704-716.
Fagiolini M, Hensch TK (2000) Inhibitory threshold for critical-period activation in primary visual cortex. Nature 404:183-186.
Fagiolini M, Fritschy JM, Low K, Mohler H, Rudolph M, Hensch TK (2004) Specific GABA-A circuits for visual cortical plasticity. Science 303:1681-1683.
Farries MA (2004) The avian song system in comparative perspective. Ann N Y Acad Sci 1016:61-76.

Farries MA, Perkel DJ (2000) Electrophysiological properties of avian basal ganglia neurons recorded in vitro. J Neurophysiol 84:2502-2513.
Farries MA, Perkel DJ (2002) A telencephalic nucleus essential for song learning contains neurons with physiological characteristics of both striatum and globus pallidus. J Neurosci 22:3776-3787.
Fortune ES, Margoliash D (1995) Parallel pathways and convergence onto HVc and adjacent neostriatum of adult zebra finches (Taeniopygia guttata). J Comp Neurol 360:413-441.
Foster EF, Bottjer SW (1998) Axonal connections of the high vocal center and surrounding cortical regions in juvenile and adult male zebra finches. J Comp Neurol 397:118-138.
Frazier WT, Kandel ER, Kupfermann I, Waziri R, Coggeshall RE (1967) Morphological and functional properties of identified neurons in the abdominal ganglion of Aplysia californica. J Neurophysiol 30:1288.
Frischknecht R, Heine M, Perrais D, Seidenbecher CI, Choquet D, Gundelfinger ED (2009) Brain extracellular matrix affects AMPA receptor lateral mobility and short-term synaptic plasticity. Nature Neurosci 12:897-904.
Galarreta M, Hestrin S (2001) Spike transmission and synchrony detection in networks of GABAergic interneurons. Science 292:2295-2299.
Gentner TQ, Hulse SH, Bentley GE, Ball GF (2000) Individual vocal recognition and the effect of partial lesions to HVc on discrimination, learning, and categorization of conspecific song in adult songbirds. J Neurobiol 42:117-133.
Gilbert CD, Sigman M (2007) Brain states: top-down influences in sensory processing. Neuron 54:677-696.
Goldberg JH, Adler A, Bergman H, Fee MS (2010) Singing-Related Neural Activity Distinguishes Two Putative Pallidal Cell Types in the Songbird Basal Ganglia: Comparison to the Primate Internal and External Pallidal Segments. Journal of Neuroscience 30:7088-7098.
Gonzalez-Burgos G, Lewis DA (2008) GABA neurons and the mechanisms of network oscillations: Implications for understanding cortical dysfunction in schizophrenia. Schizophr Bull 34:944-961.
Gray CM, Maldonado PE, Wilson M, McNaughton BL (1995) Tetrodes markedly improve the reliability and yield of multiple single-unit isolation from multi-unit recordings in cat striate cortex. J Neurosci Methods 63:43-54.
Hahnloser RH, Kozhevnikov AA, Fee MS (2002) An ultra-sparse code underlies the generation of neural sequences in a songbird. Nature 419:65-70.
Hahnloser RHR, Kozhevnikov AA, Fee MS (2006) Sleep-related neural activity in a premotor and a basal-ganglia pathway of the songbird. J Neurophysiol 96:794-812.
Hargreaves EL, Rao G, Lee I, Knierim JJ (2005) Major dissociation between medial and lateral entorhinal input to dorsal hippocampus. Science 308:1792-1794.
Harris KD, Hirase H, Leinekugel X, Henze DA, Buzsáki G (2001) Temporal interaction between single spikes and complex spike bursts in hippocampal pyramidal cells. Neuron 32:141-149.
Harris-Warrick RM, Marder E (1991) Modulation of neural networks for behavior. Annu Rev Neurosci 14:39-57.
Hasenstaub A, Yousheng S, Haider B, Kraushaar U, Duque A, McCormick DA (2005a) Inhibitory postsynaptic potentials carry synchronized frequency information in active cortical networks. Neuron 47:423-435.
Hasenstaub A, Shu Y, Haider B, Kraushaar U, Duque A, McCormick DA (2005b) Inhibitory postsynaptic potentials carry synchronized frequency information in active cortical networks. Neuron 47:423-435.
Hauser MD, Chomsky N, Fitch WT (2002) The faculty of language: what is it, who has it, and how did it evolve? Science 298:1569-1579.
Hensch TK (2004) Critical period regulation. Annu Rev Neurosci 27:549-579.

Hensch TK (2005) Critical period plasticity in local cortical circuits. Nat Rev Neurosci 6:877-888.
Hensch TK, Fagiolini M, Mataga N, Stryker MP, Baekkeshov S, Kash SF (1998) Local GABA circuit control of experience-dependent plasticty in developing visual cortex. Science 282:1504-1508.
Herfst LJ, Brecht M (2008) Whisker movements evoked by stimulation of single motor neurons in the facial nucleus of the rat. J Neurophysiol 99:2821-2832.
Herrmann K, Arnold AP (1991) The development of afferent projections to the robust archistriatal nucleus in male zebra finches: a quantitative electron microscopic study. J Neurosci 11:2063-2074.
Hessler NA, Doupe AJ (1999) Singing-related neural activity in a dorsal forebrain-basal ganglia circuit of adult zebra finches. J Neurosci 19:10461-10481.
Hill SJ, Oliver DL (1993) Visualization of neurons filled with biotinylated-lucifer yellow following identification of efferent connectivity with retrograde transport. Journal of neuroscience methods 46:59-68.
Hockfield S, McKay RDG (1983) A surface antigen expressed by a subset of neurons in the vertebrate central nervous system. PNAS, USA 80:5758-5761.
Hubel DH, Wiesel TN (1970) The period of susceptibility to the physiological effects of the unilateral eye closure in kittens. J Physiol (Lond) 206:419-436.
Immelmann K (1969) Song development in the zebra finch and other estrilid finches. Cambridge: Cambridge University Press.
Jackson JC, Johnson A, Redish AD (2006) Hippocampal sharp waves and reactivation during awake states depend on repeated sequential experience. J Neurosci 26:12415-12426.
Jarvis ED, Nottebohm F (1997) Motor-driven gene expression. Proc Natl Acad Sci U S A 94:4097-4102.
Jarvis ED, Scharff C, Grossman MR, Ramos JA, Nottebohm F (1998) For whom the bird sings: context-dependent gene expression. Neuron 21:775-788.
Jarvis ED et al. (2005) Avian brains and a new understanding of vertebrate brain evolution. Nature Reviews Neuroscience 6:151-159.
Johnson A, Redish AD (2007) Neural ensembles in CA3 transiently encode paths forward of the animal at a decision point. J Neurosci 27:12176-12189.
Johnson F, Bottjer SW (1993) Induced cell death in a thalamic nucleus during a restricted period of zebra finch vocal development. J Neurosci 13:2452-2462.
Jones SG, Vyazovskiy VV, Cirelli C, Tononi G, Benca RM (2008) Homeostatic regulation of sleep in the white-crowned sparrow (Zonotrichia leucophrys gambelii). BMC Neurosci 9:47.
Jurgens U (2009) The neural control of vocalization in mammals: a review. J Voice 23:1-10.
Kao MH, Brainard MS (2006) Lesions of an avian basal ganglia circuit prevent context-dependent changes to song variability. J Neurophysiol 96:1441-1455.
Kao MH, Doupe AJ, Brainard MS (2005) Contributions of an avian basal ganglia-forebrain circuit to real-time modulation of song. Nature 433:638-643.
Kao MH, Wright BD, Doupe AJ (2008) Neurons in a Forebrain Nucleus Required for Vocal Plasticity Rapidly Switch between Precise Firing and Variable Bursting Depending on Social Context. Journal of Neuroscience 28:13232-13247.
Karten HJ (1991) Homology and evolutionary origins of the 'neocortex'. Brain, behavior and evolution 38:264-272.
Katz LC, Gurney ME (1981) Auditory responses in the zebra finch's motor system for song. Brain Research 221:192-197.
Kelley DB, Nottebohm F (1979) Projections of a telencephalic auditory nucleus-field L-in the canary. J Comp Neurol 183:455-469.
Kimpo RR, Theunissen FE, Doupe AJ (2003) Propagation of correlated activity through multiple stages of a neural circuit. J Neurosci 23:5750-5761.

Kirn JR, Alvarez-Buylla A, Nottebohm F (1991) Production and survival of projection neurons in a forebrain vocal center of adult male canaries. J Neurosci 11:1756-1762.
Klausberger T, Magill PJ, Marton LF, Roberts JD, Cobden PM, Buzsaki G, Somogyi P (2003) Brain-state- and cell-type-specific firing of hippocampal interneurons in vivo. Nature 421:844-848.
Knudsen EI (2004) Sensitive periods in the development of the brain and behavior. J Cogn Neurosci 16:1412-1425.
Kochlamazashvili G, Henneberger C, Bukalo O, Dvoretskova E, Senkov O, Lievens PMJ, Westenbroek R, Engel AK, Catterall WA, Rusakov DA, Schachner M, Dityatev A (2010) The extracellular matrix molecule hyaluronic acid regulates hippocampal synaptic plasticity by modulating postsynaptic L-type Ca2+ channels. Neuron 67:116-128.
Konishi M (1965) The role of auditory feedback in the control of vocalization in the white-crowned sparrow. Z Tierpsychol 22:770-783.
Konishi M, Nottebohm F (1969) Experimental studies in the ontogeny of avian vocalizations. London: Cambridge University Press.
Kozhevnikov AA, Fee MS (2007) Singing-related activity of identified HVC neurons in the zebra finch. J Neurophysiol 97:4271-4283.
Kubota M, Taniguchi I (1998) Electrophysiological characteristics of classes of neuron in the HVc of the zebra finch. J Neurophysiol 80:914-923.
LeBlanc JJ, Fagiolini M (2011) Autism: A "Critical Period" Disorder? Neural Plasticity 2011:1-17.
Leonardo A, Konishi M (1999) Decrystallization of adult birdsong by perturbation of auditory feedback. Nature 399:466-470.
Leonardo A, Fee MS (2001) Minature motorized microdrive and commutator system for chronic neural recording in small animals. J Neurosci Methods 112:83-94.
Lewicki MS, Arthur BJ (1996) Hierarchical organization of auditory temporal context sensitivity. J Neurosci 16:6987-6998.
Lim HH, Anderson DJ (2007) Antidromic activation reveals tonotopically organized projections from primary auditory cortex to the central nucleus of the inferior colliculus in guinea pig. J Neurophysiol 97:1413-1427.
Lipski J (1981) Antidromic activation of neurones as an analytic tool in the study of the central nervous system. J Neurosci Methods 4:1-32.
Liu W-c, Gardner TJ, Nottebohm F (2004) Juvenile zebra finches can use multiple strategies to learn the same song. Proc Natl Acad Sci USA 101:18177-18182.
Long M, Jin… D, Fee M (2010) Support for a synaptic chain model of neuronal sequence generation. Nature 468:394-399.
Long MA, Fee MS (2008) Using temperature to analyse temporal dynamics in the songbird motor pathway. Nature 456:189-194.
Low PS, Shank SS, Sejnowski TJ, Margoliash D (2008) Mammalian-like features of sleep structure in zebra finches. Proc Natl Acad Sci U S A 105:9081-9086.
Margoliash D (1983) Acoustic parameters underlying the responses of song-specific neurons in the white-crowned sparrow. J Neurosci 3:1039-1057.
Margoliash D (1986) Preference for autogenous song by auditory neurons in a song system nucleus of the white-crowned sparrow. J Neurosci 6:1643-1661.
Margoliash D (1997) Functional organization of forebrain pathways for song production and perception. J Neurobiol 33:671-693.
Margoliash D, Konishi M (1985) Auditory representation of autogenous song in the song system of white-crowned sparrows. Proc Natl Acad Sci U S A 82:5997-6000.
Margoliash D, Fortune ES (1992) Temporal and harmonic combination-sensitive neurons in the zebra finch's HVc. J Neurosci 12:4309-4326.
Marler P (1970) Birdsong and speech development: could there be parallels? Am Sci 58:669-673.
Marler P, Peters S (1977) Selective vocal learning in a sparrow. Science 198:519-521.

McCasland JS, Konishi M (1981) Interaction between auditory and motor activities in an avian song control nucleus. Proc Natl Acad Sci U S A 78:7815-7819.
McNaughton BL, O'Keefe J, Barnes CA (1983) The stereotrode: a new technique for simultaneous isolation of several single units in the central nervous system from multiple unit records. J Neurosci Methods 8:391-397.
Mello CV, Clayton DF (1994) Song-induced ZENK gene expression in auditory pathways of songbird brain and its relation to the song control system. J Neurosci 14:6652-6666.
Mooney R (1992) Synaptic basis for developmental plasticity in a birdsong nucleus. J Neurosci 12:2464-2477.
Mooney R (2000) Different subthreshold mechanisms underlie song selectivity in identified HVc neurons of the zebra finch. J Neurosci 20:5420-5436.
Mooney R, Prather JF (2005) The HVC microcircuit: the synaptic basis for interactions between song motor and vocal plasticity pathways. J Neurosci 25:1952-1964.
Nadasdy Z, Hirase H, Czurko A, Csicsvari J, Buzsaki G (1999) Replay and time compression of recurring spike sequences in the hippocampus. J Neurosci 19:9497-9507.
Nick TA, Konishi M (2001) Dynamic control of auditory activity during sleep: correlation between song response and EEG. Proc Natl Acad Sci U S A 98:14012-14016.
Nick TA, Konishi M (2005a) Neural song preference during vocal learning in the zebra finch depends on age and state. J Neurobiol 62:231-242.
Nick TA, Konishi M (2005b) Neural auditory selectivity develops in parallel with song. J Neurobiol 62:469-481.
Nicolelis MA, Ghazanfar AA, Faggin BM, Votaw S, Oliveira LM (1997) Reconstructing the engram: simultaneous, multisite, many single neuron recordings. Neuron 18:529-537.
Nixdorf BE (1989) Ultrastructural analysis of the development and maturation of synapses and subsynaptic structures in the ectostriatum of the zebra finch. J Comp Neurol 290:472-486.
Nordeen KW, Nordeen EJ (1992) Auditory feedback is necessary for the maintenance of stereotyped song in adult zebra finches. Behav Neural Biol 57:58-66.
Nottebohm F (1968) Auditory experience and song development in the chaffinch *Fringilla coelebs*. Ibis 110:549-568.
Nottebohm F, Arnold AP (1976) Sexual dimorphism in vocal control areas of the songbird brain. Science 194:211-213.
Nottebohm F, Stokes TM, Leonard CM (1976) Central control of song in the canary, Serinus canarius. J Comp Neurol 165:457-486.
Nottebohm F, Kelley DB, Paton JA (1982) Connections of vocal control nuclei in the canary telencephalon. J Comp Neurol 207:344-357.
Nowicki S, Searcy WA (2005) Song and mate choice in birds: ow the development of behavior helps us understand function. The Auk 122:1-14.
Olveczky BP, Andalman AS, Fee MS (2005) Vocal experimentation in the juvenile songbird requires a basal ganglia circuit. PLoS Biol 3:e153.
Perkel DJ (2004) Origin of the anterior forebrain pathway. Ann N Y Acad Sci 1016:736-748.
Phan ML, Pytte CL, Vicario DS (2006) Early auditory experience generates long-lasting memories that may subserve vocal learning in songbirds. Proc Natl Acad Sci U S A 103:1088-1093.
Pizzorusso T, Medini P, Berardi N, Chierzi S, Fawcett JW, Maffei L (2002) Reactivation of ocular dominance plasticity in the adult visual cortex. Science 298:1248-1251.
Plant G, Hammarberg B (1983) Acoustic and perceptual analysis of the speech of the deafened. Speech Translation Lab Q Prog Stat Rep 2:85-107.
Podos J (1997) A performance constraint on the evolutio of trilled vocalizations in a songbird family (Passeriformes: Emberizidae). Evolution 51:537-551.
Prather JF, Peters S, Nowicki S, Mooney R (2008) Precise auditory-vocal mirroring in neurons for learned vocal communication. Nature 451:305-310.

Prather JF, Nowicki S, Anderson RC, Peters S, Mooney R (2009) Neural correlates of categorical perception in learned vocal communication. Nat Neurosci 12:221-228.
Price P (1979) Developmental determinants of structure in zebra finch song. Journal of Comparative and Physiological Psychology.
Ranck JB, Jr. (1975) Which elements are excited in electrical stimulation of mammalian central nervous system: a review. Brain Research 98:417-440.
Rauske PL, Shea SD, Margoliash D (2003) State and neuronal class-dependent reconfiguration in the avian song system. J Neurophysiol 89:1688-1701.
Reiner A (2009) Avian evolution: from Darwin's finches to a new way of thinking about avian forebrain organization and behavioural capabilities. Biol Lett 5:122-124.
Reiner A, Yamamoto K, Karten HJ (2005) Organization and evolution of the avian forebrain. Anat Rec A Discov Mol Cell Evol Biol 287:1080-1102.
Reiner A, Perkel DJ, Mello CV, Jarvis ED (2004) Songbirds and the revised avian brain nomenclature. Ann N Y Acad Sci 1016:77-108.
Richmond BJ, Optican LM, Spitzer H (1990) Temporal encoding of two-dimensional patterns by single units in primate primary visual cortex. I. Stimulus-response relations. Journal of Neurophysiology 64:351-369.
Rosenberg J, Amjad A, Breeze P, Brillinger D, Halliday D (1989) The Fourier approach to the identification of functional coupling between neuronal spike trains. Prog Biophys Mol Biol 53:1-31.
Rudolph M, Pospischil M, Timofeev I, Destexhe A (2007) Inhibition determines membrane potential dynamics and controls action potential generation in awake and sleeping cat cortex. J Neurosci 27:5280-5290.
Sakata JT, Brainard MS (2008) Online contributions of auditory feedback to neural activity in avian song control circuitry. J Neurosci 28:11378-11390.
Scharff C, Nottebohm F (1991) A comparative study of the behavioral deficits following lesions of various parts of the zebra finch song system: implications for vocal learning. J Neurosci 11:2896-2913.
Scharff C, Nottebohm F, Cynx J (1998) Conspecific and heterospecific song discrimination in male zebra finches with lesions in the anterior forebrain pathway. J Neurobiol 36:81-90.
Schmidt MF (2003) Pattern of interhemispheric synchronization in HVc during singing correlates with key transitions in the song pattern. J Neurophysiol 90:3931-3949.
Schmidt MF, Konishi M (1998) Gating of auditory responses in the vocal control system of awake songbirds. Nat Neurosci 1:513-518.
Schmitzer-Torbert N, Jackson JC, Henze D, Harris K, Redish AD (2005) Quantitative measures of cluster quality for use in extracellular recordings. Neuroscience 131:1-11.
Selverston AI, Russell DF, Miller JP (1976) The stomatogastric nervous system: structure and function of a small neural network. Prog Neurobiol 7:215-290.
Seyfarth RM, Cheney DL (2003) Signalers and receivers in animal communication. Annual review of psychology 54:145-173.
Shank SS, Margoliash D (2009) Sleep and sensorimotor integration during early vocal learning in a songbird. Nature 458:73-77.
Simpson HB, Vicario DS (1990) Brain pathways for learned and unlearned vocalizations differ in zebra finches. J Neurosci 10:1541-1556.
Sober SJ, Brainard MS (2009) Adult birdsong is actively maintained by error correction. Nat Neurosci 12:927-931.
Sohrabji F, Nordeen EJ, Nordeen KW (1990) Selective impairment of song learning following lesions of a forebrain nucleus in the juvenile zebra finch. Behav Neural Biol 53:51-63.
Solis MM, Doupe AJ (1999) Contributions of tutor and bird's own song experience to neural selectivity in the songbird anterior forebrain. J Neurosci 19:4559-4584.
Solis MM, Perkel DJ (2005) Rhythmic activity in a forebrain vocal control nucleus in vitro. J Neurosci 25:2811-2822.

Solis MM, Brainard MS, Hessler NA, Doupe AJ (2000) Song selectivity and sensorimotor signals in vocal learning and production. Proc Natl Acad Sci USA 97:11836-11842.
Sossinka R, Bohner J (1980) Song types in the zebra finch *Poephila guttata castanotis*. Z Tierpsychol 53:123-132.
Soteropoulos DS, Baker SN (2006) Cortico-cerebellar coherence during a precision grip task in the monkey. J Neurophysiol 95:1194-1206.
Stark E, Abeles M (2007) Predicting movement from multiunit activity. J Neurosci 27:8387-8394.
Steinberg RH, Schmidt R (1970) Identification of horizontal cells as S-potential generators in the cat retina by intracellular dye injection. Vision Res 10:817-820.
Stent GS, Kristan WB, Jr., Friesen WO, Ort CA, Poon M, Calabrese RL (1978) Neuronal generation of the leech swimming movement. Science 200:1348-1357.
Sur M, Frost DO, Hockfield S (1988) Expression of a surface-associated antigen on Y-cells in the cat lateral geniculate nucleus is regulated by visual experience. J Neurosci 8:874-882.
Swadlow HA (1998) Neocortical efferent neurons with very slowly conducting axons: strategies for reliable antidromic identification. J Neurosci Methods 79:131-141.
Tamas G, Buhl EH, Lorincz A, Somogyi P (2000) Proximally targeted GABAergic synapses and gap junctions synchronize cortical interneurons. Nature Neurosci 3:366-371.
Tchernichovski O, Mitra PP, Lints T, Nottebohm F (2001) Dynamics of the vocal imitation process: how a zebra finch learns its song. Science 291:2564-2569.
Tchernichovski O, Nottebohm F, Ho C, Pesaran B, Mitra P (2000) A procedure for an automated measurement of song similarity. Anim Behav 59:1167-1176.
Theunissen FE, Doupe AJ (1998) Temporal and spectral sensitivity of complex auditory neurons in the nucleus HVc of male zebra finches. J Neurosci 18:3786-3802.
Thompson JA, Wu W, Bertram R, Johnson F (2007) Auditory-dependent vocal recovery in adult male zebra finches is facilitated by lesion of a forebrain pathway that includes the basal ganglia. J Neurosci 27:12308-12320.
Thompson JA, Basista MJ, Wu W, Bertram R, Johnson F (2011) Dual pre-motor contribution to songbird syllable variation. J Neurosci 31:322-330.
Thomson D, Chave A (1991) Jackknifed error estimates for spectra, coherences, and transfer functions. Englewood Cliffs, NJ: Prentice Hall.
Thorpe W (1958) The learning of song patterns by birds, with especial reference to the song of the chaffinch *Fringilla coelebs*. Ibis 100:535-570.
Tononi G, Cirelli C (2006) Sleep function and synaptic homeostasis. Sleep Med Rev 10:49-62.
Troyer TW, Doupe AJ (2000a) An associational model of birdsong sensorimotor learning II. Temporal hierarchies and the learning of song sequence. J Neurophysiol 84:1224-1239.
Troyer TW, Doupe AJ (2000b) An associational model of birdsong sensorimotor learning I. Efference copy and the learning of song syllables. J Neurophysiol 84:1204-1223.
Vicario DS, Simpson HB (1995) Electrical stimulation in forebrain nuclei elicits learned vocal patterns in songbirds. J Neurophysiol 73:2602-2607.
Volman SF (1993) Development of neural selectivity for birdsong during vocal learning. J Neurosci 13:4737-4747.
Volman SF (1996) Quantitative assessment of song-selectivity in the zebra finch "high vocal center". J Comp Physiol A 178:849-862.
Vu ET, Mazurek ME, Kuo YC (1994) Identification of a forebrain motor programming network for the learned song of zebra finches. J Neurosci 14:6924-6934.
Wang DD, Kriegstein AR (2009) Defining the role of GABA in cortical development. J Physiol 587:1873-1879.
Wiesel TN, Hubel DH (1963) Single-Cell Responses in Striate Cortex of Kittens Deprived of Vision in One Eye. J Neurophysiol 26:1003-1017.

Wild JM (1994) Visual and somatosensory inputs to the avian song system via nucleus uvaeformis (Uva) and a comparison with the projections of a similar thalamic nucleus in a nonsongbird, Columba livia. J Comp Neurol 349:512-535.
Wild JM, Arends JJ (1987) A respiratory-vocal pathway in the brainstem of the pigeon. Brain Research 407:191-194.
Wild JM, Williams MN, Howie GJ, Mooney R (2005) Calcium-binding proteins define interneurons in HVC of the zebra finch (Taeniopygia guttata). J Comp Neurol 483:76-90.
Williams H (1990) Models for song learning in the zebra finch: fathers or others? Anim Behav 39:745-757.
Williams H (2004) Birdsong and singing behavior. Ann N Y Acad Sci 1016:1-30.
Williams H, Vicario DS (1993) Temporal patterning of song production: participation of nucleus uvaeformis of the thalamus. J Neurobiol 24:903-912.
Williams H, Mehta N (1999) Changes in adult zebra finch song require a forebrain nucleus that is not necessary for song production. J Neurobiol 39:14-28.
Witham CL, Baker SN (2007) Network oscillations and intrinsic spiking rhythmicity do not covary in monkey sensorimotor areas. J Physiol 580:801-814.
Womelsdorf T, Fries P (2007) The role of neuronal synchronization in selective attention. Curr Opin Neurobiol 17:154-160.
Woolley SM (2004) Auditory experience and adult song plasticity. Ann N Y Acad Sci 1016:208-221.
Xu X, Roby KD, Callaway EM (2010) Immunochemical characterization of inhibitory mouse cortical neurons: Three chemically distinct classes of inhibitory cells. J Comp Neurol 518:389-404.
Yoshimura Y, Callaway EM (2005) Fine-scale specificity of cortical networks depends on inhibitory cell type and connectivity. Nat Neurosci 8:1552-1559.
Yu AC, Margoliash D (1996) Temporal hierarchical control of singing in birds. Science 273:1871-1875.
Zahavi A (1975) Mate selection-a selection for a handicap. J Theor Biol 53:205-214.
Zann RA (1996) The zebra finch: a synthesis of field and laboratory studies. New York: Oxford University Press.
Zaremba S, Guimaraes A, Kalb RG, Hockfield S (1989) Characterization of an activity-dependent, neuronal surface proteoglycan identified with monoclonal antibody Cat-301. Neuron 2:1207-1219.
Zevin JD, Seidenberg MS, Bottjer SW (2004) Limits on reacquisition of song in adult zebra finches exposed to white noise. J Neurosci 24:5849-5862.
Zheng W, Knudsen EI (1999) Functional selection of adaptive auditory space map by $GABA_A$-mediated inhibition. Science 284:962-965.