Watching the Brain Learn and Unlearn: Effects of Tutor Song Experience and Deafening
on Synaptic Inputs to HVC Projection Neurons

by

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

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Abstract

The ability of young children to vocally imitate the speech of adults is critical for speech learning. Vocal imitation requires exposure to an external auditory model and the use of auditory feedback to adaptively modify vocal output to match the model. Despite the importance of vocal imitation to human communication and social behavior, it remains unclear how these two types of sensory experience, model exposure and feedback, act on sensorimotor networks controlling the learning and production of learned vocalizations. Using a combination of longitudinal in vivo imaging of neuronal structure and electrophysiological measurements of neuronal function, I addressed the questions of where, when, and how these two types of sensory experience act on sensorimotor neurons important to singing and song learning in zebra finches. The major finding of these experiments is that synaptic inputs onto neurons in HVC, a sensorimotor nucleus important to singing and song learning, are sensitive to tutor song experience and deafening. Thus, these findings for the first time link auditory experiences important to vocal imitation to synaptic reorganization in sensorimotor neurons important to behavior.

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1. Introduction

1.1 Overview

The ability to imitate the actions of others lies at the heart of human social and

cognitive development and forms the foundation for human culture (Meltzoff, 2005;

Donald, 2005). In particular, the ability of young children to vocally mimic the speech of

adults is critical for the learning of spoken language and its transmission from

generation to generation (Locke, 1993). Vocal imitation requires exposure to an external

auditory model and the use of auditory feedback to adaptively modify vocal output to

match the model. Despite the importance of vocal imitation to human communication

and social behavior, it remains unclear how these two types of sensory experience,

model exposure and feedback, act on sensorimotor networks controlling the learning

and production of learned vocalizations. Using a combination of longitudinal in vivo

imaging of neuronal structure and electrophysiological measurements of neuronal

function, I addressed the questions of where, when, and how these two types of sensory

experience act on sensorimotor neurons important to the learning and production of

learned vocalizations in songbirds.

1.2 Songbirds as a model to study vocal imitation

The songbird has proven to be an unparalleled animal model in which to

investigate the neural mechanisms of vocal learning for two reasons. First, although

evidence for vocal learning has been found in a small number of non-human animals,

including bats and marine mammals, song learning in birds is the most well documented example of non-human vocal learning in the animal kingdom (Doupe and Kuhl, 1999; Janik and Slater, 1997). In the case of the zebra finch, a seasonal songbird native to central Australia that is the subject of this study, juvenile males achieve vocal imitation by listening to, memorizing, and eventually copying the song of an adult male tutor. Second, the neural circuits in the songbird brain required for singing and song learning are relatively well-delineated. Thus, a number of experimental techniques can be exploited to investigate how sensory information required for vocal learning acts within these defined sensorimotor circuits to modify the structural and functional properties of neurons important to vocal learning and control.

1.2.1 Behavioral description of vocal learning in songbirds

Like speech learning in humans, vocal learning in songbirds requires two types of auditory experience: exposure to an auditory model and the use of auditory feedback to guide imitation of the model. The process of song learning in zebra finches is divided into two partially overlapping phases during which these two types of auditory information are used (Immelman, 1969; Konishi, 1965; Marler, 1970). During the phase of sensory learning, which begins as early as 20 days post-hatch (dph), juvenile males listen to and memorize a single song produced by an adult male tutor. The tutor is usually the father of the juvenile, but the juvenile can learn from any adult male zebra finch with which it interacts socially, or even from a taped song via operant training

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paradigms (Tchernichovski et al., 1999, 2000). Although the juvenile is typically exposed

to a tutor throughout the duration of song learning, even limited exposure to the tutor

song can result in successful song imitation in adulthood (Petrinovich, 1985; Hultsch and

Todt, 1989a,b; Tchernichovski et al., 1999). In summary, vocal learning in zebra finches

depends on the auditory experience of a tutor song during the sensory stage of song

learning.

During the phase of sensorimotor learning, which begins around 45 dph and

spans about six weeks, the juvenile male zebra finch begins singing and uses auditory

feedback from its own performance to adaptively modify its vocalizations to match the

memorized tutor song. Notably, even juveniles that have never been exposed to a tutor

(i.e., isolates) will use auditory feedback to modify their vocalizations over the period of

sensorimotor learning, although in this case, imitation does not occur because the song is

based on an innately-specified model rather than on an external tutor (Konishi, 1965).

Over song development, the juvenile's song becomes increasingly stereotyped and less

sensitive to perturbations of auditory feedback, and starting around the time of sexual

maturation (around 90 dph), the bird's song becomes highly stereotyped in its spectral

and temporal features, a process referred to as "song crystallization". Even in adult

zebra finches, however, the maintenance of stereotyped song production depends on

auditory feedback (Andalman and Fee, 2009; Brainard and Doupe, 2000; Lei and

Mooney, 2010; Leonardo and Konishi, 1999; Nordeen and Nordeen, 1992; Scharff and

Nottebohm, 1991; Williams and Mehta, 1999), and perturbations of auditory feedback, such as deafening, cause the spectral and temporal features of song to degrade (Brainard and Doupe, 2000; Horita et al., 2008; Lombardino and Nottebohm, 2000; Nordeen and Nordeen, 1992). In summary, vocal learning in juvenile zebra finches and song maintenance in adult zebra finches both depend on singing-related auditory feedback.

Zebra finch song learning is particularly amenable to behavioral studies for a number of reasons. First, because juvenile birds cannot begin the process of tutor song learning until they have heard a tutor, the experimenter has exact control over the onset of imitative learning, the total amount of exposure to the tutor song, and the exact tutor song to which the juvenile is exposed. Second, the fact that each zebra finch will only learn a single song and the fact that these songs are very stereotyped in adult birds allows for detailed quantification of the time course and quality of imitative learning. Third, the stereotypy of adult song production enables exact measurements of the effects of perturbation of auditory feedback on the stability of song structure. In sum, the time course and quality of imitative learning in zebra finches can be readily manipulated and quantified, allowing for changes in behavior to be precisely correlated with changes in the structure and function of neurons important to vocal learning and control.

1.2.2 Zebra finch song learning is limited to a sensitive period in development

Understanding the neural mechanisms of vocal learning in zebra finches is of particular interest because song learning, like speech learning in humans, is limited to a

sensitive period in development. Sensitive periods are time windows during

development when the brain exhibits heightened sensitivity to specific forms of sensory

input or experience, and it is generally thought that their purpose is to tailor the

development of an individual's brain, and ultimately perceptual and behavioral

capacity, to its environment. Although sensitive periods are a feature of neural circuits,

they are often described in terms of the corresponding behavioral changes that normally

occur during these time windows. For example, sensitive periods have been described

for the development of binocular vision (Hubel and Wiesel, 1970; reviewed by Hensch,

2005), language acquisition (Lenneberg, 1967; reviewed in Doupe and Kuhl, 1999), and

the development of social preferences (Harlow and Zimmerman, 1959; Lorenz, 1958;

Scott, 1962). Given the essential role of sensitive periods in the proper development of

neural circuits and behavior, an important goal is to understand the factors that regulate

the onset, duration, and closure of sensitive periods in development.

Song learning in zebra finches comprises a series of temporally overlapping

sensitive periods. Indeed, both the sensory and sensorimotor stages of song learning are

limited to sensitive periods in development in zebra finches.   The capacity for tutor

song learning is greatly reduced in juvenile finches older than 60 dph (Eales, 1985;

Immelman, 1969), and even if a juvenile bird hears the song of a tutor within the

sensitive period for tutor song learning, it will ultimately produce a poor copy of the

tutor song if it is prevented from hearing itself sing during the period of sensorimotor

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learning (Konishi, 1965; Price, 1979). In addition, adult birds that have been subjected to

feedback perturbation to drive song degradation show only a limited ability to recover

their pre-manipulation vocalizations or to acquire new song material once the

perturbation is stopped (Zevin et al., 2004). Thus, vocal learning in zebra finches can be

studied to understand not only how the brain changes during imitative learning but also

to gain insight into the factors that limit certain types of learning to sensitive periods in

development.

1.2.3 Neural circuits for vocal learning: a focus on HVC

The sensorimotor brain regions that are required for singing and song learning

are collectively referred to as the song system. The song system consists of two

interconnected neural circuits that originate from separate populations of projection

neurons (PNs) in the sensorimotor nucleus HVC. One HVC PN type, HVC_{RA}, innervates

the song motor pathway (SMP; shown in black in Figure 1A) that is required for song

pattering and production; lesions in this pathway disrupt and abolish the bird's ability

to produce song (Nottebohm et al., 1976; Simpson and Vicario, 1990). The SMP includes

HVC_{RA} neurons, the downstream song premotor nucleus RA, and vocal-respiratory

targets in the brainstem that control song production. The second HVC PN type, HVC_{X},

innervates Area X of the basal ganglia and is part of an anterior forebrain pathway (i.e.,

the AFP, shown in white in Figure 1A) that is required for song learning in juveniles but

not for song production in adults (Bottjer et al., 1984; Scharff and Nottebohm, 1991). The

AFP is thought to be analogous to mammalian cortico-basal ganglia-thalamo-cortical loops required for motor skill learning and refinement (Doupe et al., 2005), and it includes HVC_X neurons, the striatal region Area X, the thalamic nucleus DLM, and the telencephalic nucleus LMAN, which provides input to the SMP via its projection to RA. Thus, the AFP indirectly links HVC and RA and is ideally positioned to modulate the output of the SMP. Interestingly, the AFP is required for song variability in juveniles (Olveczky et al., 2005; Scharff and Nottebohm, 1991), social context-dependent modulation of song variability in adults (Kao et al., 2005; Kao and Brainard, 2006; Kao et al., 2008; Stepanek and Doupe, 2010) and feedback-dependent changes to song (Andalman and Fee, 2009; Bottjer et al., 1984; Brainard and Doupe, 2000; Williams and Mehta, 1999).   Although the neural circuitry required for singing and song learning has been characterized, it remains unclear how tutor song experience and auditory feedback act on this circuitry to guide imitative vocal learning.
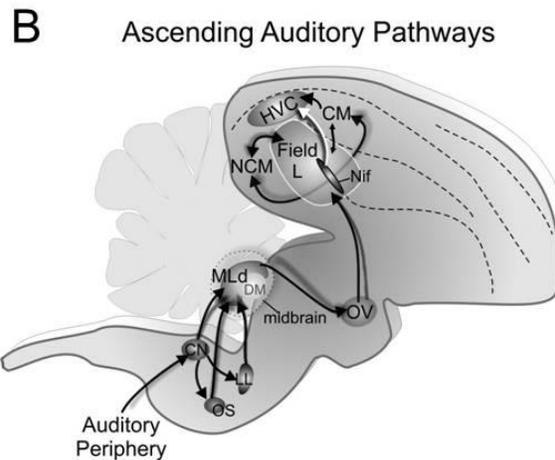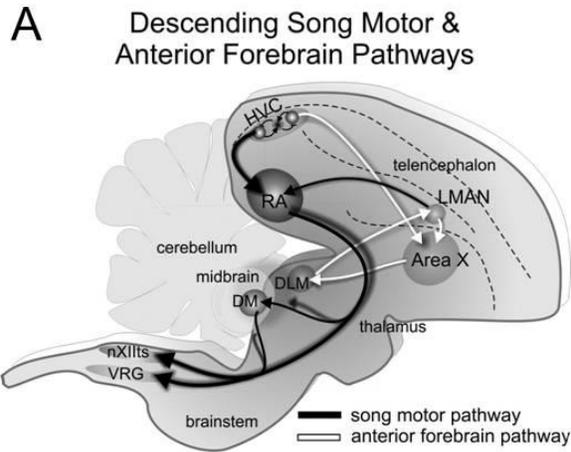
Figure 1: Simplified diagram of descending song sensorimotor pathways and ascending auditory pathways in the zebra finch brain. A) The song motor pathway (SMP) is required for song production (shown in black), and the anterior forebrain pathway (AFP) is required for song learning (white). These two pathways originate from distinct populations of PNs in HVC. B) Ascending auditory information passes from the cochlear nucleus (CN) and is relayed to the auditory hindbrain (OS and LL) and midbrain (MLd) to the thalamic nucleus ovoidalis (OV). OV projects to the auditory telencephalic area Field L, which is reciprocally connected with the secondary auditory areas NCM and the CM and also projects to NIf. NIf and CM project to HVC. Figure adapted from Bauer et al., 2008.

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This study focuses on the effects of tutor song experience and auditory feedback on HVC for two reasons. First, HVC is required for singing and song learning and is the highest site in the motor hierarchy where explicit motor representations for song can be detected. In addition, HVC receives a number of extrinsic auditory inputs, endowing HVC neurons with sensory and motor properties (Figure 1B; Bauer et al., 2008; Cardin and Schmidt, 2004; Coleman and Mooney, 2004; Nottebohm et al., 1982) and making HVC an ideal location where sensory information about the tutor song and auditory feedback could be used to shape and refine the song motor representation. Second, changes to HVC are likely to have consequences for both singing and song learning, because HVC$_{RA}$ neurons provide a precise timing signal for song patterning (Hahnloser et al., 2002; Yu and Margoliash, 1996) and HVC$_X$ neurons are likely the source of singing-related activity in the AFP (Hessler and Doupe, 1999; Kozhevnikov and Fee, 2007; Prather et al., 2008). Thus, changes to HVC$_{RA}$ neurons could directly influence song patterning, while changes to HVC$_X$ neurons could alter AFP activity and thereby alter levels of song variability. I predicted that both tutor song experience and auditory feedback act via HVC to influence song production, and therefore, that perturbation of these two types of auditory experience would alter the structural and functional properties of HVC PNs.

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1.3 Neural circuits for tutor song learning

How does the brain change during the initial stages of vocal learning, when an animal is exposed to an auditory model that it will subsequently imitate? In the case of tutor song learning in songbirds, it remains unclear where experience of the tutor song is encoded in the brain and how this representation ultimately influences the sensorimotor circuits that control song learning and production. One idea is that the neural representation of the tutor song resides in auditory brain areas. There are a number of studies supporting the idea that tutor song memory may be stored in the secondary auditory telencephalon, in areas such as the caudomedial mesopallium (CMM) or the caudomedial nidopallium (NCM) (Figure 1B). These two regions of higher auditory processing are reciprocally interconnected and receive input from the auditory thalamus and the primary auditory telencephalon (Vates et al., 1996). Additionally, CMM projects directly to HVC and indirectly to HVC via the sensorimotor nucleus NIf (Bauer et al., 2008; Vates et al., 1996), providing a route through which CMM and NCM could pass auditory information on to sensorimotor brain areas important to vocal control.

CMM and NCM have both been implicated in the neural representation of conspecific songs (Chew et al., 1996; Gentner and Margoliash, 2003; Mello et al., 1992; Mello and Clayton, 1994; Theunissen et al., 2004), and studies have also found that neural responses in these areas to playback of the tutor song to adult birds correlate with the quality of tutor song learning. A number of these studies have used the activation of

immediate early genes (IEGs), a class of genes that are rapidly and transiently activated in response to increased neural activity (Lanahan and Worley, 1998), as means to assess the strength of neural responses to playback of auditory stimuli. Interestingly, IEG expression in NCM in response to playback of tutor song to adult birds positively correlates with the number of song elements each bird shared with its tutor (Bolhuis et al., 2000), although no such correlation was found in CMM (Terpstra et al., 2004; Bolhuis et al., 2001). Also, the magnitude of electrophysiological response habituation in NCM to repeated playback of the tutor song positively correlated with the accuracy of tutor song imitation (Phan et al., 2006). Furthermore, lesions of NCM impair tutor song recognition in adults, without disrupting song production (Gobes and Bolhuis, 2007). Although these findings suggest that secondary auditory areas may be involved in the neural representation of the tutor song, an important issue not addressed by the majority of these studies is that for birds that imitated successfully, the tutor song is highly similar to the bird's own song; hence, enhanced neural responses to the tutor song in adult birds may simply reflect the bird's familiarity with its own vocalizations. Despite this potential confound, these studies raise the possibility that secondary auditory areas, and in particular NCM, may play a role in the neural representation of tutor song memory.

While these studies demonstrated correlations between imitation quality and the response properties of neurons in secondary auditory areas, they did not test directly

whether CMM or NCM are required for tutor song learning in juveniles. A study by

London and Clayton (2008) addressed this question by pharmacologically disrupting

activation of ERK, a kinase that regulates the induction of IEGs and is thought to play a

role in memory formation (Bozon et al., 2003; Sweatt, 2004). Juvenile birds treated with

the ERK inhibitor in CMM and NCM while exposed to brief, daily sessions with a tutor

produced poor copies of the tutor song as adults. Additionally, birds treated with the

ERK inhibitor were not impaired in an operant song discrimination task, suggesting that

ERK inhibition did not cause a general impairment of auditory processing. This study

suggests that a conserved, activity-dependent molecular cascade is required in

secondary auditory areas for proper formation of the tutor song memory. However, it

remains unclear whether the treated secondary auditory areas serve as the site of tutor

song memory storage or simply relay auditory information about the tutor song to

downstream areas for later storage and/or consolidation. Additionally, because

pharmacological inhibition lacks temporal precision, a remaining possibility is that

rather than disrupting memory formation, ERK inhibition impaired sensorimotor

integration during vocal rehearsal following tutoring sessions, and this integration is

essential for accurate imitation. Despite these limitations, these findings support the

idea that secondary areas of the auditory telencephalon play a role in formation of the

tutor song memory in juveniles.

As opposed to the idea that tutor song memory is stored in auditory areas, a second idea is that tutor song memory is stored in the sensorimotor circuits required for singing and song learning. In support of this idea, studies have found selective auditory responses to tutor song in two AFP nuclei, Area X and LMAN, in anesthetized, juvenile birds (Doupe and Solis, 1997). Additionally, selective responses to tutor song were also found in Area X and LMAN in juvenile birds that received peripheral vocal nerve cuts and hence had songs that were spectrally dissimilar to the tutor song (Solis and Doupe, 1999). Although these studies suggest that sensorimotor areas important to song learning are shaped by auditory experience of the tutor song, they do not address the time course over which this influence is exerted and whether song sensorimotor areas are involved in formation of tutor song memory during the initial exposure to a tutor. To test the idea that the AFP is involved in formation of the tutor song memory, Basham et al. (1996) pharmacologically blocked NMDA-type glutamate receptors in LMAN during tutoring sessions, a manipulation thought to interfere with the expression of synaptic plasticity but not with basal synaptic transmission. Interestingly, NMDA receptor blockade prevented accurate tutor song imitation, indicating that synaptic plasticity within the AFP may be important for the formation of the tutor song memory. However, the methods of this study are subject to the same criticism as those of London and Clayton (2008); the employed pharmacological manipulation lacks temporal precision and therefore may simply have impaired sensorimotor integration during

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vocal rehearsal following tutoring sessions that is critical for accurate imitation. Keeping these limitations in mind, these findings nonetheless support the idea that tutor song experience may be encoded in the sensorimotor neural circuits that are required for song learning.

A number of studies implicate the AFP in tutor song memory formation, but is there evidence that the SMP is involved in this process? A study by Shank and Margoliash (2009) addressed this question by examining the effects of tutor experience in RA, the sensorimotor nucleus downstream of HVC in the SMP. Bursting properties of RA neurons measured during sleep have been shown to reflect daytime singing experience (Dave and Margoliash, 2000), and Shank and Margoliash found that tutor song experience during the day induced changes in the bursting activity of RA neurons during the following night of sleep. The nature of these changes for each bird was related to the acoustic properties of the tutor song it heard, such that birds that had heard the same tutor exhibited similar changes in the bursting patterns of RA neurons. Additionally, these changes were largely prevented by perturbation of the birds' singing-related auditory feedback during the day. These findings suggest that auditory experience of the tutor song may be translated very rapidly into sensorimotor coordinates in areas important to song production, but this effect may depend on vocal rehearsal.

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In summary, these studies suggest that a number of secondary auditory areas and sensorimotor areas required for singing and song learning may contribute to the neural representation of tutor song, and a congruous explanation for these numerous findings is that the tutor song memory is stored in a distributed manner across both sensory and sensorimotor brain regions. Along these lines, I propose that tutor song information initially accesses the song system via the projections of secondary auditory areas onto HVC, and this interaction between auditory and sensorimotor areas is a critical step in formation of the tutor song memory. However, two important questions remain to be addressed to fully test the idea that HVC and downstream song sensorimotor areas are involved in the formation and storage of the auditory memory of the tutor song. First, does tutor song experience affect the structural and functional properties of HVC neurons? The idea that tutor song information may access the song system via HVC is supported by the findings that downstream sensorimotor regions downstream of HVC in the AFP and SMP are rapidly affected by tutor song experience and may be required for tutor song memory formation. However, although a previous study reported that HVC neurons in awake, juvenile birds responds selectively to playback of the tutor song (Nick and Konishi, 2005a), the effects of tutor song experience on the properties of the two populations of HVC PNs remain to be directly tested. Second, does auditory experience of the tutor song act directly on HVC or, alternatively, is tutor song experience translated more slowly into changes in HVC and downstream

song system areas through the process of vocal rehearsal? In my first set of experiments (Chapter 2), I used longitudinal measurements of neuronal structure and function to test the idea that HVC PNs are sensitive to auditory experience of the tutor song.

1.4 Neural circuits for singing-related auditory feedback

Auditory feedback is critical for learning and maintaining complex motor skills ranging from musical performance to speech. For example, hearing loss prevents speech learning in children and degrades speech in adults (Petitto, 1993; Waldstein, 1989). Despite its importance to motor skill learning, the neural mechanisms through which sensory feedback acts on motor circuits controlling the learning and execution of behaviors remains poorly understood. In particular, how the loss of auditory feedback causes learned skills such as speech to degrade remains unclear. In the case of songbird vocal learning, the behavioral relevance of auditory feedback to song learning and maintenance has been thoroughly described, but it remains to be determined where, when, and how auditory feedback acts within these circuits to influence vocal output.

HVC is an attractive site where auditory feedback could influence the song motor representation. In addition to being required for singing and song learning, a number of lines of evidence suggest that HVC may receive auditory feedback information. First, HVC receives a number of extrinsic auditory inputs that could in principle convey auditory feedback information to HVC (Bauer et al., 2008; Cardin and Schmidt, 2004; Coleman and Mooney, 2004; Nottebohm et al., 1982). Indeed, all three

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neuron types in HVC exhibit selective auditory responses to the bird's own song in the anesthetized state (Katz and Gurney, 1981; Lewicki and Arthur, 1996; Lewicki, 1996; Margoliash, 1983, 1986; McCasland and Konishi, 1981; Volman, 1993). This auditory selectivity emerges during sensorimotor learning and changes as the bird's song changes, indicating that response properties of HVC neurons are shaped by auditory information from the bird's recent vocal output (Nick and Konishi, 2005a, 2005b; Roy and Mooney, 2007). Second, regions of the auditory forebrain that provide either direct or indirect sources of auditory input to HVC contain neurons that are sensitive in real-time to perturbation of auditory feedback during singing (Keller and Hahnloser, 2009; Lei and Mooney, 2010). Finally, the singing-related activity of putative HVC interneurons is sensitive in real-time to perturbations of auditory feedback in Bengalese finches (Sakata and Brainard, 2008). Although this study suggests that auditory feedback affects HVC neurons in real-time, one caveat to this finding is that feedback perturbation can drive immediate changes to Bengalese finch songs, making it difficult to rule out the possibility that the observed changes in HVC interneuron activity were related to changes in song motor output. Taken together, however, these findings support the idea that the auditory inputs to HVC may relay information about singing-related auditory feedback.

In particular, HVC_X neurons are in an ideal anatomical position to convey auditory feedback-related information to downstream song sensorimotor areas. First,

HVC$_X$ neurons exhibit selective auditory responses to the bird's own song, at least under anesthesia, and the auditory afferents to this cell type could convey auditory feedback information from the bird's vocalizations during behavior. Second, HVC$_X$ neurons receive a strong feedforward projection from HVC$_{RA}$ neurons (Mooney and Prather, 2005) and hence may receive a copy of the motor signals that are sent to RA during song production. Notably, inputs from HVC$_{RA}$ to HVC$_X$ neurons are often relayed through HVC interneurons; thus, information about motor output may reach HVC$_X$ neurons with a delay and could coincide temporally with the arrival of auditory feedback information relayed to HVC$_X$ by auditory inputs. In addition to relaying motor signals from HVC$_{RA}$ neurons, HVC interneurons might relay auditory feedback information to HVC$_X$ neurons, although the sensitivity of this cell type to feedback perturbation remains to be tested in zebra finches. Finally, because the output nucleus of the AFP, LMAN, acts on RA to instructively bias song motor output (Andalman and Fee, 2009) and to enable vocal plasticity in response to deafening or other feedback perturbations (Andalman and Fee, 2009; Brainard and Doupe, 2000; Williams and Mehta, 1999), an attractive possibility is that HVC$_X$ neurons could provide the AFP with feedback-related information that is used to drive changes to song. Consistent with this idea, HVC is the source of auditory input into the AFP, at least in the anesthetized bird (Roy and Mooney, 2009). Thus, HVC$_X$ neurons could relay auditory feedback-related information,

as well as information about song motor output, to downstream areas of the AFP, where it could be harnessed to drive changes in vocal output.

To test the idea that HVC_X neurons convey auditory feedback-related information to the AFP, a number of studies have made electrophysiological measurements to determine whether the output of these neurons is sensitive to perturbations of auditory feedback. However, the singing-related activity of HVC_X neurons is unaffected by perturbation of auditory feedback, even in juvenile zebra finches and Society finches, which both exhibit relatively rapid behavioral changes (over days) in response to feedback perturbation (Kozhevnikov and Fee, 2007; Prather et al., 2008). Although the simplest interpretation of these findings is that HVC_X neurons are insensitive to perturbation of auditory feedback, the fact that a similar study also failed to detect real-time sensitivity to feedback perturbation in LMAN neurons (Leonardo, 2004) raises the alternative possibility that feedback acts within song sensorimotor circuits in a slower, cumulative manner. Indeed, a study employing peripheral vocal nerve cut, which drives immediate spectral distortion and slower temporal reorganization of song, found that LMAN neurons exhibited slow shifts (> 1 week) in auditory selectivity toward the spectrally-distorted song that preceded temporal degradation of song (Roy and Mooney, 2007). Interestingly, this process of temporal reorganization depends on an intact LMAN, suggesting that it is a form of maladaptive vocal plasticity driven by experience of chronically distorted auditory

feedback (Williams and Mehta, 1999). Taken together, these findings raise the possibility that changes to auditory feedback act on song sensorimotor neurons over relatively long timescales (hours to days) that parallel the delayed effects of feedback perturbation on vocal output.

What can we conclude about the role of HVC_X neurons in processing auditory feedback? One possibility is that HVC_X neurons convey auditory feedback-related information, but feedback perturbation only alters the output of HVC_X over long timescales (hours to days). In this scenario, perturbation of auditory feedback would drive slow changes in the properties of HVC_X neurons, but these changes would still occur prior to the onset of perturbation-induced song degradation. A second possibility is that HVC_X neurons are insensitive to changes in auditory feedback and convey only a corollary discharge of the HVC_{RA} motor signal to the AFP; in this scenario, perturbation of auditory feedback would only alter the properties of HVC neurons after changes in vocal output occurred, if at all. Thus, to determine whether HVC_X neurons are sensitive to changes in auditory feedback, a longitudinal technique is required that allows the properties of single HVC_X neurons to be tracked starting before manipulation of auditory feedback out until the onset of vocal change. To accomplish this goal, I made longitudinal measurements of neuronal structure and function in deafened zebra finches to test where, when, and how disruption of singing-related auditory feedback affects HVC PNs (Chapters 3 through 5). More specifically, I tested the hypothesis that the

properties of HVC$_X$ neurons would change following deafening but prior to the onset of deafening-induced vocal changes.

1.5 Synaptic reorganization as a neural mechanism for the encoding of experience

If tutor song experience and auditory feedback act on HVC to influence vocal output, what are the neural mechanisms through which these two types of sensory experience could influence the output of HVC neurons? More generally, how does sensory experience act on the brain to drive changes in behavioral output? In a very simplified scenario, the brain can be thought of as a collection of neurons that send information to one another via their connections at specialized structures called synapses. As early as the 19th century, Santiago Ramón y Cajal postulated the idea that neurons are separate entities that send information to one another via electrical currents through their axons and dendrites (Ramón y Cajal, 1906). This idea was echoed by Donald Hebb nearly half a century later, who suggested more specifically that neurons might undergo growth processes or metabolic changes at these points of contact to enhance communication (Hebb, 1949). At the time, little was known about the manner in which signals are conveyed from one neuron to another, but it is now a commonly accepted idea that changes in the functional and structural properties of synaptic connections between neurons are key to the encoding of experience and learning in the brain.

How is the functional efficacy of synapses regulated by experience? One simple idea is that coincident activation of a set of neurons by a particular experience would result in enhanced communication between those neurons. This idea was first tested in vitro, in a set of experiments in which repeated activation of excitatory, glutamatergic synapses from the entorhinal cortex onto granule cells in the dentate gyrus of the hippocampus was shown to cause a long-lasting (~hours) increase in strength of the synaptic connections between these two regions (Bliss and Lømo, 1973). Since the discovery of this form of synaptic enhancement, referred to as long-term potentiation (LTP), a number of forms of long-lasting strengthening and weakening of glutamatergic synapses have been characterized (Glanzman et al., 2010; Citri and Malenka, 2008). While the exact mechanisms and forms of long-term synaptic plasticity expressed vary between brain regions and even between neuron types within a single brain region, the underlying phenomenon of activity-dependent changes in synaptic strength is a common characteristic of neurons throughout the brain.

In addition to observing functional changes to synapses, in vitro studies have characterized structural changes to synapses that accompany functional changes during the induction of activity-dependent synaptic plasticity. In particular, a large amount of work has focused on the effects of neural activity on dendritic spines, small postsynaptic protrusions that constitute the major site of excitatory, glutamatergic synaptic input onto neurons in the CNS (DeRobertis and Bennett, 1955; Palay, 1965; Ramón y Cajal, 1904).

Indeed, changes in the density, stability, and size of dendritic spines have been shown to correlate with long-lasting functional alterations in functional synaptic strength. In two pioneering studies using two-photon imaging methods, Engert and Bonhoeffer (1999) and Maletic-Savatic et al. (1999) demonstrated that long-lasting functional enhancement of hippocampal synapses was accompanied by the growth of new dendritic spines. Additionally, a study using repetitive glutamate uncaging to pharmacologically activate individual dendritic spines found that spine activation caused long-lasting increases in spine size that were well-correlated with the timing, magnitude, and spatial localization of functional enhancements of synaptic activity (Matsuzaki et al., 2004). More recently, calcium imaging was used to show that individual spines activated during the induction of LTP underwent long-term stabilization, and new spines were formed and clustered near activated spines (De Roo et al., 2008). Conversely, the induction of long-term depression (LTD) is accompanied by shrinkage (Okamoto et al., 2004; Zhou et al., 2004) and retraction of dendritic spines (Nägerl et al., 2004). Thus, stabilization and growth of dendritic spines are correlated with functional enhancements in synaptic strength, while shrinkage and loss of dendritic spines are correlated with functional decrements in synaptic strength following the induction of long-term plasticity in vitro. In sum, a combination of structural and functional synaptic changes following changes in neural activation is hypothesized to be an important cellular substrate for information storage in the brain.

Although specific patterns of neural activity result in changes in synaptic function and structure in vitro, an important question is whether these changes have relevance for how experience is encoded by synapses in the brains of living animals. Because it is often technically simpler to longitudinally track structural changes to individual dendritic spines than it is to track functional changes to individual synapses, many in vivo studies have relied on measurements of the structural dynamics of dendritic spines to test the effects of experience on the synaptic organization of neural circuits. To achieve this goal, two-photon imaging methods from in vitro studies have been adapted and combined with long-term fluorescent labeling of neurons to track the size, stability, and density of dendritic spines in living animals over periods of days to months to test whether changes in sensory experience drive changes in the structural dynamics of spines in sensory cortical regions.

One particularly useful model system in which to investigate the effects of experience on the structural dynamics of dendritic spines has been the rodent barrel cortex, a region of sensorimotor cortex that has discrete columns (i.e., barrels) corresponding to the sensory representation of each of the animal's whiskers. In young rats, trimming a subset of the animal's whiskers has been shown to result in functional reorganization of response properties in the barrel cortex, with neurons that previously responded to stimulation of a trimmed whisker gaining responses to adjacent, spared whiskers (Fox, 1992; Glazewsky and Fox, 1996; Stern et al., 2001). A study of the effects

of sensory experience on dendritic spines on barrel cortex neurons demonstrated that

whisker trimming stabilizes new spines and destabilizes spines that had previously been

stable (Holtmaat et al., 2006). These effects are most pronounced in neurons at the

border between barrel columns for spared and deprived whiskers, and additionally,

these same neurons exhibit enhancement of neural responses to stimulation of the

spared whiskers (Wilbrecht et al., 2010). Finally, mice with mutations in CaMKII, an

enzyme important to the expression of long-term synaptic plasticity, show no

enhancement of neural responses to spared whiskers and also do not show increases in

spine stabilization following whisker trimming (Wilbrecht et al., 2010). These findings

indicate that dendritic spines in somatosensory cortex are sensitive to changes in sensory

experience and provide a link between the stabilization of new dendritic spines and

functional plasticity of neurons in vivo.

A second experimental paradigm that has proven useful to understand the

effects of sensory experience on the structural dynamics of dendritic spines is monocular

deprivation (MD), a manipulation in which one eye is closed for a period of time,

driving enhancement of neural responses in visual cortex to the non-deprived eye

(Hofer et al., 2006). A study examining dendritic spine dynamics in the binocular region

of visual cortex found that a single episode of MD drove increases in spine formation,

and many spines formed during this episode of MD were maintained even after

binocular vision was restored. Interestingly, a second round of MD did not change

levels of spine dynamics, although functional changes in neural responses to inputs from the two eyes could still be detected (Hofer et al., 2009). These findings suggest that spines formed during the initial period of MD provide a structural basis for subsequent functional shifts in neural responsiveness and represent a mechanism by which sensory experience could be stored in the brain. Taken together, these studies in visual and barrel cortices demonstrate that changes in sensory experience and, in particular, sensory deprivation cause the stabilization of newly-formed spines that are associated with functional changes in synaptic strength, supporting the idea that these new dendritic spines represent a structural substrate for encoding the effects of sensory experience in the brain.

While these studies demonstrate that changes in sensory experience can drive synaptic reorganization in sensory cortical areas, it remains unclear whether changes in experience that drive behavioral learning affect synapses in brain regions important to the learning and execution of behavior. To address this question, two recent studies examined how dendritic spines in motor cortex change as an animal learns a new motor skill (Yang et al., 2009; Xu et al., 2009). Yang et al. found that training on an accelerated rotarod running task caused increased formation of new spines in layer V pyramidal neurons of motor cortex. Spine elimination occurred at a later time, returning overall spine density to pre-training levels, but a small fraction of the new spines formed after training were maintained as long as 2 weeks after the initial training session. Notably,

the maintenance of new spines and pruning of previously stable spines following training were associated with the degree of behavioral improvement after training and maintenance of the learned skill. In a related study, Xu et al. found that training in a forelimb reaching task also causes the formation of new dendritic spines in motor cortex, a fraction of which are preferentially stabilized during subsequent training and persist as long as 4 months after the end of training. Again, spines that were present prior to training were selectively eliminated, resulting in spine density equivalent to baseline levels. Additionally, although later training on the same forelimb reaching task did not increase spine formation, training on novel motor tasks still drove the formation and stabilization of a new, non-overlapping set of spines. These two studies support the idea that the formation and stabilization of different sets of spines on motor cortex pyramidal neurons are closely associated with the learning of novel motor skills and that long-lasting synaptic reorganization may encode the neural representation of motor memories.

In summary, a large body of work supports the idea that synaptic reorganization is important to the encoding of sensory experience in sensory cortex and the encoding of learned motor skills in motor cortex. However, an important remaining goal is to understand how sensory experiences that drive or modify behavioral learning affect sensorimotor neurons required for execution of learned and execution of behaviors. Here, I addressed this question in the context of auditory-motor transformations

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underlying the learning and maintenance of zebra finch song. Using a combination of

longitudinal, in vivo imaging of dendritic spines and in vivo electrophysiological

measurements of the strength of synaptic inputs, I tested the idea that synaptic inputs to

HVC neurons important to singing and song learning are sensitive to two types of

auditory experience, namely tutor song experience and singing-related auditory

feedback. I hypothesized that both types of auditory information act via HVC to

ultimately drive changes to vocal output and, therefore, that perturbation of either type

of auditory experience would alter the strength and stability of synaptic inputs onto

HVC PNs.

2. Watching the brain learn: effects of tutor song experience on synaptic inputs to HVC projection neurons

It remains poorly understood how the brain changes during the initial stages of vocal learning, when an animal is exposed to an external model that it will subsequently imitate. The goal of my first set of experiments was to investigate whether auditory experience of a tutor song influences synaptic inputs to HVC neurons. To test the idea that dendritic spines on HVC PNs are sensitive to tutor song experience, I carried out in vivo imaging of dendritic spines on HVC projection neurons and asked two questions. First, are structural dynamics of HVC dendritic spines affected by isolation from tutor song experience? Second, if so, how does subsequent tutor song experience affect HVC dendritic spines? These experiments were carried out in collaboration with Dr. Todd Roberts, and our findings have been previously published (Roberts et al., 2010).

2.1 Levels of HVC structural dynamics in untutored birds predict the capacity for subsequent tutor song imitation

Measuring the effects of tutor song experience requires repeatedly imaging and measuring dendritic spines on HVC PNs. To achieve this goal, I used lentivirus-GFP constructs to fluorescently label HVC neurons, and retrograde tracers were injected into the two downstream targets of HVC, RA and Area X, to label HVC_{RA} and HVC_X projection neurons and visualize the boundaries of HVC (Figure 2A). A two-photon microscope was then used to repeatedly image dendritic spines on HVC PNs through a

surgically implanted cranial window in anesthetized, male zebra finches. To minimize

the effects of anesthesia on singing behavior and song learning, birds were placed on a

reversed day-night cycle, and all imaging sessions were carried out during the bird's

subjective nighttime. In preliminary experiments that involved repeatedly imaging

neurons in normally-tutored juvenile and adult zebra finches (5 groups of animals; 45,

60, 90, 120, or 200 dph), it was observed that the dendritic arbors of neurons were highly

stable across imaging sessions (Figure 2B). When imaging was carried out across 2 hour

intervals within single nights, I was able to detect a subset of spines that underwent

rapid turnover (Figure 2C-D; turnover is defined as the average of the total percentage

of spines gained and spines lost over a 2h interval). However, the nightly level of spine

turnover and spine density remained stable across at least 4 consecutive nights of

imaging in these normally-tutored birds (Figure 3; spine stability: $p = 0.2$, Friedman test,

1,573 spines; spine density: $p = 0.6$, Friedman test, 819 spines; N = 4 birds). These

experiments therefore indicate that my longitudinal imaging methodology does not

affect levels of HVC PN structural dynamics.
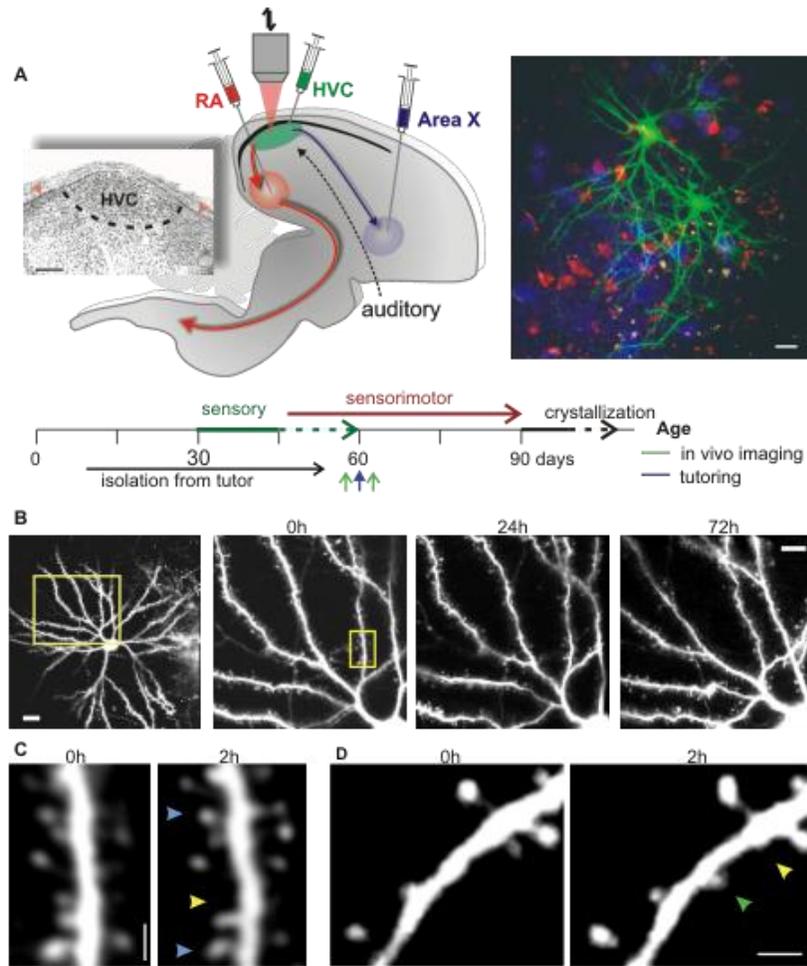
Figure 2: Longitudinal in vivo imaging of dendritic spines in the songbird sensorimotor nucleus HVC. A) Top left: schematic of the zebra finch song system and experimental design. Inset is a Nissl-stained section through HVC, showing its location on the floor of the lateral telencephalic ventricle, 60-120 μm below the pial surface. Scale bar, 200 μm. Top right: in vivo two-photon image of GFP-labeled HVC neurons amidst retrogradely labeled HVC$_{RA}$ (red) and HVC$_X$ (blue) PNs. Scale bar, 20 μm. Bottom: Time course of zebra finch song learning and experimental timeline of in vivo imaging and tutoring. B) Repeated in vivo imaging of dendritic branches from an HVC neuron of a 130 dph zebra finch over 4 nights (yellow box, left image; scale bars, 20 μm for the left image; 10 μm for the other three images. C) High magnification view of the dendritic segment shown in B (yellow box; scale bar, 2 μm). D) View of another dendritic segment of an HVC neuron showing the gain (green arrow) and loss (yellow arrow) of dendritic spines across a 2h imaging interval (scale bar, 2 μm).
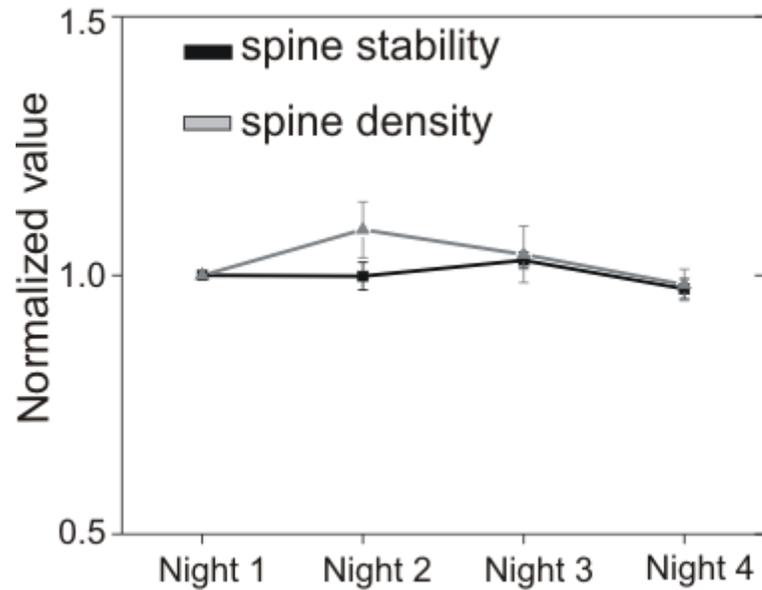
Figure 3: Longitudinal imaging does not affect HVC PN spine stability and density. Levels of spine stability and spine density measured over 2h intervals do not change across four nights of imaging (spine stability: $p = 0.2$, Friedman test, 1,573 spines; spine density: $p = 0.6$, 819 spines; $N = 4$ birds; error bars, s.e.m. unless otherwise noted)

To test whether isolation from tutor song experience influences HVC dendritic spines, levels of spine turnover in HVC PNs were measured in untutored birds that had never heard the song of an adult male (i.e., isolates). I first made measurements of spine turnover over 2h intervals in untutored birds that were aged 45, 60, or 90 dph, and these measurements were compared to those made in age-matched, normally-tutored control birds. I observed that the effects of isolation from tutor song experience on levels of HVC spine turnover depended on the age of the bird (Figure 4). All 45 dph untutored birds had higher levels of spine turnover than normally-tutored, age-matched controls. Interestingly, lack of tutor song experience had a mixed effect on 60 dph birds. On average, 60 dph untutored birds had levels of turnover that were 40% higher than age-matched controls ($p = 0.04$; untutored birds: 7% turnover, 1,879 spines, 24 cells from 18 birds; control: 4.9% turnover, 1,579 spines from 23 cells in 16 birds). Untutored 60 dph birds also had higher variance in their levels of spine turnover (F test, $p = 0.04$), with some exhibiting levels of turnover commensurate to those seen in age-matched controls and others exhibiting levels of turnover higher than those seen in age-matched controls. In contrast, 90 dph untutored birds all had levels of turnover indistinguishable from those observed in 90 dph normally-tutored controls. Thus, lack of tutor experience affects levels of HVC spine dynamics in an age-dependent manner.
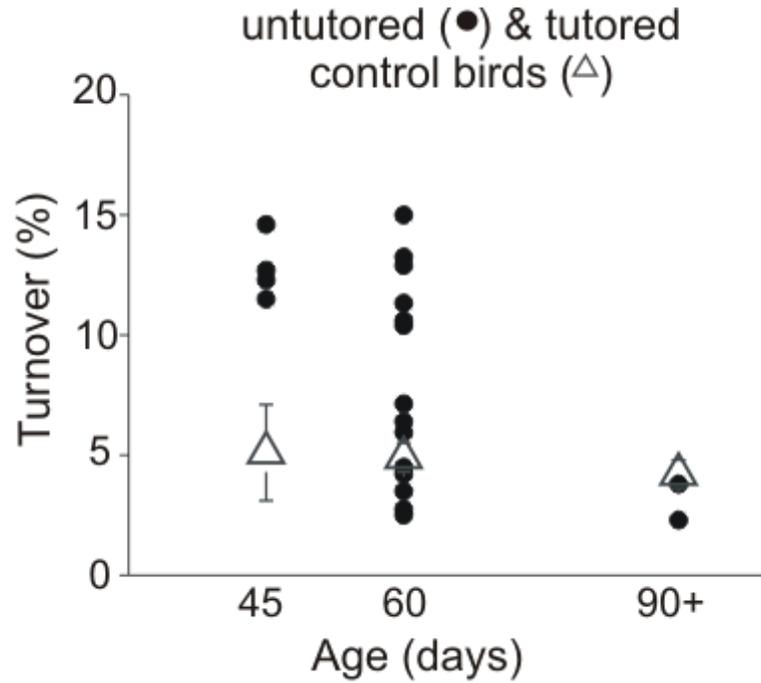
Figure 4: Age-dependent effects of isolation from tutor song experience on levels of HVC PN spine turnover. Measurements of HVC dendritic spine turnover made in normally-tutored birds at 45, 60, or 90 dph (mean values for each age group shown with open symbols; 4,145 spines, 53 cells, 30 birds) reveal that turnover levels do not change significantly over the course of sensorimotor learning. Measurements of HVC spine turnover made in untutored birds (closed symbols) reveal an age-dependent effect of isolation from tutor song experience on HVC spine turnover.

It is important to note that the three ages of untutored birds examined are at different stages of the sensitive period for tutor song learning: 45 dph birds will learn readily from a tutor, 60 dph birds are approaching the end of the sensitive period for tutor song learning, and 90 dph birds are near or beyond the end of the sensitive period and are refractory to tutor song learning (Eales, 1985; Immelman, 1969). Thus, in addition to being generally correlated with age, I wondered whether levels of HVC spine turnover in untutored birds were correlated more specifically with sensitive period timing and, therefore, the capacity to learn from a tutor. To address this relationship in individual birds, I focused on the group of 60 dph untutored birds, because they exhibited high bird-to-bird variability in levels of HVC spine dynamics. Fourteen of these birds were exposed to either a live tutor or to an operant tutoring paradigm for 3 days (see Appendix B), and song learning in these birds was then tracked into adulthood. In particular, I predicted that the 60 dph untutored birds with high levels of turnover (i.e., a more plastic HVC; referred to as HT birds) would learn more from the tutor than 60 dph untutored birds that exhibited low levels of turnover prior to tutoring (referred to as LT birds).

As expected, the ability of 60 dph untutored birds to learn from a tutor was variable; some of the birds learned well from the tutor (with a maximum of a 37.8% increase in similarity to the tutor song), while others did not show a substantial increase in similarity to the tutor song (Figure 5A). Strikingly, these birds' initial levels of HVC

spine turnover were predictive of their subsequent capacity for imitative learning (Figure 5B), with birds that had higher pre-tutoring levels of HVC spine turnover exhibiting a significantly larger capacity for tutor song imitation ($p = 0.02$, $R^2 = 0.40$). These findings indicate that levels of spine dynamics in HVC neurons important to vocal learning and control correlate with sensitive period timing in untutored birds.

I have so far shown that isolation from tutor song experience affects levels of HVC spine turnover in an age-dependent manner, and in fact, levels of HVC spine turnover predict the capacity to learn from a tutor song. Notably, 60 dph untutored birds that do not learn well from a tutor and 90 dph untutored birds, which are refractory to tutor song learning, exhibit low levels of HVC spine turnover. These findings indicate that synaptic inputs to HVC PNs stabilize as the sensitive period for tutor song learning closes, even in the absence of tutor song experience. Interestingly, 45 dph normally-tutored birds also exhibited low levels of spine turnover, despite the fact that birds at this age have not passed the end of the sensitive period and are still capable of learning from a new tutor (Yazaki-Sugiyama and Mooney, 2004). Taken together, these findings raise the possibility that tutor song experience, like closure of the sensitive period, may drive the stabilization of HVC dendritic spines.
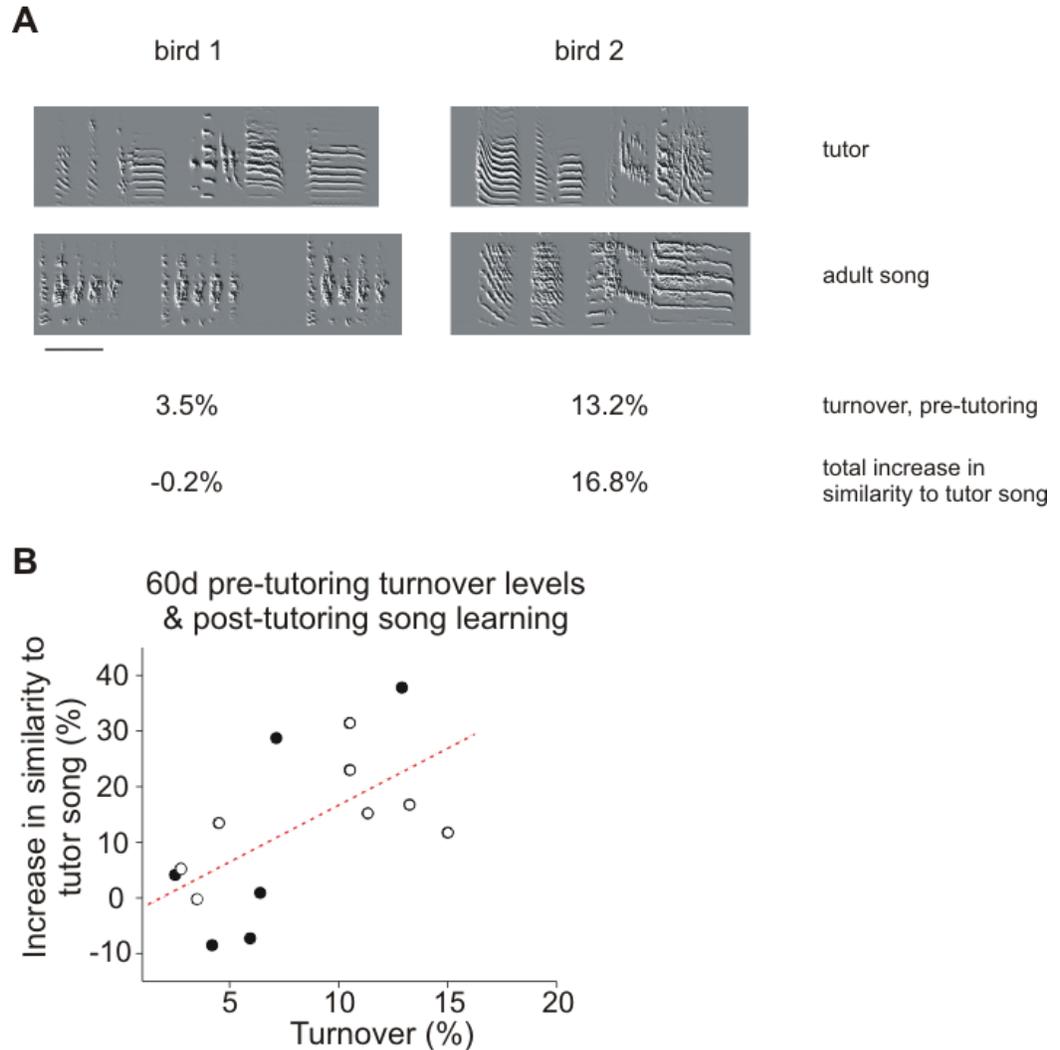
Figure 5: Levels of HVC dendritic spine turnover predict the capacity to imitate a tutor. A) Examples showing results of delayed tutoring initiated at 60 dph and lasting for 3 days in two birds. Top: sonograms of the tutor song and the adult song of the tutored bird (frequency range, 0.6- 9.0 kHz; scale bar, 150 ms). Bird 1 had initially low levels of HVC spine turnover and did not learn well from the tutor. Bird 2 initially had high levels of HVC spine turnover, and its song increased in similarity to the tutor song. B) Relation between levels of HVC dendritic spine turnover measured the night prior to the first exposure to a tutor song and the increase in similarity to the tutor song over song development (p = 0.02, R^2 = 0.40, 1,419 spines from 17 cells in 14 birds). Each circle represents a single bird. The eight empty circles correspond to birds in which post-tutoring dendritic spine turnover measurements were made.

2.2 Tutor song experience rapidly triggers the stabilization, accumulation, and enlargement of dendritic spines on HVC neurons

To test the idea that auditory experience of a tutor song stabilizes HVC dendritic spines, I tracked the effects of tutor song experience on spine turnover in a subset of the 60 dph untutored birds (N = 8). Five of the 8 birds had high pre-tutoring levels of spine turnover (HT birds, turnover = 12.2% +/- 0.86%) and the remaining 3 birds had low pre-tutoring levels of spine turnover (LT birds, turnover = 3.2 +/- 0.25%). Measurements of dendritic spines were made the night before tutor experience (0h) and the night after the first tutor experience (24h). I found that tutor song exposure rapidly and dramatically decreased levels of HVC spine turnover in all 5 HT birds (Figure 6; p < 0.01, 12.2 +/- 0.86% pre-tutoring compared to 7.0 +/- 1.2% post-tutoring, 468 spines). In contrast to the HT birds, LT birds showed no significant change in spine turnover (p = 0.3). Thus, in untutored birds with high levels of HVC spine turnover, tutor song experience results in a structural correlate of strengthening of synaptic inputs onto HVC PNs.

In addition to stabilization of dendritic spines, two additional structural hallmarks of synaptic strengthening are increased density and size of dendritic spines (De Roo et al., 2008; Engert and Bonhoeffer, 1999; Hofer et al., 2009; Holtmaat et al., 2005; Kopec et al., 2006; Maletic-Savatic et al., 1999; Matsuzaki et al., 2004). Following tutoring, HT birds exhibited an increase in spine density within 24h of the initial tutor song experience (Figure 7; p = 0.03, 14.0 +/- 4.4% increase in spine density, N = 4 birds). In contrast, LT birds showed no significant change in spine density (p = 0.5) following

tutor song exposure (Figure 7A). These findings are consistent with the idea that

tutoring causes the rapid stabilization and accumulation of dendritic spines on HVC

PNs.

To test whether tutor song experience affected the size of stable spines,

individual spines were tracked pre-tutoring (0h) to post-tutoring (24h), and relative

brightness was used to estimate spine size, since this measurement is monotonically

related to spine volume (Holtmaat et al., 2005). A size index was calculated for each

measured spine (time 24 size/time 0 size), with values greater than 1 indicating an

increase in spine size and values less than 1 indicating a decrease in spine size. The size

of stable spines increased an average of 28% following tutoring in HT birds but did not

change significantly in LT birds (Figure 8A-C; HT birds: $p = 0.0001$, 1.08 +/- 0.14%

compared to 1.39 +/- 0.16%, N = 47 spines, Wilcoxon-Signed rank test for paired samples;

LT birds: $p = 0.4$, 1.68 +/- 0.2% compared to 1.61 +/- 0.2%, n = 34 spines). Additionally,

prior to tutoring, stable spines were 52% smaller in HT birds than in LT birds ($p = 0.02$),

and this difference was abolished after the first tutoring session (Figure 8B; $p = 0.3$).

Thus, in birds that will subsequently learn a tutor song, tutor song experience causes the

stabilization, accumulation, and enlargement of dendritic spines in HVC. Importantly,

HT birds learned more from tutor song exposure than LT birds (HT birds: 19.6 +/- 3.5%

increase in similarity to the tutor song over development; LT birds: 6.2% +/- 4.0%

increase). Thus, instructive tutor song experience that triggers behavioral imitation also

causes the stabilization, accumulation, and enlargement of excitatory synaptic inputs on HVC neurons important to singing and song learning.

One alternative explanation is that the observed changes in HVC dendritic spines are driven by social experience with the tutor, as opposed to auditory experience of the tutor song. Three lines of evidence suggest that this is not the case. First, none of the 60 dph untutored birds had previous social experience with an adult male, and yet only HT 60 dph untutored birds exhibited changes to dendritic spines following tutoring. Second, three HT birds were tutored using an operant tutoring paradigm in which playback of a pre-recorded tutor song is triggered via the pecking of a key, a method that does not involve social interaction with a tutor. Tutor exposure still decreased spine turnover in these 3 birds (pre-tutoring turnover = 12.9 +/- 1.3% compared to post-tutoring turnover = 7.7 +/- 2.0%, 220 spines), demonstrating that effects of tutor song on HVC dendritic spines can occur independently of social experience with a live tutor. Third, in a single case, an untutored juvenile was introduced to a live tutor that did not sing for the first two days of the experiment. Spine turnover in the juvenile did not decrease until after the tutor began to sing on day 3 (Figure 9; turnover was 11.4% on night 1, 6.8% on night 2, and 3.5% on night 3). These findings suggest that changes in spine turnover are linked to auditory experience of the tutor song and not to social experience with the tutor.
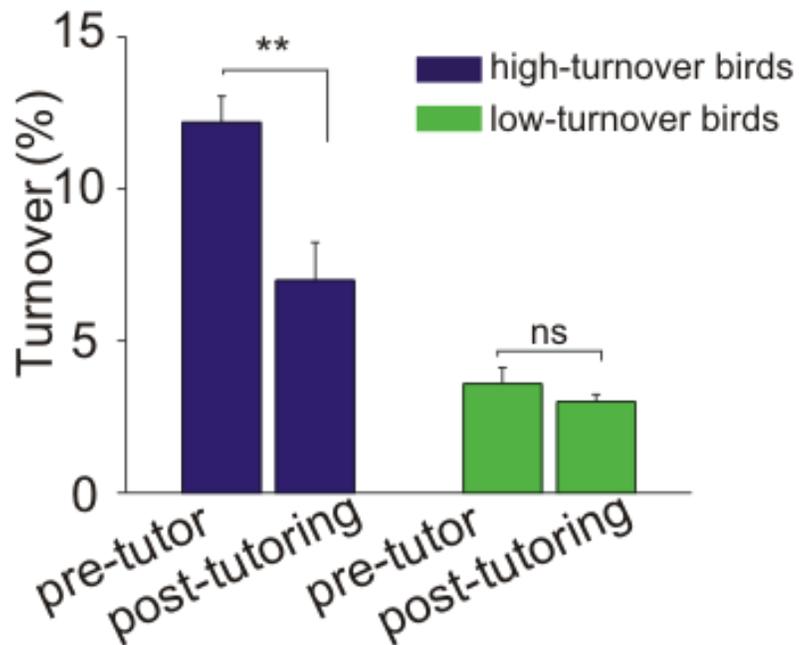
Figure 6: Tutor song experience triggers stabilization of dendritic spines on HVC PNs. Levels of HVC dendritic spine turnover measured the night before and the night after the first day of tutoring in HT and LT birds (HT birds: $p < 0.01$, from 12.2 +/- 0.8% to 7.0 +/- 1.2%, pre and post-tutoring turnover, respectively, 468 spines from 5 birds; LT birds: $p = 0.3$, spine turnover = from 3.6 +/- 0.5% to 3.0 +/- 0.2%, pre and post-tutoring, respectively, 449 spines from 3 birds).
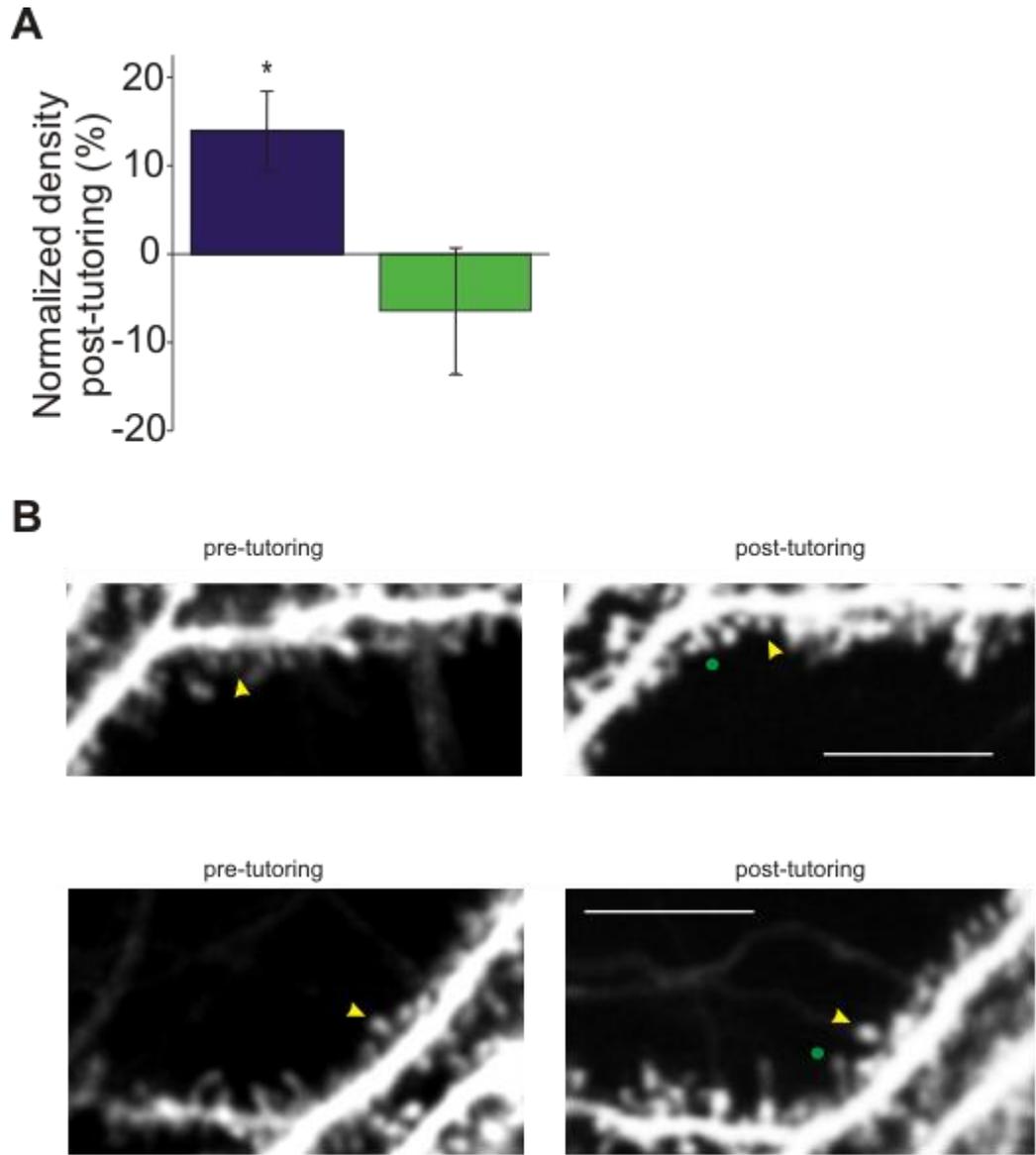
Figure 7: Tutor song experience triggers accumulation of dendritic spines on HVC PNs. A) Percent change in dendritic spine density by the first night following tutoring (post-tutoring density/pre-tutoring density; HT birds: p = 0.03, 14.0 +/-4.4 % increase in spine density, N = 4 birds; LT birds: p = 0.5, -6.4 +/- 7.0% increase in spine density, N = 3 birds). B) Examples of two neurons from HT birds that underwent increases in spine density following tutoring. New spines are indicated by green dots, and yellow arrowheads indicate stable spines, for reference. Scale bar, 10 μm.
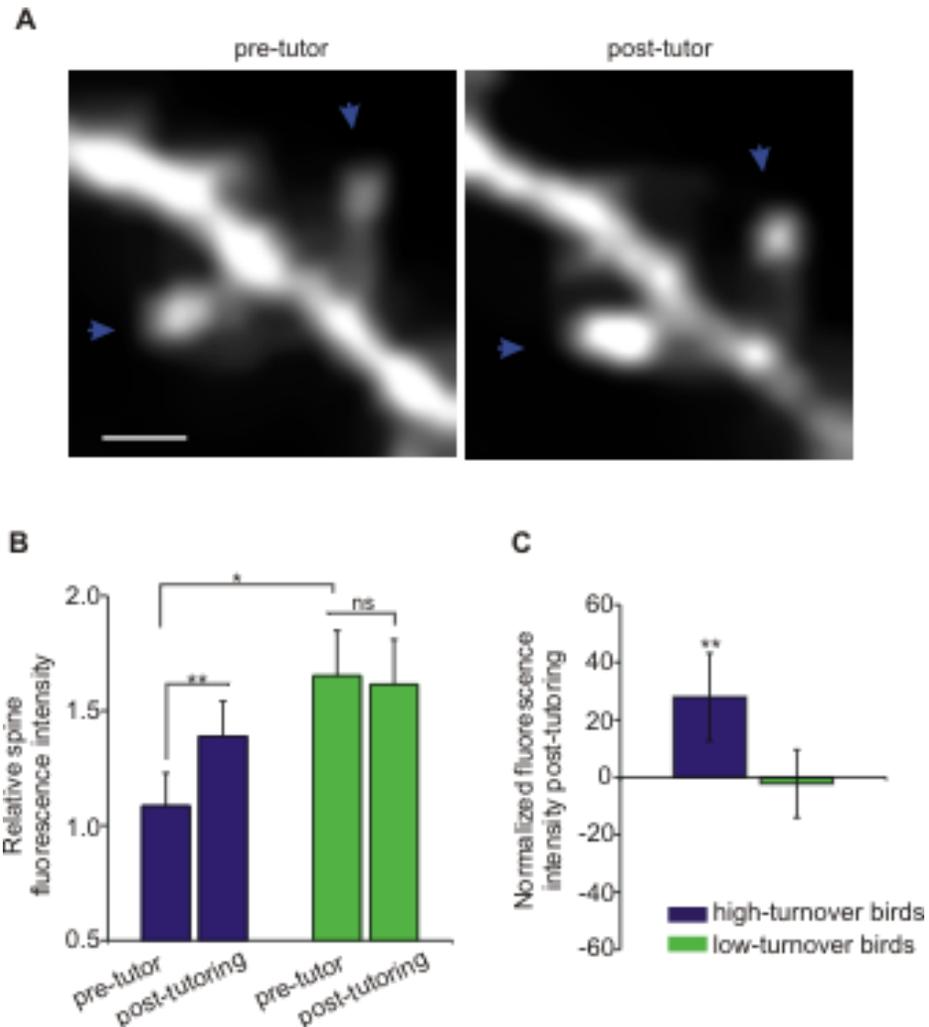
Figure 8: Tutor song experience triggers enlargement of stable dendritic spines on HVC PNs. A) Example of two stable spines that exhibited increased fluorescence intensity following tutoring, indicating an increase in dendritic spine volume. Scale bar, 2 μm. B) The size of stable spines increased in HT (blue) but not LT (green) birds by the first night following tutoring (81 spines, 8 birds; HT birds: ** p = 0.001, N = 47 spines, Wilcoxon signed-rank test for paired samples; LT birds: p = 0.4, N = 34 spines). Moreover, HT birds had smaller stable dendritic spines before tutor exposure (* p = 0.02), but this difference is abolished by the first night post-tutoring. C) HT, but not LT, birds show increases in the size of stable spines by the first night following tutoring (HT birds: ** p = 0.001; LT birds: p = 0.4).
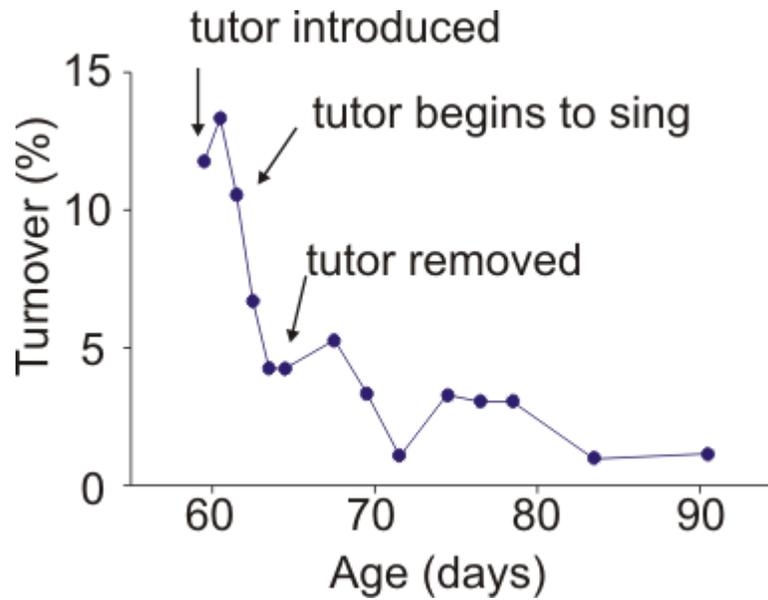
Figure 9: Stabilization of HVC dendritic spines following tutor experience cannot be attributed to social experience with the tutor. Changes in spine turnover in an isolate bird subjected to delayed tutoring do not occur until the tutor begins to sing (day 3), showing that structural changes are not due to social experience with the tutor.

How do the effects of tutor song experience on levels of HVC spine dynamics compare to the changes that occur over the remainder of sensorimotor learning? In a single juvenile bird (Figure 9), I was able to track dendritic spines on the same HVC neuron out until 90 dph, near the end of sensorimotor learning. Surprisingly, the effects of tutor song experience on levels of HVC spine turnover accounted for almost 70% of the change in turnover measured across almost a month of sensorimotor learning. These data reiterate my previous observation that levels of spine turnover are not significantly different between 45-90 dph normally-tutored birds (Figure 4) and support the idea that the effects of tutor song experience account for the majority of changes in levels of spine turnover in HVC that occur over course of song learning.

While these findings suggest that experience with the tutor can drive stabilization of HVC dendritic spines, it remains unclear whether these changes are due to auditory experience of the tutor song (i.e., sensory-driven) or, alternatively, are associated with initial attempts at song imitation (i.e., sensorimotor-driven). To address this issue, I analyzed songs from juveniles following the first tutoring session to determine whether changes in vocal output occurred prior to the first post-tutoring imaging session. I assessed syllable entropy variance (EV), a measure that reflects transitions from noisy to structured song elements and which has been previously described as an early indicator of tutor song imitation (Tchernichovski et al., 2000; Derégnaucourt et al., 2005). Changes in syllable EV could be detected by the afternoon

of the first tutoring day in HT but not LT birds (Figure 10; Post-hoc Tukey test, alpha =

0.05, 3 of 4 HT birds), prior to the first post-tutoring imaging session. Thus, because

tutor experience affected vocal output so rapidly, the design of this experiment does not

allow me to distinguish whether the observed changes in HVC dendritic spines are

dependent on vocal rehearsal or can be driven by auditory experience of the tutor song

alone (but see Section 2.3).

In summary, I have observed three structural hallmarks of synaptic

strengthening—increased spine stability, increased spine density, and increased size of

stable spines—following tutor song experience that results in imitative learning. These

changes are driven by auditory experience of the tutor song and not simply by social

interactions with the tutor, suggesting that tutor song experience acts via HVC to

influence the downstream song sensorimotor network and, ultimately, song output.
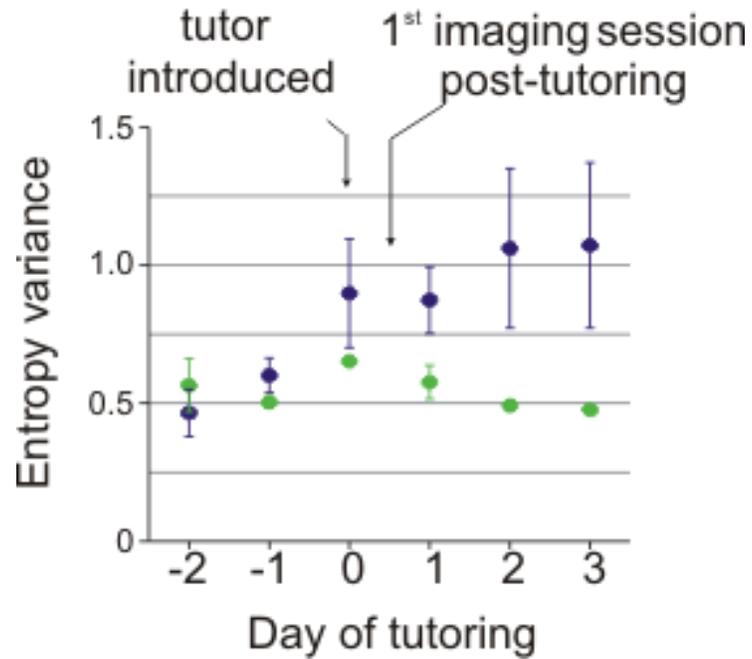
Figure 10: Entropy variance of vocal output in HT birds increases during the first day of tutor exposure. Mean afternoon EV scored for birds with high (blue) and low (green) pre-tutoring turnover levels reveal an increase in HT bird EV by the afternoon of the first day of tutoring.

2.3 Tutor song experience rapidly triggers functional strengthening of synaptic inputs onto HVC neurons

A remaining question is whether the observed structural changes to dendritic spines on HVC PNs are accompanied by functional changes in the strength of excitatory synaptic inputs. In a separate set of experiments carried out by Dr. Todd Roberts, electrophysiological measurements of synaptic strength were made before and after tutoring to test whether functional changes could be detected that paralleled the structural changes observed in the imaging experiments. To maximize the likelihood that HVC dendritic spines of all birds were in a high turnover state, experiments were conducted in ~45 dph juveniles previously raised without a tutor. By adapting the windowing methods used for in vivo imaging, sharp intracellular recordings were carried out in anesthetized birds from electrophysiologically-identified PNs in the same small region of HVC one night before and one night after a juvenile bird's initial exposure to a tutor.

Indeed, tutoring rapidly enhanced synaptic activity in HVC, consistent with the idea that structural changes to spines elicited by tutoring are associated with functional changes to synapses. A comparison of spontaneous synaptic activity recorded before and after the first day of tutoring revealed a marked increase in the amplitude of depolarizing synaptic activity (Figure 11A-B; 24 cells in 3 birds, $p < 0.00001$, KS test) and the emergence of prolonged (~1 sec) bursts of synaptic activity. These functional

changes were not paralleled by changes in resting membrane potential ($p = 0.6$) or action

potential firing rate ($p = 0.3$). Therefore, the synaptic enhancement was unlikely to arise

from increased driving force on synaptic currents or increased firing of HVC PNs, which

are a major source of synaptic input onto other HVC PNs. Rather, the functional and

structural changes observed in HVC following tutoring are suggestive of rapid synaptic

strengthening, although another mechanism that cannot be excluded is increased

excitability of neurons afferent to HVC. Additionally, the cumulative distributions of

spontaneous synaptic events recorded in HVC the night following the first tutoring

session were similar to those from age-matched juveniles raised with access to a tutor

(Figure 11B; $p = 0.1$), consistent with the idea that tutoring results in a rapid

physiological strengthening of initially weak synaptic inputs onto HVC PNs. Notably,

tutor song experience drove functional enhancements of spontaneous synaptic activity

in both HVC PN types, indicating that tutor song experience rapidly influences HVC

neurons that provide input to the SMP and the AFP (data not shown). Finally, the rapid

enhancement of synaptic activity was even detected in a juvenile that failed to sing

during its first tutoring session (data not shown, $p < 0.00001$), suggesting that it could

occur in the absence of vocal rehearsal and its associated auditory feedback.
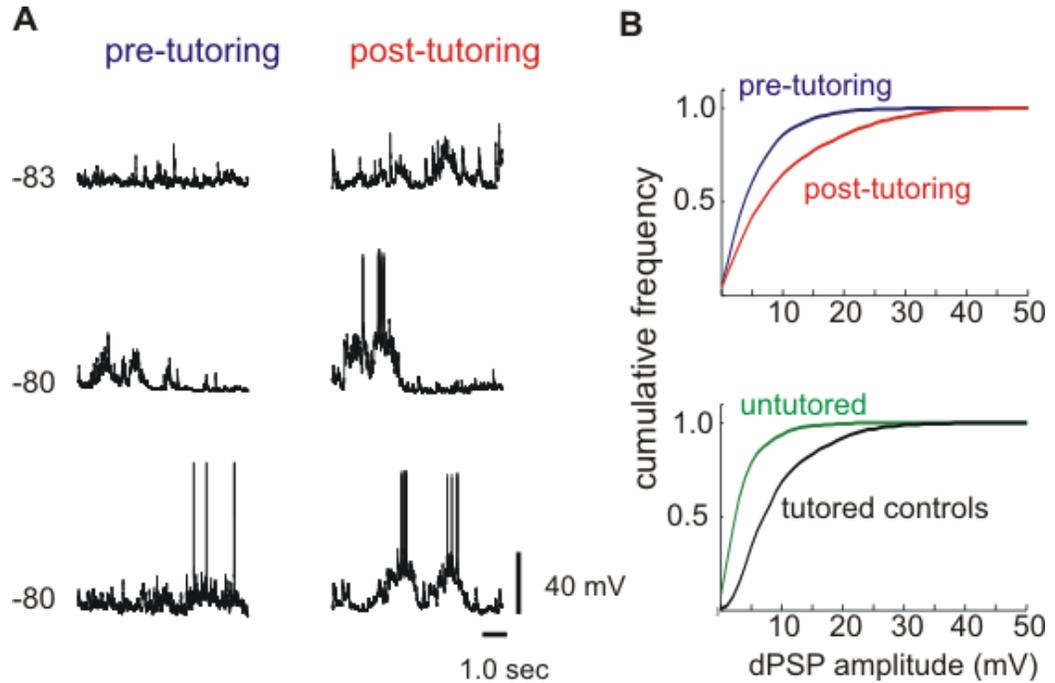
Figure 11: Tutor song experience triggers enhancement of spontaneous synaptic activity in HVC. A) In vivo intracellular membrane potential recordings made from six different HVC neurons in a juvenile bird one night before (left) and one night after (right) initial tutor song exposure show that tutoring drives a rapid increase in the amplitude of spontaneous depolarizing synaptic activity. B) Top: Cumulative frequency distributions of spontaneous synaptic activity showing the amplitude of depolarizing synaptic events recorded intracellularly in HVC neurons immediately before (blue) and after (red) tutoring ($p < 0.001$). Data collected from 24 cells in 3 birds. Bottom: Cumulative frequency distribution of spontaneous synaptic activity showing the amplitude of depolarizing synaptic events recorded intracellularly in HVC neurons on two consecutive days from a 45 dph untutored bird (green, 5 cells on each night) and a normally-tutored 45 dph bird (black, 2 cells on each night).

2.4 Chapter 2 conclusions

I have demonstrated that isolation from tutor song experience can affect the structural dynamics of HVC dendritic spines, and further, that pre-tutoring levels of HVC structural dynamics predict the capacity for tutor song learning. These findings indicate that synaptic stability within the song sensorimotor network correlates with the timing of the sensitive period for tutor song learning. Subsequent tutor song experience that results in imitative learning causes rapid stabilization, accumulation, and enlargement of dendritic spines on HVC PNs. Additionally, these structural changes are associated with functional enhancement of spontaneous synaptic activity, suggesting that tutor song experience causes rapid and dramatic strengthening of synaptic inputs onto HVC neurons. The location of HVC at the sensorimotor interface makes it likely that these observed structural and functional changes to synaptic inputs have direct consequences for how HVC translates auditory and motor-related activity into song. In addition, the rapidity of these tutoring-induced changes suggests that tutor song information is being translated rapidly (< 24h) into sensorimotor coordinates and raises the intriguing possibility that the auditory memory of the tutor song may even be stored, at least partially, within the HVC network. Overall, these findings suggest that experience acts in the juvenile brain to rapidly stabilize and strengthen a structurally dynamic sensorimotor network, providing a foundation for learning new behaviors.

3. Watching the brain unlearn: effects of deafening on structural dynamics of HVC dendritic spines

How does sensory feedback act on sensorimotor brain circuits that control the learning and maintenance of motor skills? In particular, it remains poorly understood how the loss of auditory feedback causes learned skills, such as songbird vocalizations, to degrade. In this second set of experiments, I carried out in vivo imaging of dendritic spines on HVC PNs before and after deafening to test the idea that synaptic inputs to HVC PNs are sensitive to perturbation of auditory feedback. Specifically, I hypothesized that auditory feedback acts via HVC_X neurons to drive changes in vocal output and, therefore, that deafening would cause changes in the strength and stability of dendritic spines on this cell type that preceded the onset of deafening-induced changes in vocal output.

3.1 Deafening causes a cell type-specific decrease in the size of stable spines in HVC_X neurons

To label and identify HVC projection neurons for in vivo imaging, a GFP-lentivirus was injected into HVC, and retrograde tracers were injected into the two downstream targets of HVC, Area X and RA, in adult male zebra finches (80 to 150 dph). Birds were maintained on a reversed day-night cycle, and imaging sessions were conducted (2 sessions per night over a 2h interval) during the birds' subjective nighttime, to minimize interference with singing behavior. Images were obtained through a small cranial window and collection of imaging data was restricted to neurons

with dendritic spines, because both populations of HVC PNs are spinous (Mooney, 2000). Neurons were identified as either HVC_X or HVC_{RA} cells by the presence of retrograde label or, in the absence of retrograde label, by the measurement of soma size, which differed significantly for the two PN types (Figure 12). After collecting 1-2 nights of baseline imaging data, birds were deafened by bilateral removal of the cochleae, and data collection was continued for as long as possible (13 birds were imaged for an average of 7.2 +/- 4.1 nights post-deafening).
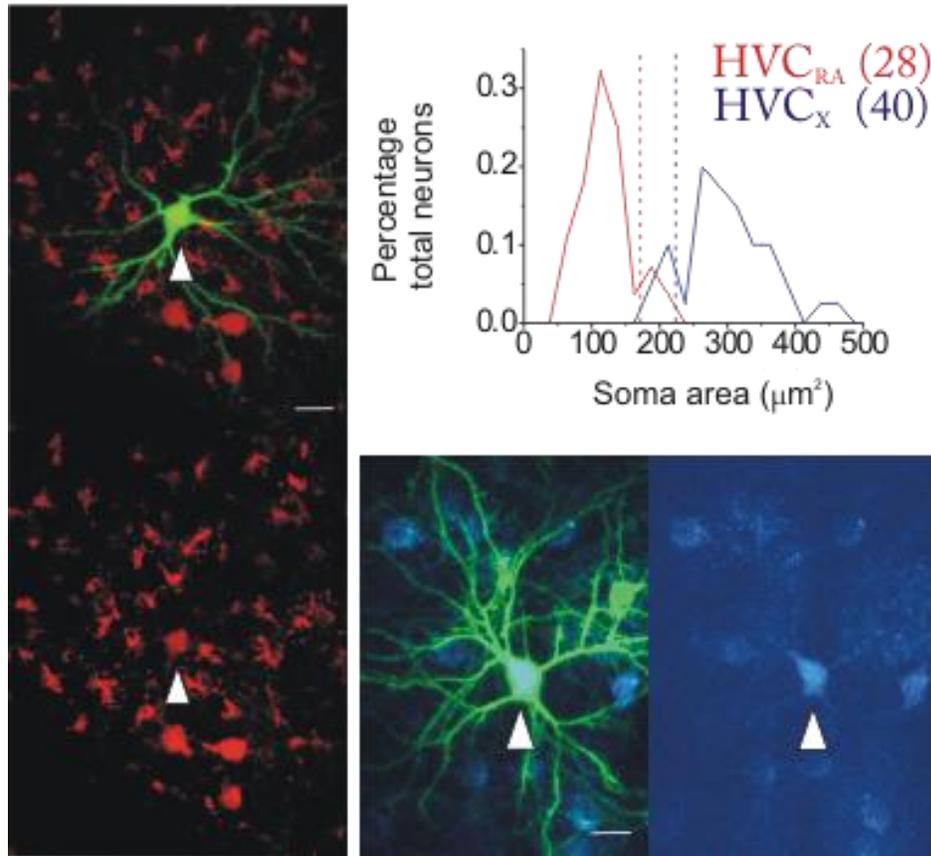
Figure 12: Classification of HVC PN types. Images show HVC PNs that are double-labeled with GFP and retrograde tracers (red, HVC_RA neuron; blue, HVC_X neuron, scale bars, 20 μm). Upper right panel shows histogram of soma area measurements for HVC_X and HVC_RA neurons. Soma measurements were made for each cell from a single plane of a z-stack of images, in which the largest amount of the soma was in focus. Any neuron with a soma area greater than 222 μm² (mean HVC_RA soma area plus two standard deviations; blue dashed line) was categorized as an HVC_X neuron, and any neuron with a soma area less than 173 μm² (mean HVC_X soma area minus two standard deviations; red dashed line) was categorized as an HVC_RA neuron.

An initial assessment was made of the effects of deafening on the size of stable spines in HVC PNs, because changes in spine size have been shown to correlate with functional changes in synaptic strength (Kopec et al., 2006; Matsuzaki et al., 2004; Okamoto et al., 2004; Zhou et al., 2004), and I previously observed that the size of dendritic spines on HVC PNs is sensitive to sensory experience important to imitative learning (Roberts et al., 2010; Chapter 2). Stable spines were defined as those that were present across two nights of imaging (over a 24h interval), and a size index was calculated for each measured spine (time 24 size/time 0 size), with values greater than 1 indicating an increase in size and values less than 1 indicating a decrease in size. Prior to deafening, stable spines in HVC_{RA} neurons tended not to change in size over 24h periods, while stable spines in HVC_X neurons tended to increase slightly in size (Figure 13, size index = 1.00 +/- 0.02 for HVC_{RA} neurons: 94 spines from 9 cells in 8 birds; size index = 1.07 +/- 0.03 for HVC_X neurons: 106 spines from 10 cells in 9 birds; p = 0.05 for difference between cell types, Mann Whitney U test). Interestingly, comparing spines in a subset of these cells over an initial 24h window and during the last 24h time window following deafening revealed that spine size index decreased significantly following deafening in HVC_X neurons but not in HVC_{RA} neurons (Figure 13; HVC_X: 152 spines from 7 cells in 6 birds, p = 0.03; HVC_{RA}: 146 spines from 8 cells in 6 birds, p = 0.67, Wilcoxon ranked-signs tests). Thus, deafening causes a cell type-specific decrease in the size of stable spines on HVC_X neurons.
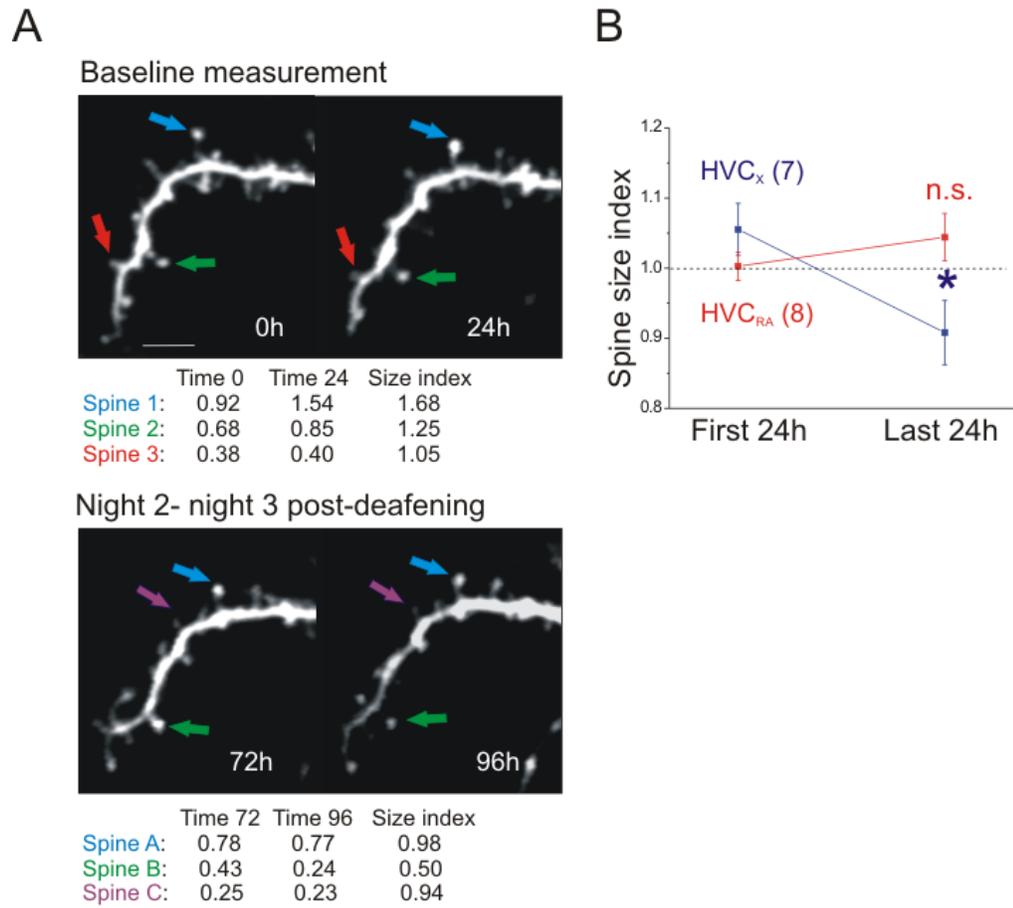
Figure 13: Spine size index decreases following deafening in HVC$_X$ neurons but not in HVC$_{RA}$ neurons. A) Images showing measurements of spine size for an HVC$_X$ neuron before and after deafening. The size of stable spines was measured (see Appendix B) across 24h intervals, and a size index for each spine was calculated (time 24 size/time 0 size). Prior to deafening, stable spines tend to increase in size (size index > 1), while following deafening, stable spines tend to decrease in size (size index < 1). B) The first spine size index measurement for each cell (baseline) was compared to the last measurement made from the same cell following deafening, and a significant decrease over time in average spine size index was observed for HVC$_X$ neurons (p = 0.03) but not for HVC$_{RA}$ neurons (p = 0.67).

3.2 Deafening causes subtle but significant degradation of spectral features of song with 1-4 days

If HVC$_X$ neurons are sensitive to changes in auditory feedback, then changes in to dendritic spines on these neurons should precede the onset of deafening-induced vocal change. Establishing when these structural changes occur relative to deafening-induced song degradation depends on detecting initially subtle changes in song features following deafening. To this end, I analyzed two spectral features, Wiener entropy and entropy variance (EV), of each syllable in a bird's song over time (see Appendix B). These parameters respectively measure the uniformity of a sound's power spectrum and intra-syllabic transitions from tonal to broadband sounds (Tchernichovski et al., 2000) and were chosen because they remain stable in normal adults and change in predictable directions following deafening (Figure 14). Using this analysis, subtle but significant effects of deafening on syllable spectral features were detected in the majority of birds (18/19) within the first four days of post-deafening song, with ~ 50% of birds (10/19) showing significant degradation over the first day of post-deafening song (Figure 15). Notably, the changes I detected occur days to weeks earlier than those reported in previous studies (Brainard and Doupe, 2000; Horita et al., 2008; Lombardino and Nottebohm, 2000; Nordeen and Nordeen, 1992), suggesting that the analysis method used here is extremely sensitive to early deafening-induced changes to song. The number of days to the onset of this subtle degradation was not predicted by the age of the bird (Figure 15C), although older birds sang a larger number of motifs before their

songs degraded (Figure 15D, see Appendix B). Overall, syllable entropy tended to be slightly more sensitive to deafening than EV (40 syllables scored from 18 birds; 45% of syllables showed earliest changes in entropy, 33% showed earliest EV changes, and 22% changed in entropy and EV on the same day).

The effects of deafening on syllable sequencing were also assessed, by analyzing sequence consistency (adapted from Scharff and Nottebohm, 1991). Briefly, the most common ordering of syllables within a motif were defined (ABC, for example), typical syllable transitions were then defined (start to A, A to B, B to C, C to end), and the effects of deafening on the average percentage of typical transitions per motif were assessed. In contrast to the early effects of deafening on spectral features of song, the effects of deafening on the sequencing of syllables were slower and much more variable across birds. Notably, in all of the birds examined, deafening caused earlier changes to syllable spectral features than to syllable sequencing. This finding is in agreement with previous studies indicating that song spectral features are more sensitive to feedback perturbation than syllable sequencing (Lombardino and Nottebohm, 2000; Horita et al., 2008) and thus serve as the most reliable early marker of deafening-induced song degradation.
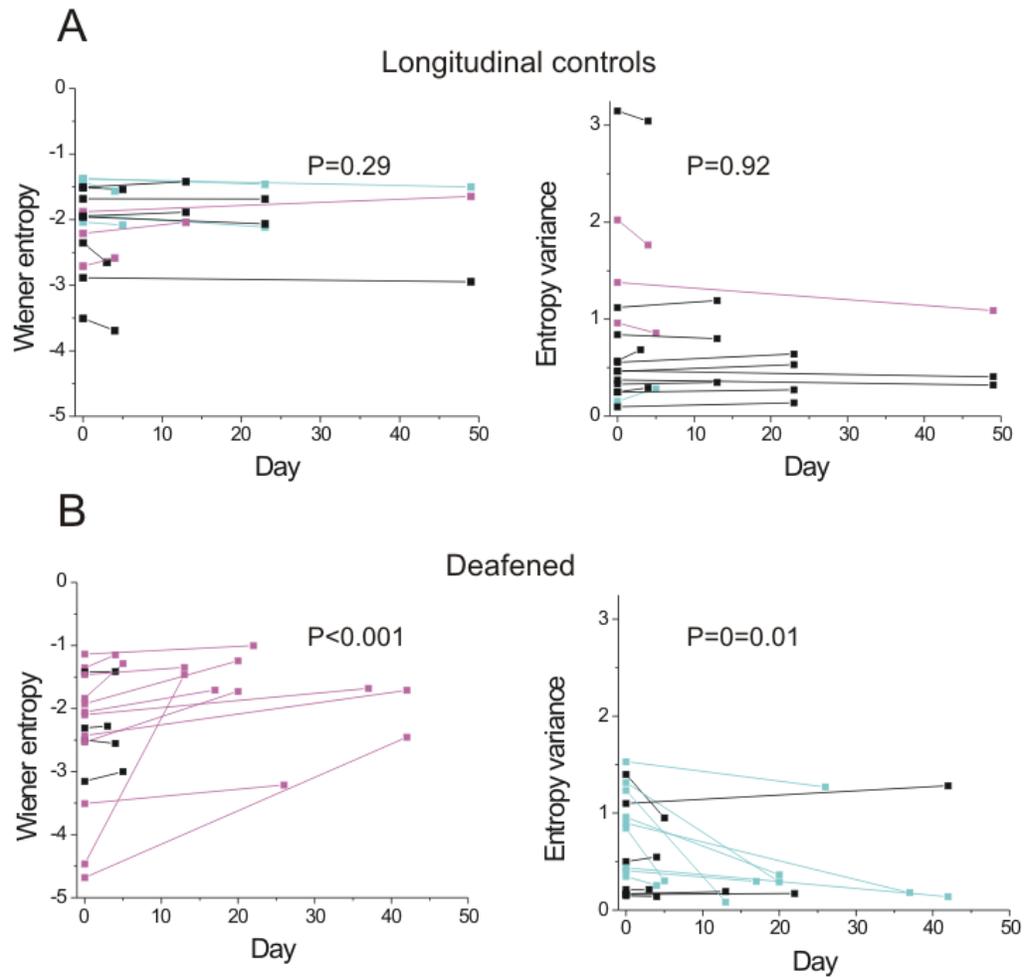
Figure 14: Syllable entropy and entropy variance remain stable over time in adult birds and change in predictable directions following deafening. (A) Syllable entropy and EV tend to remain stable over time in adult birds (p values shown for Wilcoxon ranked-signs tests, time 0 vs. last time point measured for all syllables; 16 syllables from 6 adults birds were measured longitudinally for periods ranging from 4-49 days). Black symbols represent individual syllables that did not change significantly over time, pink=significant increase, and cyan= significant decrease. (B) Syllable entropy increases following deafening (p < 0.01), while syllable EV decreases (p = 0.01; 16 syllables, 7 adult birds, 4-42 days).
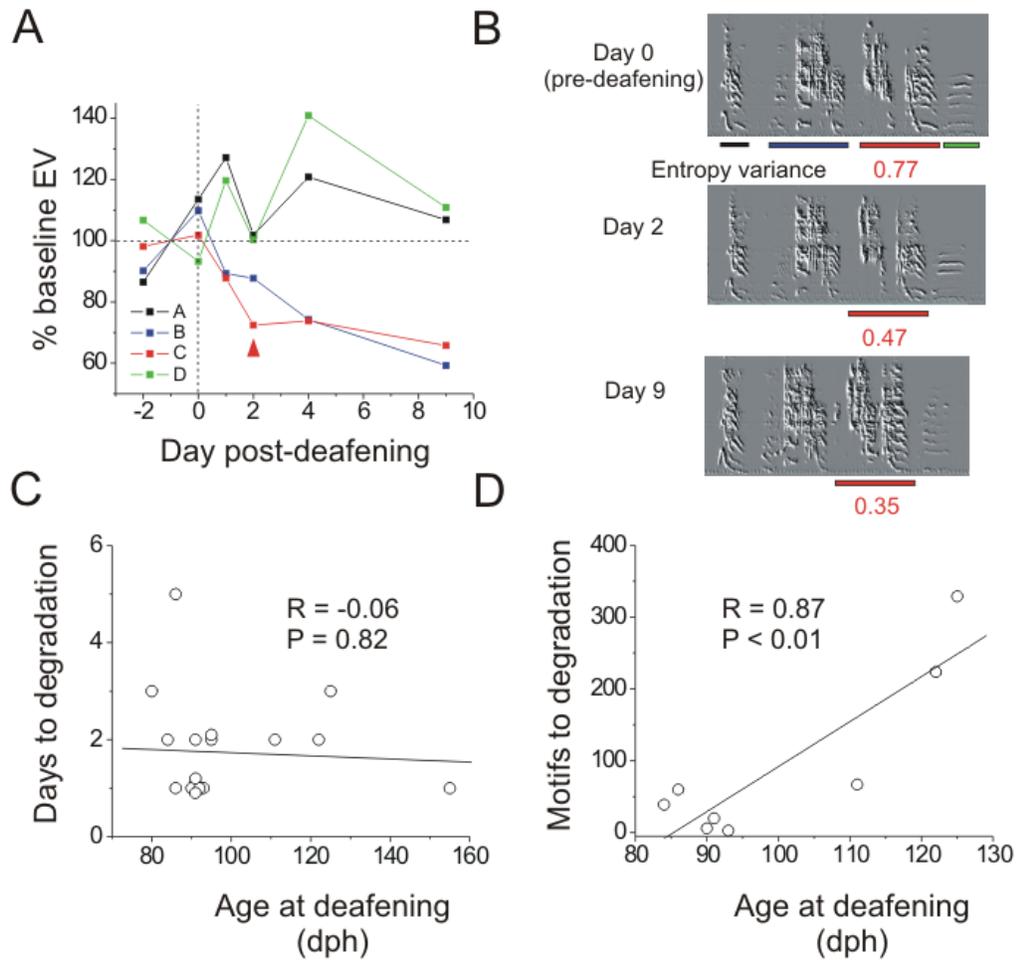
Figure 15: Deafening causes significant degradation of spectral features of song within 1-4 days. A) Example of syllable-by-syllable analysis of EV carried out for a bird whose song contains four syllables, A-D. Song behavior is shown for pre-deafening days (-2 and 0) and post-deafening days 1, 2, 4, and 9. Red arrowhead indicates the first syllable to undergo significant spectral degradation (syllable A, shown in red, defined as onset of degradation). B) Representative spectrograms of the same song analyzed in A. Syllable A (red lines) underwent degradation starting at 2d post-deafening. EV values are included below each rendition of the two syllables. C) The day of the earliest deafening-induced spectral changes to song was not related to the age at which birds were deafened (p = 0.82). D) Older birds sang a larger number of motifs before their songs underwent significant degradation (p < 0.01). These data come from a subset of the birds analyzed in C (9/19).

3.3 Effects of deafening on HVC$_X$ neuron dendritic spines precede the onset of song degradation

The onset of song degradation estimated in this manner was then used to temporally align in vivo imaging data collected from different birds. To facilitate comparison between HVC$_X$ and HVC$_{RA}$ neurons and take into account different pre-deafening values of spine size index, each cell's last pre-deafening size index value was used to normalize its subsequent size index values (Figure 16, top and middle panels), and these normalized size index values were pooled separately for the two cell types (Figure 17B; Figure 16, bottom panel). Interestingly, these pooled comparisons revealed that the spine size index of HVC$_X$ neurons decreased prior to the onset of song degradation, whereas the spine size index of HVC$_{RA}$ neurons did not change before or after songs began to degrade (Figure 17A-B; 495 spines from 7 HVC$_X$ neurons in 6 birds; 428 spines from 8 HVC$_{RA}$ neurons in 6 birds, time > 0 is post-degradation). Although I also attempted to assess whether changes in HVC$_X$ neuron spine size occurred prior to the onset of song degradation on a bird-by-bird basis, size index data from individual neurons were noisy (Figure 16), and decreases in size index were rarely significantly different from baseline for individual cells. In summary, deafening causes a cell type-specific decrease in the size of stable spines of HVC$_X$ neurons that on average precedes the onset of song degradation.
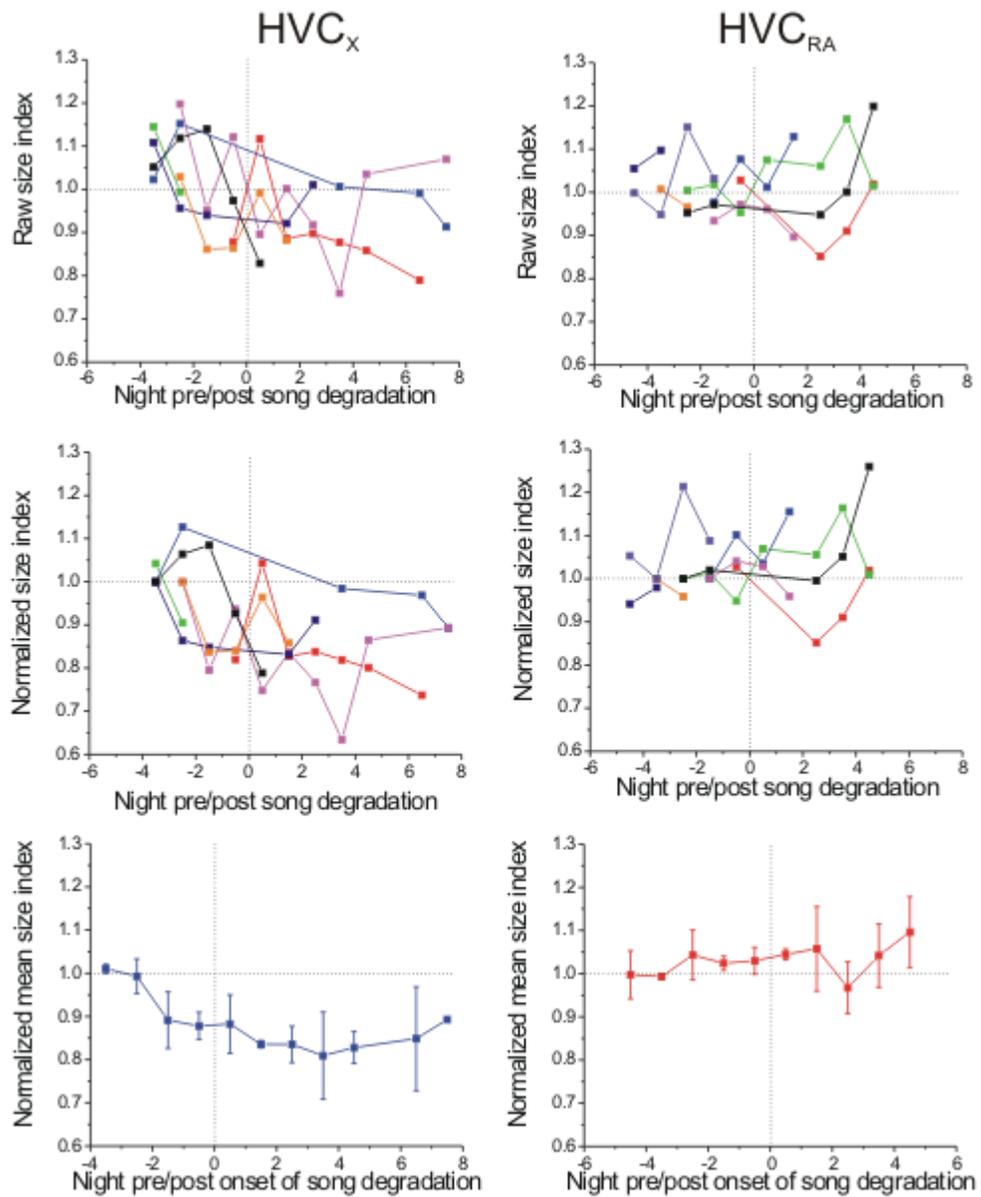
Figure 16: Normalization and pooling of spine size index measurements from individual HVCₓ and HVCRA neurons. Measurements of raw spine size index are shown for all HVCₓ and HVCRA neurons in top panel. Measurements for each cell were normalized to the baseline size index for that cell (middle panel), and the normalized size index measurements were pooled for each PN type (bottom panel) for analysis.
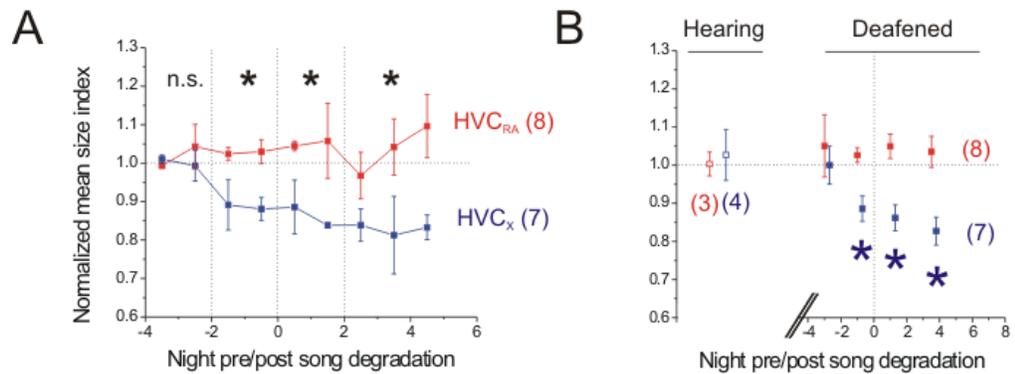
Figure 17: Deafening causes a cell type-specific decrease in the size of stable spines in HVCx neurons prior to the onset of song degradation. A) Deafening causes a cell type-selective decrease in the size index of stable spines in HVCx neurons, and this effect occurs on average prior to the onset of song degradation (time > 0 on the x axis is post-degradation). Data are grouped into 4 time bins (2 bins before onset of song degradation and 2 bins after), the average time of deafening is night -2, and asterisks indicate a significant ($p < 0.05$) difference between the two PN types for a particular time bin. B) Stable spines from HVCx neurons in longitudinally-imaged, age-matched controls do not show a decrease in size index. Data from control neurons are represented as the average of all non-baseline, longitudinal measurements, collapsed across time for each PN type. Asterisks indicate a significant difference for a given PN type between the measurements from deafened birds and hearing controls in a particular time bin.

Various control measurements ensured that decreases in HVC_X neuron spine size were not related to imaging methodology. First, decreases in HVC_X spine size index were not due to effects of longitudinal imaging, because HVC_X neurons from longitudinally-imaged, age-matched hearing birds never underwent a decrease in spine size index (Figure 17C and Figure 18; control HVC_X: 115 spines from 4 cells in 4 birds; control HVC_{RA}: 77 spines from 3 cells in 3 birds). In fact, when HVC_X neurons from deafened birds were compared to HVC_X neurons from longitudinally-imaged control birds, significant post-deafening decreases in spine size index were again detected prior to the onset of song degradation (Figure 17C). Moreover, the same pattern of significant differences was obtained whether normalized or raw spine size index values were used for comparison. Second, decreases in HVC_X size index were unrelated to variable sampling of dendritic branches over time (Figure 19). Additionally, raw spine size decreased in slightly more than half of the individual spines that were tracked for multiple nights following deafening, suggesting that decreases in size index were also unrelated to variable sampling of individual dendritic spines over time (Figure 20). Interestingly, the change in size for individual spines was negatively and significantly correlated with their intial, pre-deafening size, suggesting that deafening may preferentially weaken previously strong excitatory synaptic inputs (Figure 20C, $p = 0.003$, $R = -0.44$). These various measurements are consistent with the idea that deafening selectivly weakens synaptic inputs onto HVC neurons that innervate the AFP.
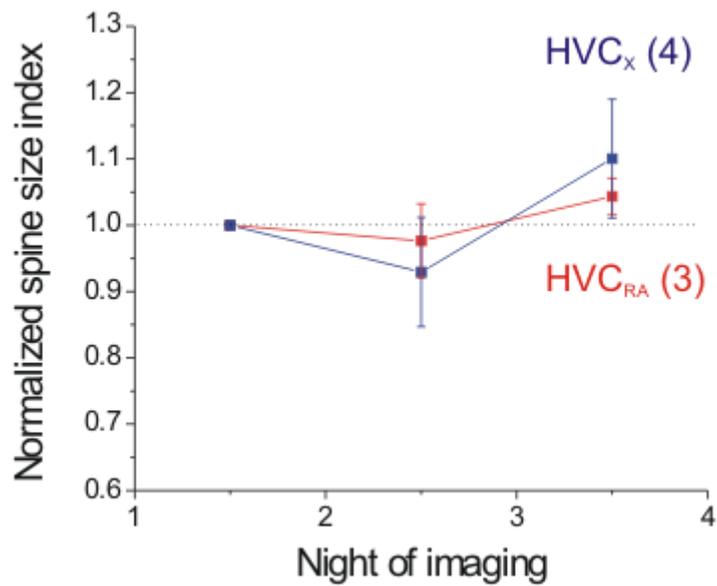
Figure 18: Spine size index does not decrease in longitudinally-imaged HVC PNs from hearing controls. Mean size index measurements for longitudinally-imaged HVC$_X$ and HVC$_{RA}$ neurons in non-deafened control birds do not decrease significantly over time.
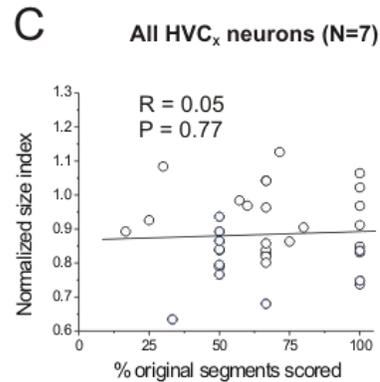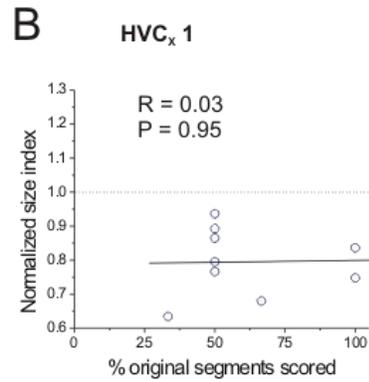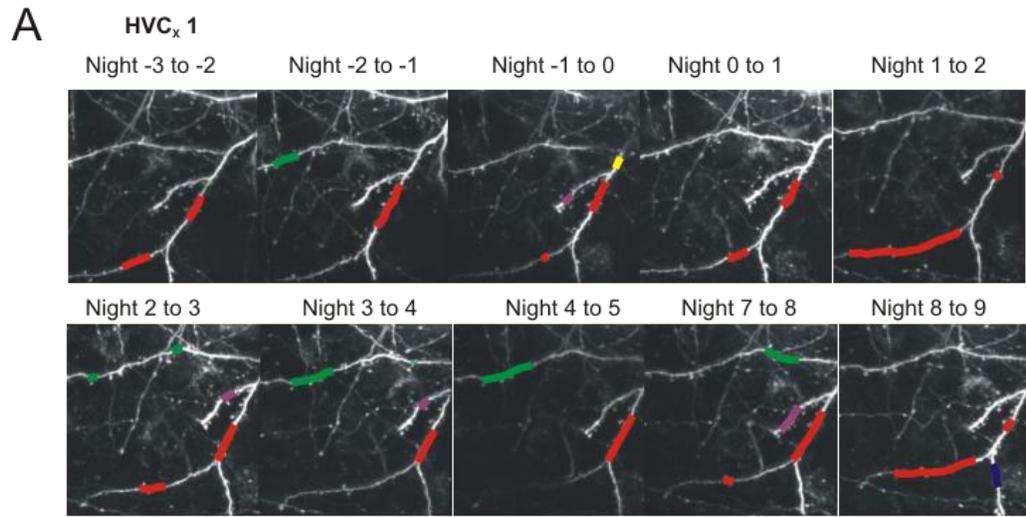
Figure 19: Decreased spine size index in HVCₓ neurons following deafening is unrelated to spatial variability in dendrite sampling over time. A) Images are shown for an HVCₓ neuron whose spine size index was tracked over 13 nights (total of ten 24h comparisons, images shown for first time point of each comparison). Dendritic branch segments containing the spines scored in the baseline 24h comparison (i.e., original segments) are indicated in red, and additional segments scored in subsequent comparisons are shown in other colors. B) For this same cell, decreases in normalized size index values for non-baseline, post-deafening 24h comparisons are not predicted by differences in dendritic segment sampling. C) Same as (B), with data included from all HVCₓ neurons (N=7).

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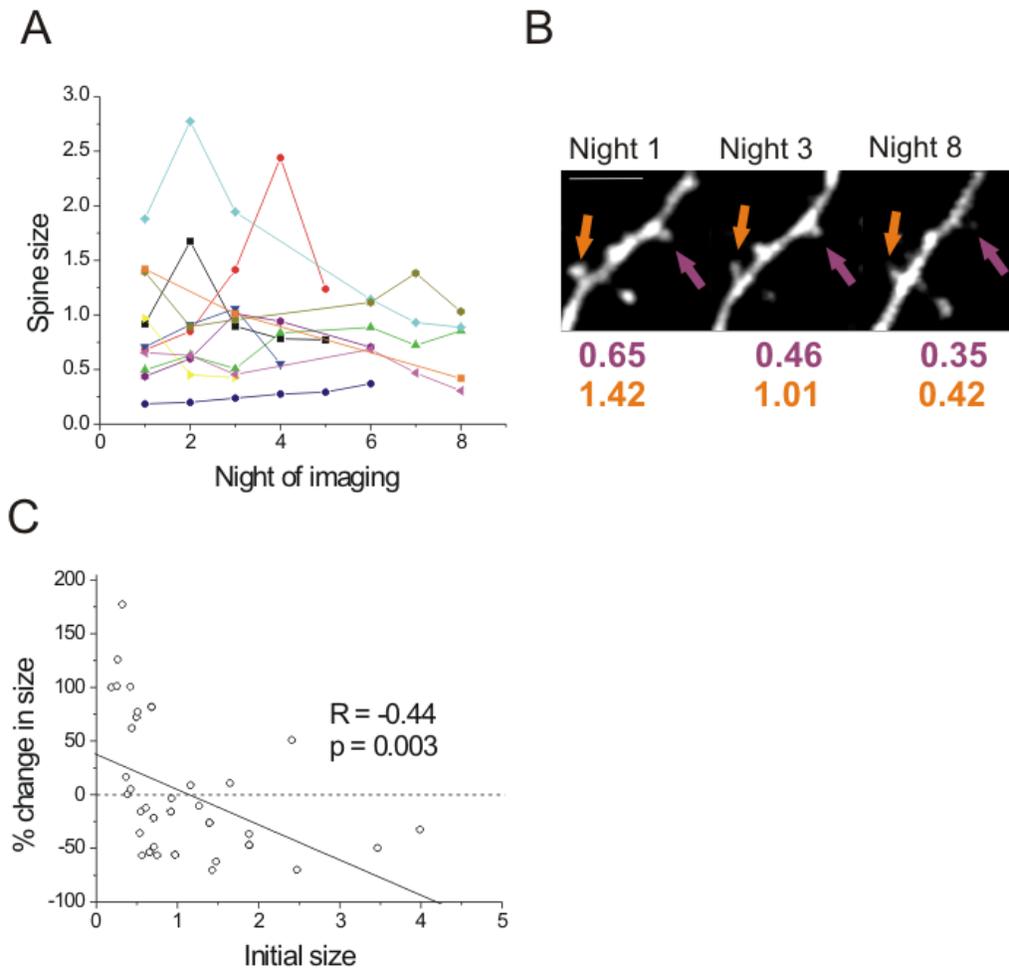
Figure 20: Individual spines from HVCx neurons decrease in size following deafening. A) Size measurements from individual spines from HVCx neurons that were tracked for multiple days. More than half of these (20/35; measurements shown only for a subset) show post-deafening decreases in size, suggesting that decreased size index was unrelated to variable sampling of individual dendritic spines over time. B) Representative images and size measurements from two spines from an HVCx neuron that decreased in size following deafening. Colors for individual spines correspond to those used in A. C) Change in spine size over time is negatively and significantly correlated with initial spine size, indicating that deafening may preferentially decrease the size of large stable spines. Percentage change in spine size was calculated by comparing size on the first night of imaging to size on the last night of imaging for each spine.

3.4 Deafening causes a cell type-specific decrease in spine stability in HVC$_X$ neurons

Because spine stability is another structural correlate of synaptic strength (De Roo et al., 2008; Engert and Bonhoeffer, 1999; Hofer et al., 2009; Maletic-Savatic et al., 1999; Nägerl et al., 2004) and changes in levels of spine stability were previously found to correlate with changes in synaptic strength in HVC following tutor song experience (Roberts et al., 2010, Chapter 2), I next tested the idea that deafening destabilizes spines in HVC. Spine stability was relatively high in both cell types prior to deafening (HVC$_{RA}$: 93.9 +/- 1.0% spines stable over 2h, 789 spines from 10 cells in 7 birds; HVC$_X$: 92.0 +/- 1.6% spines stable over 2h, 731 spines from 13 cells in 8 birds; p = 0.33 for difference between PN types). However, tracking spine stability before and after deafening revealed that spine stability decreased in HVC$_X$ but not HVC$_{RA}$ neurons (Figures 21). This destabilization reflected increased spine gain as well as spine loss (Figure 21C), consistent with my observation that deafening did not affect spine density in HVC$_X$ neurons (data not shown). In contrast to the rapid effects of deafening on the size of stable spines, however, the effect of deafening on spine stability was delayed and only occurred after the onset of song degradation (Figure 21B and 21D, as compared with Figure 17A-B).
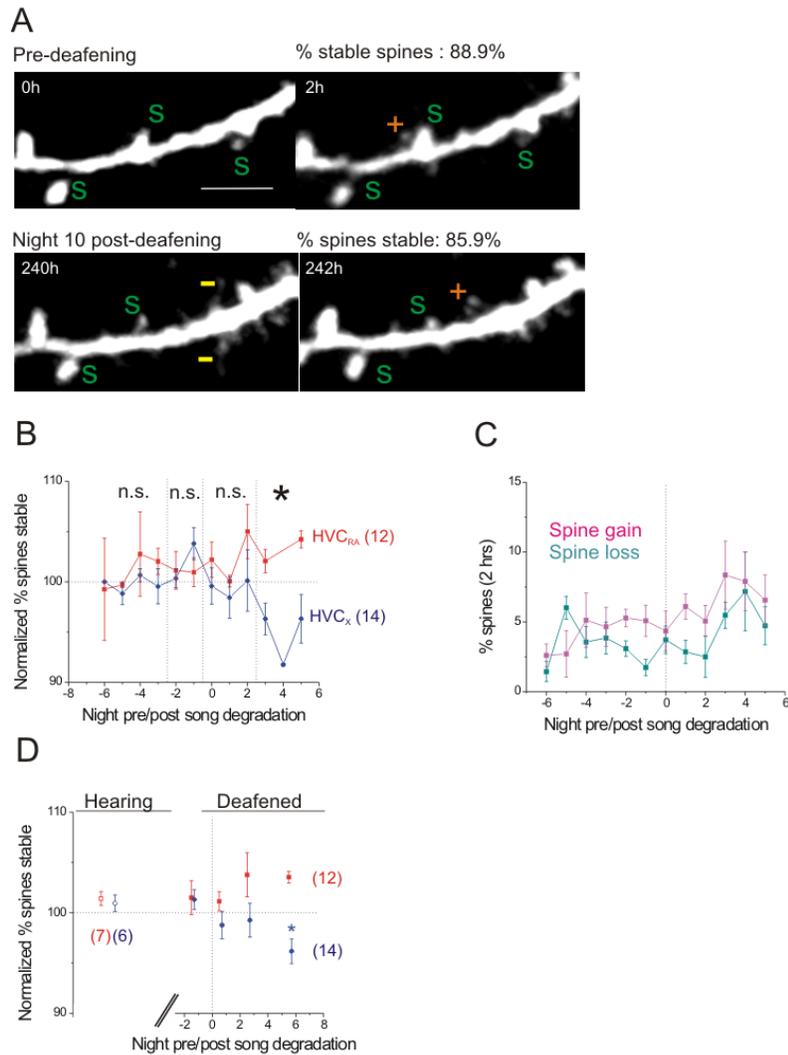
**Figure 21: Deafening causes a cell type-specific decrease in spine stability in HVCx neurons that follows the onset of song degradation.** A) Images showing measurements of spine stability for an HVCx neuron before and after deafening. B) Deafening decreases spine stability in HVCx neurons after the onset of song degradation (time > 0 on the x axis is post-degradation). C) Spine gain and loss both increase following deafening. D) Spines from HVCx neurons in longitudinally-imaged, age-matched controls do not show decreased stability. Data from control neurons are represented as the average of all non-baseline, longitudinal measurements, collapsed across time for each PN type. Asterisks indicate a significant difference for a given PN type between the measurements from deafened birds and hearing controls in a particular time bin.

Decreases in spine stability were not attributable to effects of longitudinal imaging, because HVC$_X$ neurons from longitudinally-imaged, age-matched hearing birds never underwent a decrease in spine stability (Figure 21D; Figure 22A; control HVC$_X$: 1964 spines from 6 cells in 4 birds; control HVC$_{RA}$: 1168 spines from 7 cells in 4 birds). Although there was a slight, negative relationship between the variability of dendritic sampling and levels of spine stability (i.e., post-deafening measurements including dendrites that were not scored on the pre-deafening, baseline night tended to have lower stability values), subsequent re-sampling of the data to include only post-deafening measurements in which > 50% of the dendrites samples were the same as those sampled in the baseline measurement did not support the idea that variable sampling account for decreased spine stability in HVC$_X$ neurons (Figure 22-C). Thus, deafening induces decreases in HVC$_X$ spine size and stability, which are two structural correlates of synaptic weakening (Nägerl et al., 2004; Okamoto et al., 2004; Zhou et al., 2004), but these structural changes differ in when they first appear relative to the onset of song degradation.

A

B

C
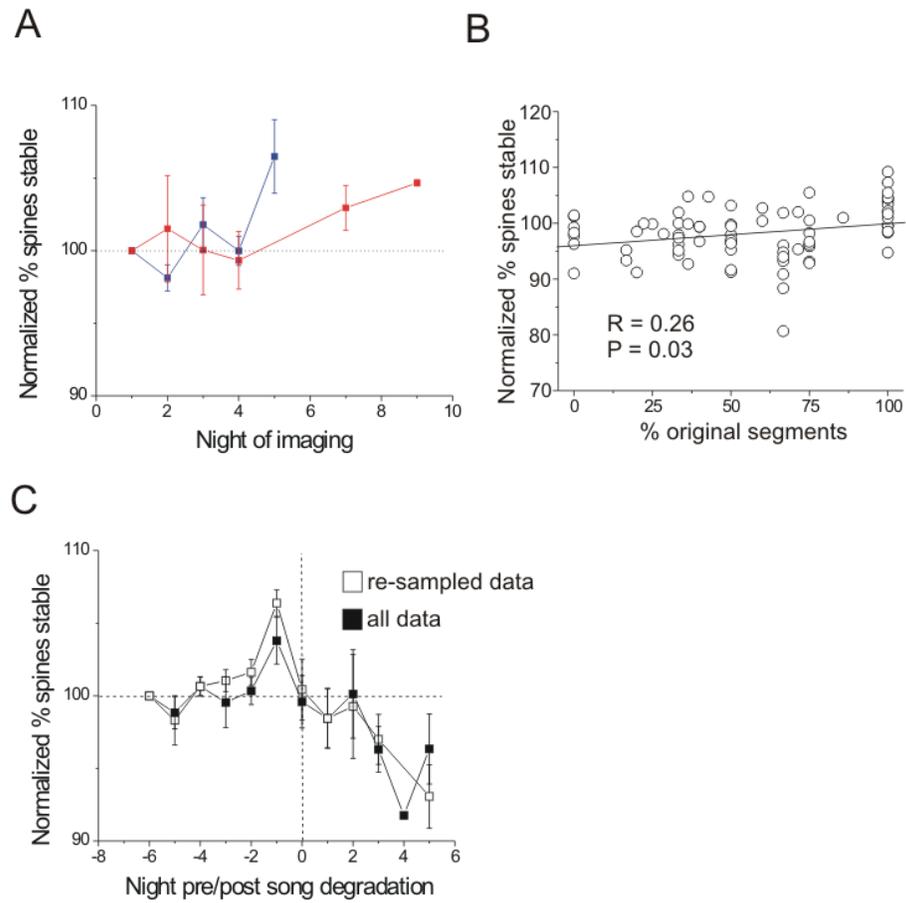
Figure 22: Decreases in HVCx spine stability following deafening are not due to imaging methodology. A) Decreases in spine stability are not observed in longitudinally-imaged HVC PNs from hearing controls (control HVCx: 1964 spines from 6 cells in 4 birds; control HVCRA: 1168 spines from 7 cells in 4 birds). B) There is a slight, negative relationship between variability of dendritic sampling and spine stability. C) Spine stability data that have been re-sampled to include only post-deafening measurement in which > 50% of the dendrite segments sampled were the same as those sampled in the baseline measurement still clearly show a decrease in HVCx neuron spine stability after deafening.

Does the magnitude of the deafening-induced decrease in spine size index predict the magnitude of decrease in stability for an individual HVC$_X$ neuron? To test whether the magnitudes of these two structural changes were correlated, the minimum post-deafening value of spine stability was compared to the minimum post-deafening values of spine size index for each HVC$_X$ neuron. While HVC$_X$ neurons with larger decreases in spine stability also tended to have larger changes in spine size index following deafening, these two measures were not significantly correlated (Figure 23; p = 0.39).

The fact that deafening-induced changes in HVC$_X$ neuron spine size index precede the onset of song degradation suggests that structural changes are driven by removal of auditory feedback following deafening. However, a remaining question is whether these changes are a general effect of auditory deafferentation or, alternatively, are driven selectively by perturbation of signing-related auditory feedback. One HVC$_X$ neuron was imaged in a bird that did not sing for the first 7 days post-deafening, and in this cell, deafening did not drive decrease spine size index or spine stability (Figure 24). Although the interpretation of this single experiment is limited, these data suggest that deafening-induced changes may be driven specifically by perturbation of singing-related auditory feedback.

Finally, I wondered whether the deafening-induced decreases in spine size index and stability were long-lasting. Although there was no evidence for a reversal of

deafening-induced decreases in spine size index or spine stability within the time frame

of the initial imaging experiments (~ 7d post-deafening), a remaining possibility is that

levels of structural dynamics may return to baseline levels at long times post-deafening.

To test this idea, I conducted imaging experiments in an additional set of animals that

were deafened around 90 dph and were imaged at 130 dph (3 HVC_X neurons imaged in

3 birds, 8 HVC_{RA} neurons imaged in 5 birds). Because the majority of the measurements

made in these experiments consisted of a single pair of imaging sessions (0-2h), only

spine stability measurements were considered. When measurements of 2h spine

stability made in deafened birds were compared to those obtained from age-matched,

hearing controls (7 HVC_X imaged in 6 birds, 12 HVC_{RA} imaged in 8 birds), no significant

difference was detected for either cell type (HVC_X: 95.5 +/- 0.1 % spines stable in

deafened group, 94.3 +/- 0.0 % spines stable in control group, p = 0.42; HVC_{RA}: 93.8 +/-

1.2% spines stable in deafened group, 95.5 +/- 0.8% spines stable in control group, p =

0.37). Thus, deafening-induced synaptic reorganization in HVC does not last

indefinitely, and levels of HVC spine stability return to baseline levels at long time

points (~40d) post-deafening.
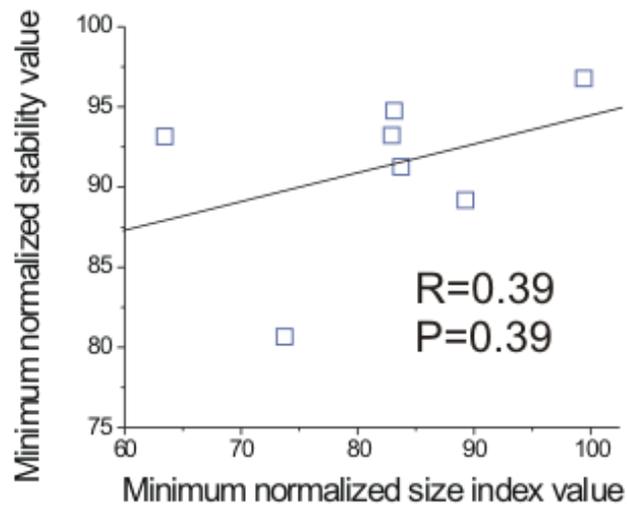
Figure 23: The magnitude of deafening-induced changes to HVCx neuron spine size and stability are not significantly correlated. HVCx neurons than underwent larger decreases in spine size index tended to undergo larger decreases in spine stability, but these two measures were not significantly correlated (p = 0.39).
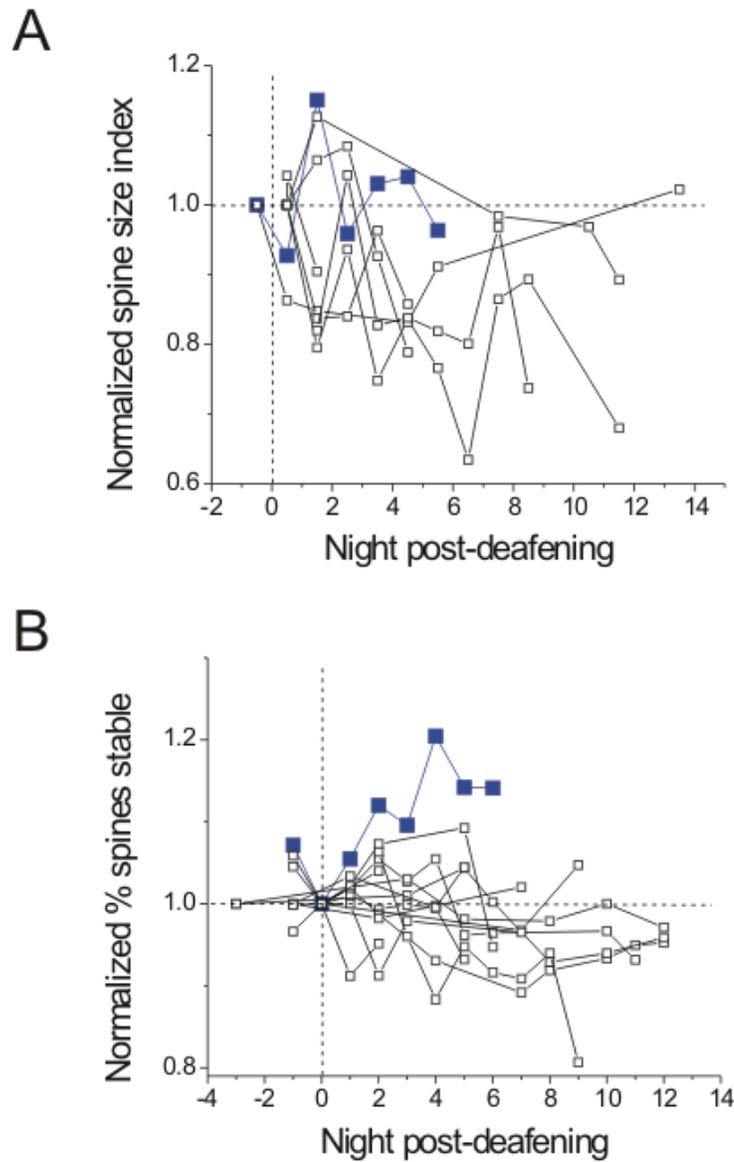
Figure 24: Deafening does not drive decreases in spine size index or spine stability in an HVCx neuron from a bird that did not sing for the first week post-deafening. A) Spine size index in an HVCx neuron imaged in a bird that did not sing for a period of 1 week following deafening (shown in blue) is compared to data from HVCx neurons imaged in other birds that did sing during this time period. B) Same as A, except data for spine stability are shown.

3.5 Chapter 3 conclusions

In summary, deafening causes two cell type-specific structural correlates of weakening of excitatory synaptic inputs onto HVC_X neurons, with decreases in spine size index occurring prior to song degradation. In contrast, decreases in the spine size index or spine stability were never observed in HVC_{RA} neurons. These findings constitute the first observation of sensitivity to deafening in a song sensorimotor nucleus and implicate HVC_X neurons in the processing of auditory feedback-related information.

4. Effects of deafening on spontaneous synaptic activity, spontaneous spiking, and intrinsic membrane properties of HVCx neurons

Do the structural changes to HVCx dendritic spines observed in Chapter 3 reflect functional changes in the strength of excitatory synaptic inputs onto these neurons, as was shown to be true following tutor song experience? If so, are these changes associated with alterations in the action potential output or intrinsic membrane properties of HVCx neurons? To address these questions, I next carried out in vivo electrophysiological recordings to test the effects of deafening on the strength of synaptic inputs to and the action potential output of HVCx neurons. In addition, in vitro electrophysiological recordings were carried out to examine whether deafening affects the intrinsic membrane properties of HVCx neurons.

4.1 Deafening causes functional weakening of synaptic inputs onto HVCx neurons

To determine whether deafening-induced structural changes to HVCx dendritic spines reflect functional changes in the strength of excitatory synaptic inputs onto these neurons, sharp intracellular recordings were made from HVCx neurons in anesthetized adult male zebra finches several days after deafening (16 HVCx from 3 birds, 88-114 dph, mean age of 97 dph, recorded on average at 2.8 +/- 0.8 days post-deafening,), within the time range when structural changes to HVCx dendritic spines were observed. Similar recordings were also carried out in a second group of age-matched, hearing control

birds (22 HVC$_X$ from 14 birds, 88-143 dph, mean age of 105 dph). Hyperpolarizing

current was injected into the impaled cells to facilitate the measurement of depolarizing

postsynaptic potentials (dPSPs) without contamination from action potentials (Figure

25A, membrane potential was -87.5 +/- 2.1 mV in deafened group, -85.8 +/- 1.9 in control

group, p = 0.78 for difference between groups). Deafening significantly decreased

median dPSP amplitude in HVC$_X$ neurons (Figure 25B, left; p = 0.03, Mann Whitney U

test) and also caused a significant leftward shift in the distribution of dPSP amplitudes

(Figure 25B, right; p < 0.0001, KS test). The average decrease in median dPSP amplitude

(16.4%) was comparable in magnitude to the average decrease in HVC$_X$ spine size index

observed between 1 and 4 days post-deafening (11.2%), consistent with the

interpretation that deafening decreases the strength of excitatory synaptic input to HVC$_X$

neurons. However, because the neurons were hyperpolarized to values near or beyond

the reversal potential for inhibitory synaptic currents (near -85 mV; evidenced here by

the lack of hyperpolarizing potentials), the dPSPs I measured likely represent a mixture

of excitatory and inhibitory inputs, and thus one or both types of inputs may be affected

by deafening.
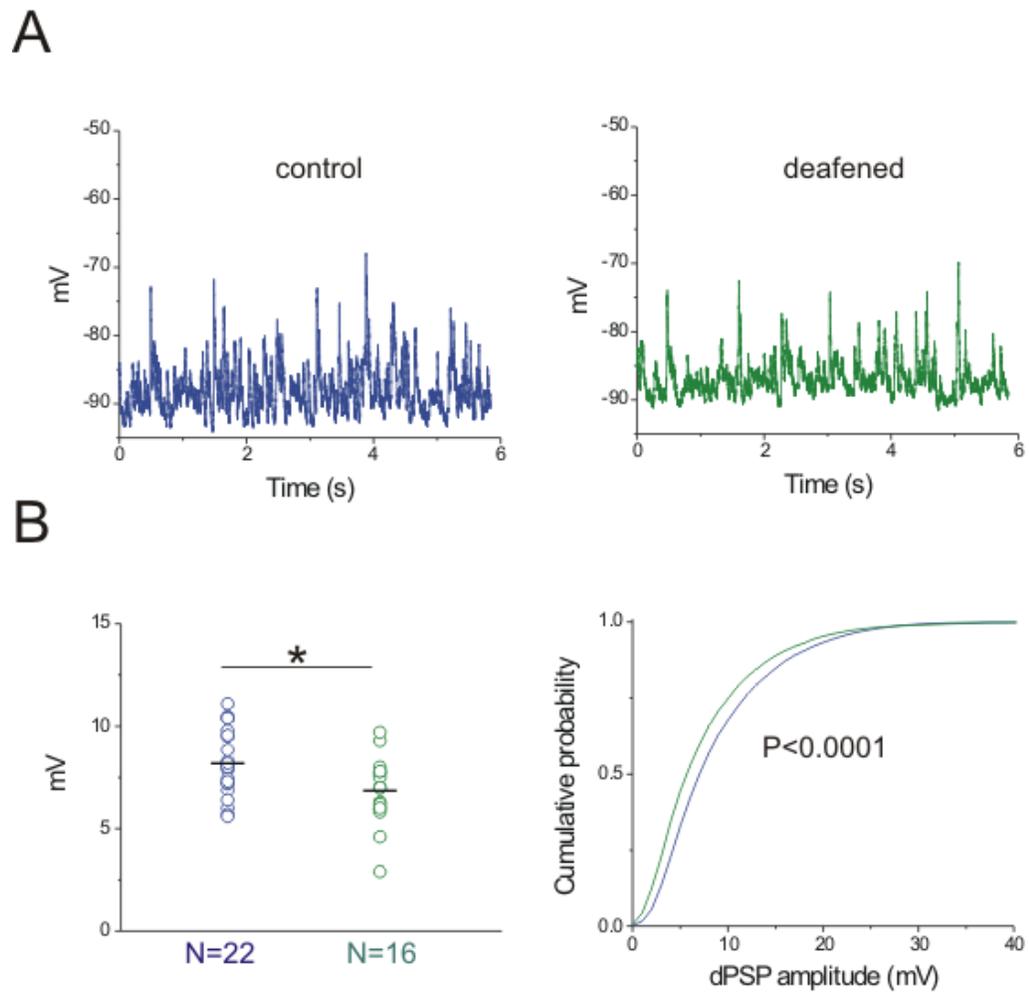
Figure 25: Deafening causes electrophysiological weakening of synaptic inputs onto HVCx neurons. Top: example traces of depolarizing postsynaptic potentials (dPSPs) from HVCx sampled from control (blue) and deafened (green) birds. Bottom: Deafening significantly decreases the median amplitude of dPSPs (left panel) and causes a significant leftward shit in the distribution of dPSP amplitudes (right panel).

One possibility is that HVC$_X$ neurons are losing synaptic input following deafening, resulting in weaker and less frequent spontaneous synaptic activity. However, the rate of spontaneous dPSPs in HVC$_X$ neurons was not affected by deafening (mean frequency of 16.0 +/- 1.0 events per second in deafened group, 14.7 +/- 0.6 in control group, p = 0.3, Mann Whitney U test). Taken together, these observations suggest that the average strength of synaptic inputs to HVC$_X$ neurons decreases following deafening without an apparent loss of synaptic input. In summary, electrophysiological and structural correlates of weakened synaptic inputs onto HVC$_X$ neurons can be detected following deafening, suggesting that HVC$_X$ neurons are sensitive to deafening.

4.2 Deafening tends to increase spontaneous spiking and bursting in HVC$_X$ neurons

Do deafening-induced changes in the amplitude of spontaneous synaptic activity affect the action potential output of HVC$_X$ neurons? When spontaneous firing rates of HVC$_X$ neurons were measured in vivo, there was a slight trend toward increased firing frequency following deafening (example traces in Figure 26A; 9.5 +/- 2.0 Hz for 18 deafened bird HVC$_X$; 5.8 +/- 1.0 Hz for 23 control bird HVC$_X$; p = 0.24, Mann Whitney U test), and a nearly significant decrease in median inter-spike interval (Figure 26B, left panel; 154.0 +/- 25.1 ms for 18 deafened bird HVC$_X$; 194.1 +/- 24.9 for 23 control bird HVC$_X$; p = 0.07, Mann Whitney U test), which was highly significant when the entire

distribution of inter-spike intervals was considered (Figure 26B, right panel; KS test, p <

0.0001). These observations suggest that although the average strength of synaptic

inputs to HVC_X neurons decreases following deafening, HVC_X neurons tend to exhibit

increases in levels of spontaneous spiking, a finding inconsistent with the idea that

deafening causes a substantial loss of synaptic input to HVC_X neurons.
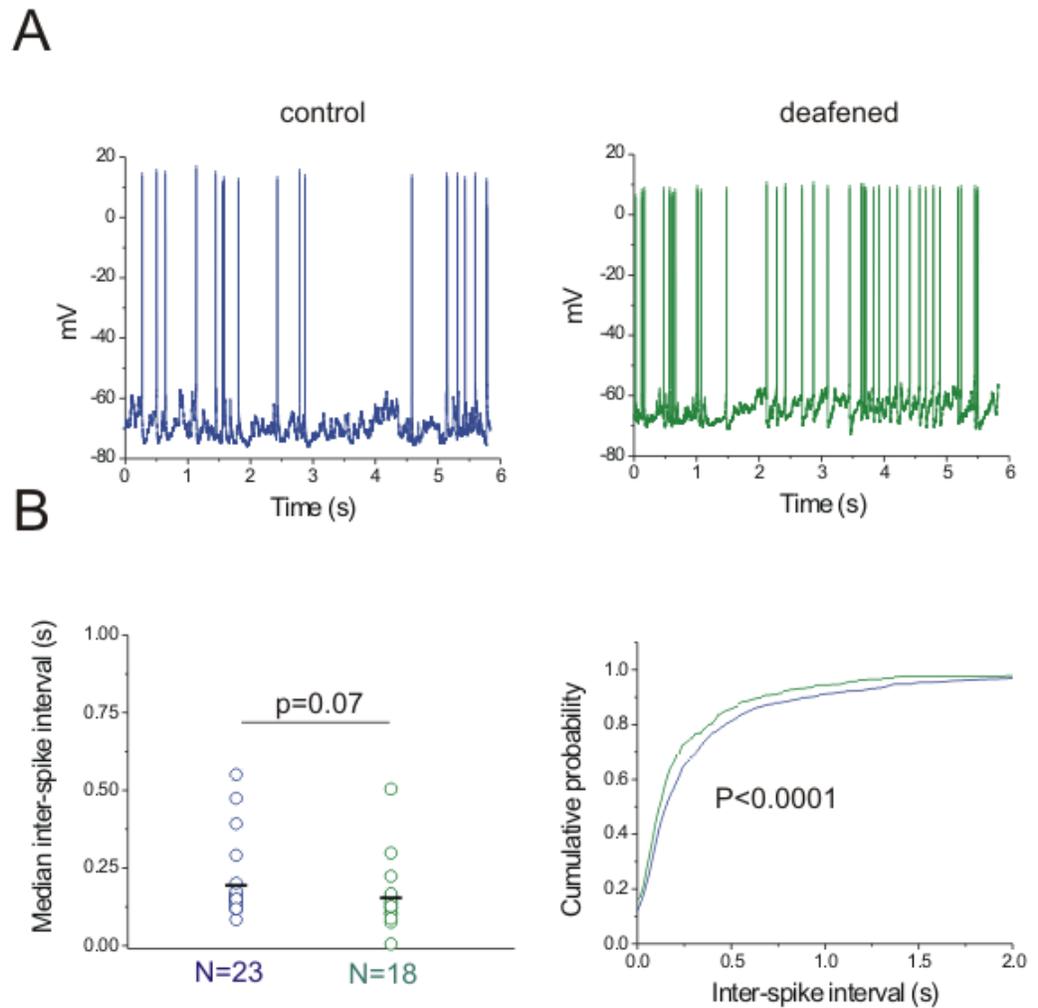
Figure 26: Deafening tends to increase levels of spontaneous spiking in HVCx neurons. A) Example traces of spontaneous firing of HVCx sampled from control and deafened birds. B) Deafening tends to decrease the median amplitude of inter-spike intervals (left panel). The leftward shit in distribution of all inter-spike intervals lengths is highly significant (right panel).

4.3 Deafening increases the intrinsic excitability of HVC$_X$ neurons

One possible explanation for the finding that deafening does not decrease spontaneous firing rates of HVC$_X$ neurons is that the strength of inhibitory inputs onto HVC$_X$ neurons has decreased in parallel with the decrease in the strength of excitatory inputs. In this manner, the net amount of excitatory drive to HVC$_X$ neurons would remain unchanged, as would levels of spontaneous spiking. A second, not mutually exclusive possibility is that HVC$_X$ neurons undergo changes in intrinsic membrane properties, and increased excitability following deafening allows HVC$_X$ neurons to maintain the same firing output in response to weaker synaptic drive. Indeed, a number of previous studies have shown that neurons homeostatically modulate their intrinsic excitability in the face of changed levels of synaptic input in order to maintain the same range of spiking output (Desai at al., 1999; Nelson and Turrigiano, 2008; Turrigiano and Nelson, 2004). I investigated this second possibility by measuring the effects of deafening on the intrinsic membrane properties of HVC$_X$ neurons.

To test the idea that HVC$_X$ neurons deafening affects intrinsic membrane properties of HVC$_X$ neurons, I made sharp electrode recordings of HVC$_X$ neurons in brain slices, because levels of spontaneous synaptic activity are known to be lower in vitro than in vivo and thus would be less likely to contaminate the measurements of intrinsic properties (Paré et al., 1998; Destexhe and Paré, 1999). Slices were made from

adult zebra finches at one week post-deafening, a time at which both structural and functional synaptic changes were already evident, and additional recordings were carried out in slices from age-matched hearing birds (33 HVC$_x$ neurons from 5 deafened birds, 93-98 dph, and from 30 HVC$_x$ neurons in 4 control birds, 89-97 dph). Families of negative and positive currents were injected into neurons, and the resulting changes in membrane potential were used to calculate a number of intrinsic properties (see Appendix B). Using this experimental design, I found that HVC$_x$ neurons from deafened birds exhibited increased spike frequency in response to current injection, decreased spike duration, decreased afterhyperpolarization (AHP) time-to-peak, and increased resting membrane potential (Figure 27). In contrast, no significant changes were observed in the threshold current required to elicit spiking, inter-spike interval, latency to spike, AHP amplitude, AHP time-to-decay, input resistance, membrane potential sag after hyperpolarization, or membrane time constant. Thus, deafening affected intrinsic membrane properties of HVC$_x$ neurons in a manner consistent with increased excitability, and these changes may contribute to the maintenance of stable firing rates following deafening.
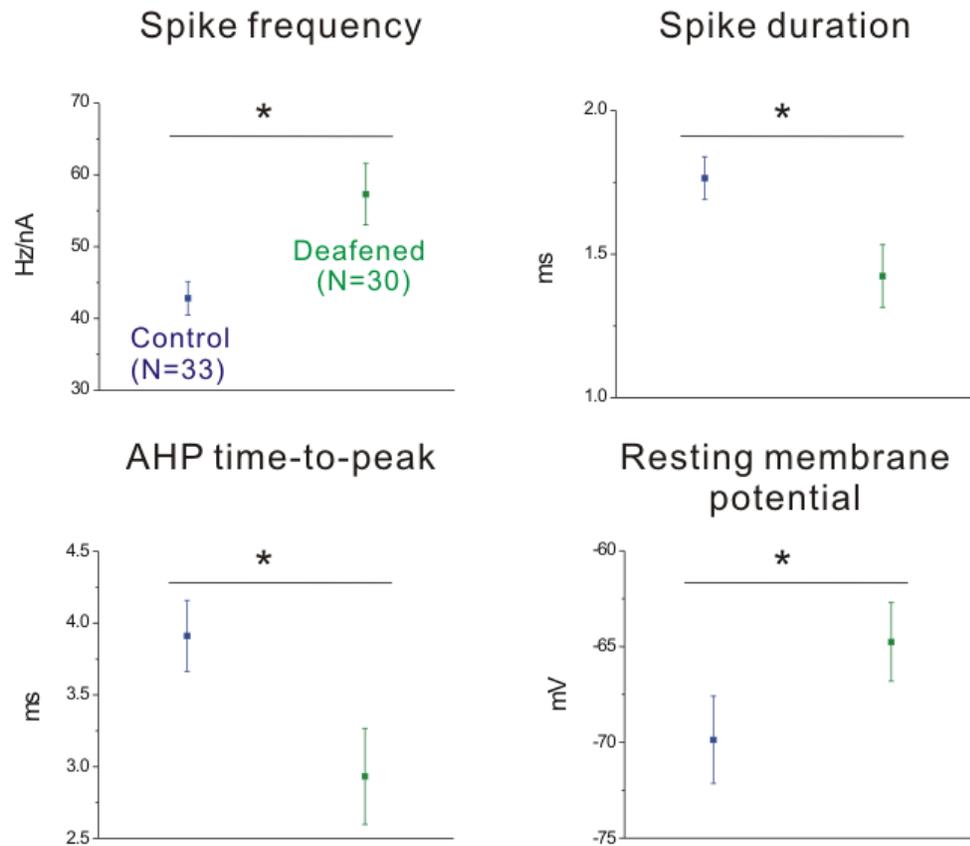
Figure 27: Deafening affects intrinsic membrane properties of HVCₓ neurons in a manner consistent with increased excitability. Measurements of intrinsic properties were made from 30 HVCₓ neurons in deafened birds (1 week post-deafening) and from 33 HVCₓ neurons in age-matched, hearing controls. Deafening significantly increased spike frequency and resting membrane potential and significantly decreased spike duration and AHP time-to-peak.

4.4 Chapter 4 conclusions

Deafening significantly decreased the amplitude of spontaneous synaptic activity in HVC$_X$ neurons, indicating that functional decrements in the strength of synaptic inputs accompany deafening-induced decreases in spine size and stability in this cell type. However, the observed changes in electrophysiologically-measured synaptic strength cannot be explained by a decrease in the overall amount of synaptic input, since the frequency of spontaneous synaptic events was not significantly affected by deafening. Surprisingly, levels of spontaneous firing do not decrease following deafening, suggesting that additional functional changes occur in HVC$_X$ neurons to maintain stable firing rates in the face of deafening-induced decreases in the strength of excitatory synaptic inputs. Indeed, in vitro recordings revealed that deafening drives changes in the intrinsic membrane properties of HVC$_X$ neurons that are consistent with increased excitability, suggesting that HVC$_X$ neurons may undergo changes in both synaptic inputs and intrinsic membrane properties at long times post-deafening. In summary, I have now observed both structural and functional changes to synaptic inputs onto HVC$_X$ neurons following deafening, supporting the hypothesis that HVC$_X$ neurons are sensitive to perturbation of auditory feedback.

5. Deafening-induced changes to dendritic spines on HVC$_X$ neurons require an intact AFP

The deafening-induced structural and functional changes to HVC$_X$ neurons observed here indicate that synaptic inputs onto these cells are sensitive to changes in auditory feedback-related information. However, these observations cannot distinguish whether changes in synapses on HVC$_X$ neurons are driven solely by the impingement of altered auditory feedback on the motor network or whether changes to these synapses depend on feedback-sensitive error correction circuitry elsewhere in the brain (i.e., downstream neurons of the AFP). The findings of the current study could be explained by three scenarios. In the first scenario, auditory feedback information acts directly on HVC. Thus, weakened synapses on HVC$_X$ neurons following deafening reflect diminished auditory drive and would occur even in the absence of downstream error correction circuitry. In this feedforward scenario, any deafening-induced changes to downstream neurons of the AFP would depend on and occur after changes to HVC$_X$ neurons.

An alternative possibility is that deafening-induced changes to HVC depend on the output of downstream error correction circuits (i.e., the AFP). There are two ways in which LMAN, the output nucleus of the AFP, could influence deafening-induced synaptic reorganization in HVC. LMAN is a source of feedback-related error signals to RA that are used to implement motor corrections following perturbation of auditory

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feedback (Andalman and Fee, 2009). Thus, although LMAN does not project directly to

HVC, it is possible that feedback-related error signals from LMAN access HVC through

recurrent circuitry and actively drive the deafening-induced changes to HVC$_X$ dendritic

spines. In this second scenario, LMAN could influence HVC via the recurrent

connections from RA to HVC (Roberts et al., 2008), through a brainstem pathway

indirectly linking RA and HVC (Striedter and Vu, 1998), or indirectly through midbrain

dopaminergic inputs to HVC (LMAN to Area X to ventral pallidum to VTA; Bottjer,

1993; Gale et al., 1998; Nixdorf-Bergweiler et al., 1995; Soha et al., 1996).

In a third scenario, LMAN could act permissively to maintain HVC in a

competent state to respond to feedback-related information conveyed by auditory areas.

LMAN lesions in very young birds (~20 dph) cause cell death in RA which can be

rescued by infusions of BDNF into RA (Johnson and Bottjer, 1994; Johnson et al., 1997),

suggesting that LMAN may be a source of BDNF that provides trophic support to RA at

early stages in development. In older juveniles, LMAN lesions drive a premature

stabilization of song output, and these behavioral changes are accompanied by

structural and functional consolidation of inputs from HVC to RA (Johnson and Bottjer,

1994; Kittelberger and Mooney, 1999). Interestingly, injections of BDNF into RA

reversibly disrupt song stability and increase synaptic density in RA in adult birds,

indicating that trophic factors continue to regulate song structure and synaptic

organization in the adult brain (Kittelberger and Mooney, 2005). Although these

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previous studies have focused on the effects of LMAN output and/or trophic signaling

on RA, an untested possibility is that LMAN might also influence synaptic organization

in HVC, either through retrograde effects in RA or through recurrent connections to

HVC. Thus, in this third scenario, auditory feedback information would still impinge

directly on HVC but LMAN would act permissively to allow synaptic reorganization in

HVC following deafening.

To determine whether deafening-induced changes to HVC_X dendritic spines

require an intact AFP, in vivo imaging experiments were conducted using a new group

of zebra finches that received bilateral LMAN lesions five to six days prior to deafening

(3 birds, 120-145 dph). I anticipated that if auditory feedback acts on HVC in a strictly

feedforward manner that is independent of the AFP, deafening-induced changes to

HVC_X dendritic spines would still be detected following deafening in birds that received

LMAN lesions. Alternatively, if LMAN output either actively drives or is permissive for

deafening-induced changes to HVC_X dendritic spines, these changes would be abolished

in birds that received LMAN lesions prior to deafening.

5.1 LMAN lesions prevent the effects of deafening on HVC_X dendritic spines

In vivo imaging of HVC PNs was carried out in three adult male zebra finches

with LMAN lesions to measure the effects of LMAN lesions on deafening-induced

structural changes to HVC_X neurons (these three were a subset of the birds that were

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subjected to behavioral analyses and post mortem histological analysis of lesion extent, as detailed in the following section). A GFP-lentivirus was injected into HVC to label neurons for in vivo imaging, and 4-5 days prior to the first imaging session, LMAN was lesioned bilaterally with ibotenic acid and a retrograde tracer was injected bilaterally into Area X. Only a single night of imaging data was collected prior to deafening in order to maximize post-deafening data collection; therefore, raw, non-normalized spine size index and stability measurements were used in all comparisons. Song behavior was recorded starting at least two days prior to LMAN lesion and until at least 1 week post-deafening. Following deafening, birds that received LMAN lesions still showed subtle but significant song degradation (see next section), and data from different birds were aligned to song behavior and pooled as in previous experiments.

Surprisingly, LMAN lesions prevented the decrease in HVC_X neuron spine size index that normally follows deafening (Figure 28A, 471 spines from 7 HVC_X in 3 LMAN lesion-deafened birds, p = 0.001 for difference between groups across all time bins). In fact, post-deafening spine size index values from HVC_X neurons in deafened birds with LMAN lesions did not differ from those observed in HVC_X neurons from age-matched, hearing controls (data not shown). Lesions to LMAN also prevented deafening-induced decreases in HVC_X neuron spine stability, and spine stability measurements from HVC_X neurons in LMAN lesion-deafened birds were significantly higher than those from LMAN intact-deafened HVC_X neurons in all post-degradation time bins (Figure 28B,

2401 spines from 7 HVC$_X$ in 3 LMAN lesion-deafened birds, $p \leq 0.02$ for difference

between groups in last 3 time bins). Additionally, LMAN lesions did not affect baseline

values of HVC$_X$ spine size index or stability, suggesting that LMAN lesions did not

mimic the effects of deafening or, conversely, prevent the detection of deafening-

induced morphological changes by driving HVC into a hyper-stable state (HVC$_X$ neuron

spine size index: 1.10 +/- 0.06 in LMAN lesion group, 1.07 +/- 0.03 in control group, p =

0.62; stability: 94.0 +/- 1.1% stable in LMAN lesion group, 92.0 +/- 1.6% in control group,

p = 0.46). In summary, LMAN lesions abolished both structural correlates of synaptic

weakening in HVC$_X$ neurons following deafening. These findings suggest that although

auditory feedback-related information can rapidly influence synaptic inputs to HVC$_X$

neurons, these deafening-induced changes ultimately depend on input from

downstream sensorimotor areas.
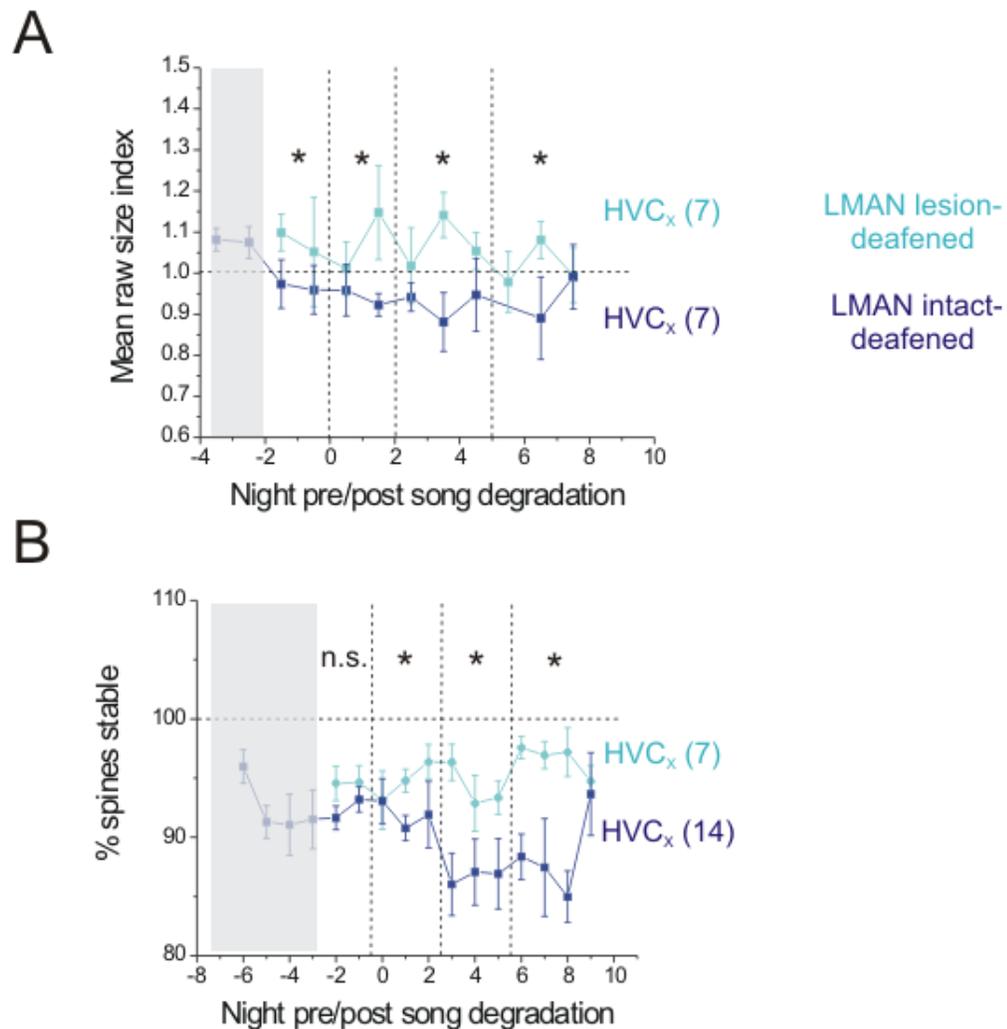
**Figure 28: LMAN lesions prevent deafening-induced decreases in HVC$_X$
neuron spine size index and spine stability.** A) LMAN lesion prevents deafening-
induced decreases in HVC$_X$ neuron spine size index across all 4 time bins. Asterisks
indicate significant differences between groups within time bins (p = 0.001 for difference
between groups across all time bins, LMAN intact-deafened HVC$_X$ in dark blue, LMAN
lesion-deafened HVC$_X$ in cyan). Gray shaded regions indicate time points excluded from
comparisons. B) LMAN lesions prevent deafening-induced decreases in HVC$_X$ neuron
spine stability (p ≤ 0.02 for difference between groups in each of last 3 time bins).

5.2 LMAN lesions do not affect baseline song behavior and significantly reduce deafening-induced song degradation

I also conducted additional analyses to fully characterize the anatomical extent of the LMAN lesions and their effects on singing. Three additional birds that were used for the imaging experiments were included in these histological and behavioral analyses. At least one week after birds received LMAN lesions and 4-5 days prior to sacrifice, a retrograde tracer was injected bilaterally into RA to label any remaining RA-projecting LMAN neurons. Confocal images were taken of any tissue sections that contained remaining retrogradely-labeled LMAN neurons, the tissue sections were Nissl-stained, and the confocal images were used to guide the identification and tracing of intact portions of LMAN in the Nissl-stained tissue sections.

Using this method, post hoc histological measurements showed that the average extent of LMAN lesion was 82.3 +/- 3.4% (12 hemispheres from 6 birds, ranging from 57% - 100% lesion, example of a representative LMAN lesion shown in Figure 29). Previous studies that have relied on Nissl staining alone have reported more extensive LMAN lesions (83-100% lesion extent, Thompson et al., 2011). However, the accuracy of this method is questionable, because it can be difficult to identify small areas of remaining, intact LMAN neurons using Nissl stain alone. Indeed, the lesion estimates in the current study are in the same range as those reported in studies using immunocytochemistry to stain for calcitonin gene-related peptide (40-100% lesion

extent; Brainard and Doupe, 2000; Kao and Brainard, 2006), a protein that is highly expressed in LMAN and RA (Bottjer et al., 1997). Thus, the LMAN lesions made in the current study are comparable in magnitude to those reported in previous work.

After estimating the extent of LMAN lesions, behavioral analyses were used to assess the effects of LMAN lesions on singing, because a previous study reported increased variability in song spectral features following LMAN lesions, including syllable entropy (Thompson et al., 2011). Because entropy and EV were measured to assess deafening-induced song degradation, the effects of LMAN lesions were tested on the mean or variability of these two parameters. Although the mean and spread of entropy and EV were significantly affected for some syllables, overall, LMAN lesions had no significant effect on these parameters (Wilcoxon signed-ranks test used to compare pre-lesion to post-lesion values for 25 syllables from 6 birds; $p = 0.09$ for mean entropy, $p = 0.92$ for entropy coefficient of variation (CV), $p = 0.25$ for mean EV, $p = 0.31$ for EV CV). Although a number of factors could have contributed to the differences between my findings and those of Thompson et al., including lesion efficacy (average size of LMAN lesion was 93% in Thompson et al., although different methods to estimate lesion extent were employed, as noted above), average number of birds and syllables included in the analysis, and other methodological differences, LMAN lesions had no consistent effect on the mean values or spread of syllable entropy or EV in the present study.
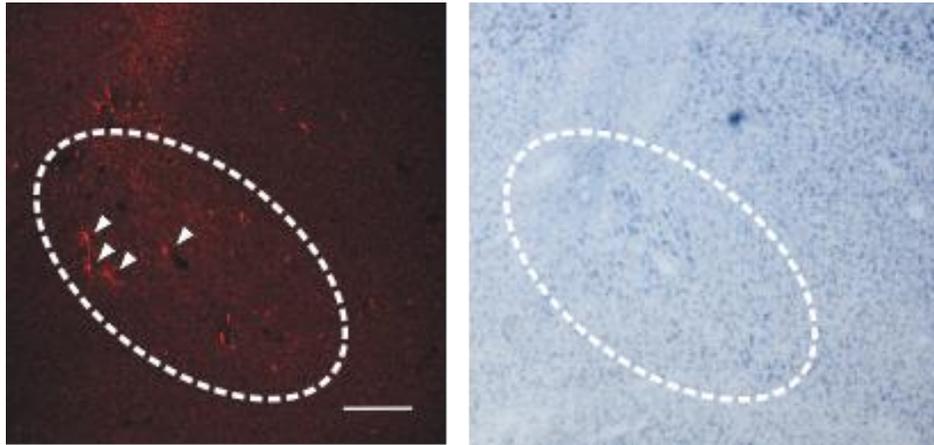
Figure 29: Representative LMAN lesion. Remaining retrogradely-labeled LMAN neurons (indicated by white arrowheads) from an injection of retrograde tracer into RA are shown in the left panel, and the right panel shows the same section after Nissl staining. Approximate borders of LMAN are indicated by the white dashed line. Scale bar, 100 μm.

As a second step to assess the effects of LMAN lesions on singing behavior, I recorded and analyzed song produced by LMAN-lesioned bird before and after deafening. Previous studies have reported that LMAN lesions prevent deafening-induced song degradation (Brainard and Doupe, 2000), and behavioral analyses were carried out to determine if that finding could be replicated in the current study. A global analysis of song degradation (see Appendix B) demonstrated that LMAN lesions significantly reduced long-term song degradation following deafening (1-2 weeks post-deafening; Figure 30A). However, song degradation could still be detected in birds that received LMAN lesions, although it was much more subtle than the degradation observed following deafening in birds without LMAN lesions (Figure 30). In particular, my analysis indicated that LMAN lesions failed to prevent all spectral changes following deafening, because post-deafening entropy and EV values for songs from LMAN-lesioned birds tended to fall below baseline levels (Figure 30A).

I also examined whether LMAN lesions prevented the subtle, early changes to song that occur within the first week after deafening. To examine whether early spectral degradation (1-7d post-deafening) could also still be detected in birds that received LMAN lesions, the syllable-by-syllable analysis that was used previously to assess post-deafening song degradation (Chapter 3) was employed again here. Surprisingly, for 6 out of 6 birds examined, at least one syllable of the bird's song showed small but significant spectral degradation within the first 3 days following deafening, and this

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time course was indistinguishable from the onset of degradation measured previously

for deafened-LMAN intact birds (percentage of syllables that degraded ranged from

25% to 100% in deafened-LMAN lesion birds). Although early degradation of spectral

features still occurred following deafening, LMAN lesions significantly reduced the

magnitude of these changes (Figure 30B; the change from baseline value of these

degraded syllables was measured on the third day of post-deafening song and

compared between the two groups).

Interestingly, even a bird with complete, bilateral LMAN lesions still exhibited

subtle song degradation at both early and late times following deafening (Figure 31),

suggesting that a small amount of song degradation may occur independently of

LMAN. In summary, the LMAN lesions made in the current study were extensive

enough to dramatically reduce deafening-induced song degradation, indicating that the

output of the AFP has been greatly reduced. However, subtle spectral degradation of

song features could still be detected in birds that received LMAN lesions, in contrast to

previous reports (Brainard and Doupe, 2000), indicating that the method of behavioral

analysis used in the current study is highly sensitive to deafening-induced changes to
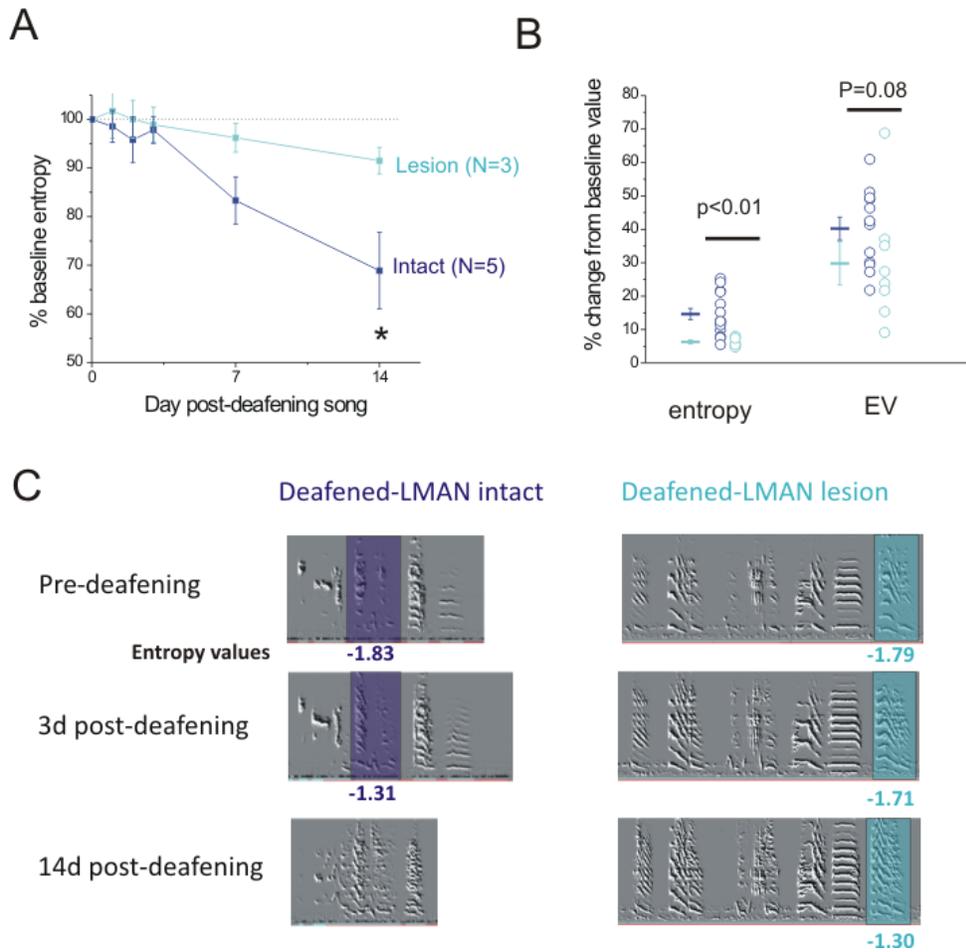
song.

Figure 30: Bilateral LMAN lesions reduce deafening-induced spectral degradation of song. A) Global song analysis reveals that LMAN lesions significantly reduce long-term (1-2 wks) spectral song degradation (data shown for entropy only). B) Syllable-by-syllable analysis reveals that LMAN lesions reduce, but do not prevent, early spectral degradation of syllables. The entropy and EV of syllables that degraded significantly within the first 3d post-deafening were measured at the end of this period, and the percentage difference from baseline was calculated. Song behavior data from 3 additional LMAN lesion-deafened birds that were not used for imaging experiments are included (total of 12 syllables from 6 deafened-LMAN birds). LMAN lesions significantly reduce the magnitude of entropy changes (p < 0.01) and tend to reduce the magnitude of EV changes (p = 0.08). C) Sonograms are shown from a LMAN intact-deafened bird and a LMAN lesion-deafened bird. Entropy measurements of highlighted syllables are indicated below each motif. Note that the highlighted syllable from the deafened-LMAN intact bird can no longer be identified at 14d post-deafening.
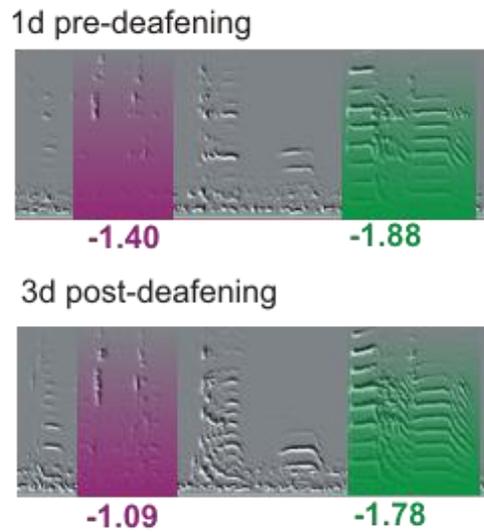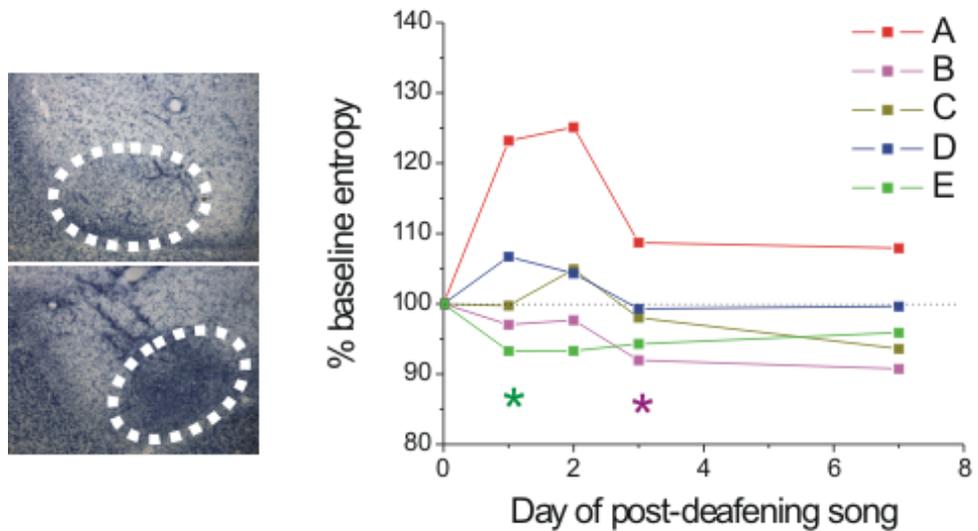
Figure 31: Subtle but significant song degradation is still observed in a bird with complete, bilateral LMAN lesions. Left panel: histology shown for bilateral LMAN lesions. Dashed white lines indicate the borders of LMAN. Three electrode tracts can be seen over LMAN's location in the image from the right hemisphere (bottom). Middle panel: analysis of entropy is shown for five syllables of the bird's song. Note that for two of the five syllables, entropy decreases significantly within the first 3d of post-deafening song (colored asterisks). Right panel: representative spectrograms are shown, the two degraded syllable, B and E, highlighted in green and purple, respectively.

5.3 Chapter 5 conclusions

In summary, the deafening-induced changes to HVC$_X$ dendritic spines require an intact LMAN. While these findings do not allow us to distinguish between permissive and instructive roles for LMAN, they do allow us to rule out a strictly feedforward view of auditory feedback-processing in the songbird brain. While the exact role of the AFP in deafening-induced synaptic reorganization in HVC$_X$ neurons remains to be determined, these findings for the first time demonstrate that HVC is sensitive to deafening and implicate HVC$_X$ neurons in the processing of auditory feedback.

6. Conclusions and future directions

The major finding of these experiments is that synaptic inputs to HVC are sensitive to tutor song experience and deafening. Hence, this study for the first time links sensory experiences to synaptic reorganization in a sensorimotor nucleus important for vocal learning. In the following sections, I will re-summarize the specific findings of each set of experiments, discuss the implications of the observed neural changes to singing and song learning, and discuss the potential role of HVC in the comparison of tutor song information and auditory feedback.

6.1 Synaptic inputs onto HVC PNs are sensitive to tutor song experience

In the first set of experiments (Chapter 2), I tested the idea that synaptic inputs onto HVC neurons are sensitive to tutor song experience. First, in vivo imaging of HVC dendritic spines demonstrated that isolation from tutor song experience had an age-dependent effect on levels of spine turnover in HVC PNs. Second, the initial levels of spine turnover correlated with an untutored bird's capacity for subsequent tutor song learning, suggesting that levels of HVC spine turnover were related specifically to the capacity for imitative learning and not just developmental age. Third, in birds that subsequently learned from a tutor, tutor song experience drove increased stability, density, and size of dendritic spines on HVC PNs. Finally, electrophysiological

measurements made by Dr. Todd Roberts showed that these structural changes were paralleled by electrophysiological strengthening of synaptic inputs following tutoring.

The significance of these experiments is four-fold. First, this study was the first to employ in vivo imaging of dendritic spines in the songbird brain, advancing the songbird as an exceptional model to study how changes in sensory experience drive the synaptic reorganization of neural circuits important to behavioral learning. Second, the finding that levels of HVC spine turnover correlate with the quality of subsequent imitative learning indicates that HVC structural dynamics decrease in concert with the closure of the sensitive period for tutor song learning and, more generally, sheds light on potential factors controlling the timing and duration of sensitive periods for behavioral learning. Third, tutor song experience that results in behavioral learning causes rapid strengthening of synaptic inputs onto sensorimotor neurons important to learned vocal control, establishing for the first time a direct link between instructive sensory experience and sensorimotor networks that control the learning and performance of imitative behavior. Fourth, tutor song experience affects synaptic inputs on both types of HVC PNs, indicating that downstream sensorimotor circuits for song learning and song motor output may be rapidly affected by instructive sensory experience that drives behavioral learning. In summary, these findings indicate that sensory experience can rapidly strengthen and stabilize a structurally dynamic network in the juvenile brain, providing a substrate for the learning of new behaviors.

6.1.1 Levels of HVC spine dynamics are sensitive to isolation from tutor song experience and predict the capacity for tutor song learning

Lack of tutor song experience had an age-dependent effect on HVC structural dynamics, with young (45 dph) untutored birds exhibiting higher levels of spine turnover than age-matched controls and older (90 dph) untutored birds exhibiting levels of spine turnover indistinguishable from age-matched controls. In 60 dph untutored birds, the effects of isolation from tutor song experience were variable and were correlated on a bird-by-bird basis with the capacity for tutor song learning. Indeed, untutored birds with high HVC turnover learned more from the tutor than untutored birds with low levels of turnover. These observations support two ideas. First, isolation from sensory experience during sensitive periods in development may alter levels of structural dynamics in brain regions important to the representation of the sensory experience. Second, levels of structural dynamics in these brain regions return to control levels, even in the absence of appropriate sensory stimulation, and this return to control levels may correlate with a reduction in behavioral sensitivity to the sensory stimulus and the closure of the sensitive period.

In agreement with the first idea, a number of previous studies have reported that sensory deprivation during sensitive or critical periods in development can alter levels of structural dynamics of dendritic spines. In the visual cortex, binocular visual deprivation starting at birth causes an up-regulation of spine motility during the critical period for ocular dominance plasticity without affecting spine morphology or density

(Majewska and Sur, 2003), while a brief period of monocular visual deprivation during the critical period drives increases in spine motility and the later pruning of dendritic spines (Mataga et al., 200; Oray et al., 2004). Studies of avian sexual imprinting, a two-stage process that involves the acquisition of mating preferences early in development and consolidation of these preferences through mating experience later in life, also indicate that sensory deprivation during sensitive periods can affect dendritic spines. Indeed, visual isolation from females during development drives an increase in spine density in two forebrain regions thought to be important to the imprinting process, the lateral and medial neo/hyperstriatum (LNH and MNH; Bischof and Rollenhagen, 1999). Thus, although the exact effects of sensory deprivation on spine structural dynamics may depend on the sensory domain studied, the onset and duration of deprivation, and additional factors, these studies support the idea that sensory deprivation during sensitive periods can alter structural dynamics of dendritic spines and are consistent with my finding that isolation from tutor song experience influences spine dynamics in HVC. Notably, although many previous studies have employed complete or extensive deprivation within a single sensory domain, I found that the exclusion of a specific type of auditory experience dramatically reduced the stability, size, and density of dendritic spines in HVC, indicating that the HVC sensorimotor network in juvenile male birds is extremely sensitive to this single auditory stimulus.

Additionally, previous studies support the idea that changes in levels of spine dynamics mark the end of sensitive periods in development. In visual cortex, decreases in spine motility parallel the end of the sensitive period for the development of binocular vision (Grutzendler et al., 2002). Also, studies of avian sexual imprinting reported that even in the absence of visual exposure to a female, spine density in LNH and MNH eventually declines to control levels, and this decrease correlates with the inability to consolidate a clear sexual preference, even after subsequent exposure to a female (Bischof et al., 2002). These studies are in agreement with the finding that untutored birds refractory to tutor song learning had levels of spine turnover indistinguishable from normally-tutored control birds, and taken together, these studies indicate that levels of spine structural dynamics may correlate with the end of a variety of sensitive periods in development.

Given that levels of HVC spine turnover in untutored birds correlate with the capacity for tutor song learning and, hence, with closure of the sensitive period, an intriguing idea is that levels of HVC spine dynamics are a factor that regulate the duration and timing of the sensitive period. If this is true, a simple prediction is that the sensitive period for tutor song learning could be reopened or extended simply by increasing levels of spine turnover in HVC. This idea has not been tested directly, primarily because there is not a straightforward method to directly and specifically manipulate levels of structural dynamics of dendritic spines.

Interestingly, studies in visual cortex suggest that although changes to dendritic spines are driven by sensory deprivation during and parallel the end of the critical period for ocular dominance plasticity, these spine changes are ultimately downstream of other functional and structural changes that are essential for critical period timing. In particular, the maturation of inhibitory neurotransmission, marked by the postnatal emergence of a set of parvalbumin (PV)-positive interneurons, appears to be essential for the onset of the critical period (del Rio et al., 1994). Reductions and enhancements of GABAergic transmission respectively delay and speed up the onset of the critical period (Hensch et al., 1998; Fagiolini and Hensch, 2000), demonstrating that the maturation of inhibition plays an essential role in critical period timing in visual cortex. Additionally, PV-positive neurons are surrounded by perineuronal nets (PNNs) composed of extracellular matrix (ECM) proteins (Härtig et al., 1999), and genetic disruption of PNNs reduces inhibition onto excitatory neurons (Saghatelyan et al., 2001), implicating the ECM in the regulation of inhibitory neurotransmission. Notably, reductions of GABA synthesis or enzymatic degradation of the ECM can reactivate ocular dominance plasticity in the adult visual cortex (Harauzov et al., 2010; Pizzorusso et al., 2002), indicating that levels of inhibition and ECM stability both regulate critical period timing. Finally, the effects of MD on spine motility are occluded by degradation of the ECM (Oray et al., 2004), and MD-induced spine loss is prevented in mice with genetic disruption of an enzyme that degrades the ECM (Mataga et al., 2004), indicating that

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changes in dendritic spines during the critical period likely lie downstream of changes in the balance of excitation and inhibition and expression of ECM proteins in visual cortex. In summary, these studies suggest that critical period timing in visual cortex is driven by changes in the balance of inhibition and excitation, and accompanying changes in the expression of ECM proteins regulate the structural consolidation of synapses and potentially link changes in activity to structural dynamics of dendritic spines.

Is there evidence that maturation of the ECM or inhibitory circuits in HVC regulates the timing of the sensitive for tutor song learning? PNNs are present in HVC and other song sensorimotor nuclei in zebra finches, and their development correlates well with behavioral measures of song maturity (Balmer et al., 2009). Additionally, lack of tutor song experience causes a decrease in PNN staining and also in PV expression (Balmer et al., 2009), suggesting that extension of the sensitive period is correlated with a delay in PNN maturation and decreased inhibition in HVC. Interestingly, a study using a sequential tutoring paradigm found that treatment with diazepam, which enhances GABAergic transmission, caused early closure of the sensitive period for tutor song learning (Yazaki-Sugiyama et al., 2007). Normally during sequential tutoring, juvenile zebra finches that are exposed to a first tutor at an early age and a second tutor at a later stage during the sensitive period for tutor song learning will usually copy the song of the second tutor. However, juvenile birds treated with diazepam learned more from the

first tutor than controls, suggesting that accelerated maturation of inhibitory circuits could regulate sensitive period timing in songbirds. Because diazepam was administered systemically, it remains unclear whether the locus of its effects on sensitive period timing resided in HVC. However, systemic diazepam treatment did increase HVC staining for calretinin, a calcium-binding protein expressed by HVC interneurons (Wild et al., 2005). Given the findings of Yazaki-Sugiyama et al., an interesting idea is that a coordinated functional and structural stabilization of excitatory and inhibitory synapses renders HVC, and perhaps other song sensorimotor areas, unresponsive to the tutor song and accounts for closure of the sensitive period. It remains to be seen whether pharmacological treatments that alter sensitive period timing also cause a corresponding regulation of levels of HVC spine dynamics. In summary, while these studies raise the possibility that changes in inhibitory function and levels of ECM proteins may modulate sensitive period timing in songbirds, whether changes to HVC spines are associated with these changes or are sufficient to shape the timing of the sensitive period for tutor song learning remains as an important question for future study.

6.1.2 Instructive tutor song experience triggers the stabilization, accumulation, and enlargement of dendritic spines on HVC PNs

In untutored birds that subsequently imitated the tutor song, initial tutor song experience drove the stabilization, accumulation, and enlargement of dendritic spines on HVC PNs. Additionally, these structural changes were accompanied by enhancements

in the amplitude of spontaneous synaptic activity in HVC. In summary, this study was
the first to directly examine the effects of tutor song experience on HVC neurons, and in
vivo imaging was used to establish a link between changes in sensory experience and
synaptic reorganization in sensorimotor neurons important to singing.

How do these findings relate to changes to dendritic spines reported during
other forms of behavioral learning? Tutor song experience shares many behavioral
similarities with avian filial imprinting, the process through which newly hatched birds
learn the characteristics of and develop a social preference for a stimulus (Bolhuis, 1999).
In particular, both tutor song memorization and filial imprinting are restricted to
sensitive periods in development, require only very limited exposure to the sensory
stimulus, and result in the formation of a long-lasting memory that influences adult
behavior. However, in contrast to the finding that tutor song experience drives
increases in spine density, filial imprinting in birds is reported to drive a dramatic loss of
spines in a forebrain region important to imprinting (Bischof et al., 2002; Bock and
Braun, 1999a; Bock and Braun, 1999b).   Thus, although tutor song learning and filial
imprinting both result in a long-lasting neural representation of a salient sensory object,
the nature of the underlying structural changes to dendritic spines may vary according
to the form of behavioral learning, the brain region involved, and the time in
development during which learning takes place.

Interestingly, the effects of tutor song experience on HVC dendritic spines were similar to those reported in motor cortex following learning of a new motor task (Yang et al., 2009; Xu et al., 2009). In both studies, motor skill learning drove the formation of new spines, as did tutor song experience. However, following motor skill learning, spines that were present prior to training were selectively eliminated over time, resulting in spine density equivalent to pre-training levels. In the current study, new spines that formed following tutoring were not tracked longitudinally, so it remains unclear whether the increase in spine density observed within the first 24h of tutor song experience was maintained indefinitely. However, the finding that decreases in spine turnover driven by tutor song experience were maintained for the remainder of sensorimotor learning suggest that at least some subset of the effects of tutor song experience were long-lasting.

Another finding from studies of motor skill learning was that the stabilization and maintenance of a small fraction of new spines correlated with the degree of behavioral improvement following training on the motor task, supporting the idea that long-lasting synaptic reorganization encodes the neural representation of motor memories. A related question that remains unanswered in the current study is whether individual spines formed during tutor song experience are maintained throughout the duration of sensorimotor learning. Alternatively, these spines could be lost as the bird begins modifying its own song to match the tutor song. In a related vein, it would be

interesting to examine the effects of sequential tutoring on HVC dendritic spines. Would the HVC circuit de-stabilize and then re-stabilize following experience with the second tutor song? If so, would the spines formed during experience with the first tutor song be "overwritten," or would the second tutor song drive changes in a non-overlapping population of dendritic spines? Interestingly, swamp sparrows have been shown to retain a neural representation in HVC of tutor songs that they imitated as juveniles but did not perform as adults (Prather et al., 2010). In zebra finches, it remains poorly understood whether the tutor song is represented stably in the adult brain or is overwritten by the bird's own vocal experience during sensorimotor learning. Thus, an important goal of future studies will be to determine the fate of synapses that are formed on HVC neurons during tutor song experience.

Another important question raised by the findings of the current study is whether the observed changes to HVC spines are driven by auditory experience of the tutor song or, alternatively, by vocal rehearsal that followed tutor song experience. Because birds exhibited vocal changes within the first day of tutor song experience, prior to the first post-tutoring imaging session, the in vivo imaging experiments were unable to distinguish whether structural changes are driven solely by auditory experience of the tutor song or by initial attempts at tutor song imitation. However, the finding that a bird that didn't sing during the first day of tutoring still exhibited functional enhancements of synaptic activity indicate that at least a portion of the changes I

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observed may occur independently of vocal rehearsal. To address this question with greater precision, recent studies have manipulated HVC activity specifically during auditory experience of the tutor song to test whether the precise pattern of HVC activity during auditory experience of the tutor song is critical for formation of an accurate tutor song memory. In these experiments, a juvenile was placed in a chamber with a live adult tutor, and electrical microstimulation of the juvenile's HVC was yoked to the production of a specific syllable by the tutor. Thus, neural activity in the juvenile's HVC was disrupted specifically as it listened to a specific portion of the tutor song but remained unperturbed during the juvenile's vocal rehearsal. Strikingly, subsequent imitation of the tutor song syllable that was blanketed by the microstimulation pulse was poor, although juveniles subsequently copy the non-stimulated portions of the tutor song with high accuracy (Roberts and Mooney, unpublished observations). These findings indicate that neural activity in HVC is critical to the online encoding of tutor song experience, and similar experiments will be useful to test the role of downstream regions of the SMP and the AFP in tutor song memory formation.

Finally, the identity of the presynaptic partners to the HVC dendritic spines that were stabilized, strengthened, and gained following tutor experience remains to be established. Two areas in the auditory forebrain, NIf and CM, have been shown to provide HVC with direct, excitatory auditory input (Bauer et al., 2008; Cardin and Schmidt, 2004; Coleman and Mooney, 2004). Interestingly, tutor song experience drove

the emergence of bursting in HVC PNs, and NIf has been shown to drive bursting in HVC during sleep (Hahnloser and Fee, 2007). Thus, an attractive possibility is that NIf may convey tutor song information to HVC. In support of this idea, microstimulation of NIf, but not CM, during tutor song experience blocks subsequent tutor song imitation in juveniles, implicating NIf to HVC synapses in the encoding of tutor song memory (Roberts and Mooney, unpublished observations).

In addition to these microstimulation experiments, in vivo imaging of axons of NIf, CM, and other afferents to HVC may be a useful approach to identify inputs to HVC that undergo increased structural stability and growth following tutor song experience. A previous study found that axonal inputs to the barrel cortex exhibited cell type-specific structural plasticity (De Paola et al., 2006), and I have preliminary data demonstrating the feasibility of imaging axonal inputs to the songbird HVC (Appendix A). Another possibility is that tutor song experience drives structural changes in local excitatory axon collaterals in HVC that contribute to the observed changes to HVC dendritic spines. This possibility could be addressed by injecting RA or Area X with a retrogradely-infecting GFP-virus in order to label only a single population of HVC PNs and their axons collaterals. These types of correlative imaging studies could be paired with functional manipulations of afferents to HVC during tutor song experience to shed light on the identity of the auditory inputs that convey tutor song information to HVC.

6.1.3 Both populations of HVC PNs are sensitive to tutor song experience that results in subsequent imitation

The finding that tutor song experience drives functional enhancements of spontaneous synaptic activity in both populations of HVC PNs suggests that tutor song experience may rapidly influence neural activity in areas downstream of HVC in the SMP and the AFP. While I did not measure the effects of tutor song experience on singing-related neural activity of HVC neurons, the pivotal role of HVC at the sensorimotor interface makes it quite likely that changes in the synaptic inputs to HVC PNs have direct consequences for song learning and song patterning. Indeed, although average rates of firing were unaffected by tutor song experience, I observed that spontaneous bursting in HVC PNs increased following tutoring. Spontaneous bursting in RA neurons immediately downstream of HVC has been shown to increase during the initial stages of tutor song imitation, and the temporal pattern of the bursting was related to the acoustic features of the tutor song heard by the juvenile (Shank and Margoliash, 2009). These effects are likely driven by changes in the output of HVC as a result of the changes to dendritic spines that were observed in the current study, because bursting activity in RA depends on input from HVC (Hahnloser et al., 2002, 2006). Furthermore, inactivation of NMDA-type glutamate receptors in LMAN during tutoring sessions has been shown to impair subsequent imitation (Basham et al., 1996), suggesting that HVC conveys tutor song information to downstream areas of the AFP areas during the initial stages of imitation. In this light, an important future direction

will be to understand whether the effects of tutor song in HVC and downstream areas of the AFP and SMP serve to encode the sensory representation of the tutor song, to transform this auditory information into changes in song motor output, or serve a combination of these two functions.

6.1.4 Behavioral relevance of effects of tutor song experience on HVC dendritic spines

The in vivo imaging experiments in the current study revealed that prior to tutor song experience, HVC is structurally dynamic, and subsequent tutor song experience causes a dramatic stabilization and strengthening of synaptic inputs onto both populations of HVC PNs. The stabilizing effects of tutor song experience peaked within ~48h and were accompanied by increases in the entropy variance of the juvenile's song. However, despite the fact that significant changes in song behavior can be detected within the first few days of tutor song experience, juvenile's songs continue to change substantially over the remainder of sensorimotor learning. What then is the behavioral consequence of the rapid, stabilizing effects of tutor song experience on HVC dendritic spines?

One idea is that tutor song experience sets up timing signals in HVC that are used to guide subsequent sensorimotor song learning. During singing in adult zebra finches, HVC$_{RA}$ neurons fire only a single short burst of action potentials at a single time during each song motif (Hahnloser and Fee, 2002), and an influential idea is that the sparse firing patterns of these neurons serve as timing signals to drive ensembles of

neurons downstream in RA. More specifically, it has been speculated that "chains" of

HVC$_{RA}$ neurons burst sequentially during song production and that this organized

propagation of activity drives the stereotyped timing and sequence of syllables and gaps

during the production of each motif (Li and Greenside, 2006; Jin et al., 2007).

Interestingly, focal cooling of HVC with a small Peltier device causes a uniform

stretching of song tempo, lending additional support to the idea that song timing is

generated (at least partially) within the local HVC circuit (Long and Fee, 2008). Thus,

tutor song experience could drive the organization of synaptic connections between

groups of HVC$_{RA}$ neurons, leading to the emergence of sequential and precise bursting

that specifies the timing of song elements. Indeed, tutor song experience leads to the

emergence of bursting in HVC neurons in the anesthetized bird (Roberts et al., 2010) and

also drives changes in the bursting properties of RA neurons (Shank and Margoliash,

2009).

These electrophysiological findings are interesting in light of the behavioral

finding that one of the earliest song changes following tutoring is the repetitive

production of "prototype" syllables within the first couple days of tutor song experience

(Tchernichovski et al., 2001). Although the spectral content of these prototype syllables

is later modified to give rise to the serial production of distinct syllables in the bird's

adult song, the early emergence of repeated sequences of prototype syllables indicates

that tutor song experience rapidly influences the timing of song production. Taken

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together, these findings raise the possibility that tutor song experience drives the emergence of precise bursting in HVC$_{RA}$ neurons that conveys timing signals for song production, although a direct test of this idea would require the measurement of the singing-related activity of individual HVC$_{RA}$ neurons in juvenile birds before and after tutor song experience.

Given that HVC$_X$ neurons exhibit auditory responses (Lewicki, 1996; Prather, 2008) and also receive strong feedforward input from HVC$_{RA}$ neurons (Mooney and Prather, 2005), I propose that the effects of tutor song experience on HVC$_X$ neurons serve to link song timing signals produced by HVC to the generation of song variability by the AFP. Very young juveniles produce subsong, a variable and unstructured form of vocal output which requires DLM (the thalamic nucleus of the AFP), LMAN (the output nucleus of the AFP), and RA, but not HVC (Aronov et al., 2008; Goldberg and Fee, 2011). Thus, prior to sensorimotor learning, there exists a circuit independent of HVC that generates vocal output. A great deal of evidence supports the idea that LMAN is essential for the generation of song variability during sensorimotor learning. For example, inactivation of LMAN reduces the trial-to-trial variability in juvenile songs (Olveczky et al., 2005), LMAN activity is more variable during the production of more variable song and is required for socially-modulated increases in song variability (Hessler and Doupe, 1999; Kao et al., 2005; Kao and Brainard, 2006; Kao et al., 2008), and microstimulation of LMAN drives spectral changes to song features (Kao et al., 2005).

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Additionally, experiments showing that LMAN is required for adaptive shifts in syllable pitch during contingent white noise playback suggest that LMAN is capable of driving instructive song variability that is precisely linked to a specific portion of the bird's song (Andalman and Fee, 2008). Hence, a parsimonious idea is that following tutor song experience, HVC_X neurons convey timing signals to the AFP that are used to entrain the song variability generated by the AFP to the timing of the song motor sequence generated in HVC. Interestingly, untutored birds produce more variable songs than normally-tutored birds and also take longer to produce stereotyped songs as adults (Eales, 1985; Price, 1979), indicating that the coupling between HVC and LMAN may be weaker in these birds. Future studies could test this idea by comparing the efficacy of signal transmission from HVC to different nuclei of the AFP in tutored and untutored birds.

If the rapid stabilizing effects of tutor song experience on HVC spines serve to set up timing signals for song, what is the relevance of the low level of HVC spine turnover that persists throughout the remainder of sensorimotor learning? One possibility is that these low levels of turnover are the minimum that can be achieved, essentially represent "noise" within the circuit, and are unrelated to sensorimotor learning. This scenario seems unlikely, though, because levels of HVC spine stability continue to increase in adult birds, even after the end of sensorimotor learning (personal observation). Perhaps a more likely possibility, then, is that the low level of HVC spine turnover contributes to

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a continued sculpting of the HVC network, which drives synaptic reorganization downstream in the AFP and in RA and supports ongoing gradual changes to the spectral and temporal features of song over development. Although there currently exists no experimental method to manipulate levels of spine dynamics in HVC, it would be interesting to test the effects on a juvenile's song of further reducing levels of HVC spine turnover after tutor song experience.

In summary, the finding that synaptic inputs onto HVC PNs are sensitive to tutor song experience allows us to distinguish between two general models of how tutor song information is used to guide song learning. In the first model, tutor song memory is stored solely in forebrain auditory areas; after the tutor song memory is encoded in auditory areas, it gradually influences the output of song sensorimotor circuits in a manner dependent on vocal rehearsal. In the second model, formation of the tutor song memory depends on auditory and song sensorimotor areas, and tutor song experience acts directly on these song motor circuits in a manner independent of vocal rehearsal. The finding that tutor song experience causes the rapid stabilization, accumulation, and enlargement of dendritic spines on HVC neurons supports the second model and highlights the sensitivity of in vivo imaging as a method to detect neural sensitivity to auditory experience. In conclusion, the findings of the current study for the first time link changes in sensory experience that drive imitative learning to synaptic reorganization of sensorimotor circuits important to singing. Thus, these findings

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implicate HVC in the neural representation of the tutor song and clarify our understanding of where, when, and how tutor song experience acts on song sensorimotor circuits to guide song learning.

6.2 Synaptic inputs onto HVC PNs are sensitive to deafening

In my second set of experiments (Chapters 3-5), I tested the hypothesis that deafening modifies synaptic inputs onto HVC neurons that provide input to a corticostriatal pathway important to vocal learning and feedback-dependent maintenance. Using longitudinal, in vivo imaging of dendritic spines, I first determined that deafening induces two cell type-specific structural correlates of weakening of synaptic inputs onto HVC_X neurons, namely decreased spine size and stability. Next, a highly sensitive method of behavioral analysis was used to show that a subset of these structural changes occur prior to the onset of deafening-induced vocal change. Using in vivo sharp electrode recordings, I demonstrated that these structural changes are paralleled by electrophysiologically-measured weakening of synaptic inputs in this same cell type. Finally, in vivo imaging experiments conducted in birds that received LMAN lesions prior to deafening demonstrated that deafening-induced changes in HVC_X neurons depend on the output of the AFP.

There are three important aspects to these findings. First, this is the first study to use in vivo, two-photon imaging to assess the effects of deafening on synaptic connectivity in the central nervous system. In so doing, this study provides the first

observation of sensitivity to deafening in any song sensorimotor area and, to my

knowledge, the first description of the effects of hearing loss on synaptic inputs to

sensorimotor neurons important to vocal control. Second, the timing of the observed

synaptic changes implicates HVC in the processing of auditory feedback-related

information. Third, deafening-induced changes in HVC require an intact AFP,

suggesting that a strictly feedforward model of the effects of feedback-related

information on song sensorimotor circuits is overly simplistic. Taken together, these

findings indicate that although deafening-induced synaptic reorganization in HVC_X

neurons ultimately depends on the output of downstream error correction circuits,

HVC_X neurons are sensitive to deafening and may play a role in the processing of

auditory feedback.

6.2.1 Deafening exerts cell type-specific effects on dendritic spines on HVC_X neurons

This study highlights the technical strengths of longitudinal in vivo imaging as a

method to track effects of auditory feedback on sensorimotor neurons important to

singing and song learning. While previous studies employing chronic

electrophysiological recordings failed to detect feedback-driven changes in neuronal

activity in sensorimotor neurons at the input or output of the AFP (Hessler and Doupe,

1999; Kozhevnikov and Fee, 2007; Leonardo, 2004; Prather et al., 2008), in vivo imaging

allowed me to detect subtle changes in dendritic spines of HVC neurons that provide

input to the AFP within the first few days of deafening. By tracking dendritic spines on

single HVC neurons, I was able characterize the cell type-specificity and time course of deafening-induced changes in synaptic inputs with a degree of precision that would be difficult to achieve using electrophysiological methods.

Can I be certain that deafening only drives changes in synaptic inputs to HVC_X neurons? Because I only tracked changed to HVC PNs, a remaining possibility is that HVC interneurons are sensitive to deafening. Indeed, a previous study in Bengalese finches found that the output of putative HVC interneurons was slightly suppressed during feedback perturbation (Sakata and Brainard, 2008), raising the possibility that this cell type may be sensitive to deafening in zebra finches. However, a major caveat to the interpretation of the Sakata and Brainard study is that fact that acute feedback perturbation drives immediate changes to Bengalese finch song. Therefore, even though HVC interneuron activity was decreased by feedback perturbation on trials in which song output was not affected, it remains possible that all changes to HVC interneuron activity were motor-related and the difference in motor signal on these trials was simply below the threshold necessary to drive a change in vocal output. Despite these limitations, an appealing idea is that HVC interneurons might act as real-time sensors of auditory feedback, while HVC_X neurons act as accumulators of feedback-related information and only change their neural output more slowly. In this scenario, one would expect that changes in inhibitory inputs onto HVC_X neurons would precede and perhaps even drive changes to dendritic spines. However, measurements of sensitivity

to feedback perturbation would need to be made in HVC interneurons in zebra finches to fully test this idea. Regardless of these additional possibilities, the fact that structural changes to dendritic spines occurred only in HVC_X neurons supports the idea that this cell type is sensitive to deafening.

While the current study is the first to resolve synaptic-level consequences of deafening in a sensorimotor area important to vocal learning, it is useful to consider how the current findings compare to previous studies of the effects of hearing loss on the central auditory system. At lower stages of the central auditory system, hearing loss can have especially dramatic effects, especially if it occurs very early in development. For example, cochlear removal or damage decreases spontaneous activity in the auditory nerve (Cook et al., 2002) and in the cochlear nucleus (Born et al., 1991; Park et al., 1999; Tucci et al,. 2001). Additionally, cochlear removal results in a loss of nearly one-third of the neurons in the cochlear nucleus and a reduction in size of the remaining neurons (Born and Rubel, 1985); however, these effects appear to be highly age-dependent and are more pronounced when hearing loss occurs prior to the normal time of hearing onset (Hashisaki and Rubel, 1989; Tierney et al., 1997).

Dramatic effects such as cell death have not been reported outside of the auditory brainstem following hearing loss. Rather, in auditory regions from the midbrain to the cortex, hearing loss alters the balance of excitation and inhibition (Sanes and Bao, 2009). In the inferior colliculus, for example, unilateral deafening increases

neural responses to acoustically-driven activity (Kitzes and Semple, 1985; McAlpine et al., 1997; Szczepaniak and Møller, 1995). Increases in levels of spontaneous activity and excitability have also been described in the auditory cortex following hearing loss (Kotak et al., 2005; Noreña and Eggermont, 2003; Seki and Eggermont, 2003), along with reductions in the amplitude of spontaneous and evoked inhibitory currents and GABA release probability (Kotak et al., 2008). Importantly, although precise measurements of excitatory and inhibitory activity are impractical in human subjects, imaging studies in humans show that deaf or hearing impaired subjects exhibit larger ratios of gray matter to white matter in auditory cortical areas (Emmorey et al., 2003; Kim et al., 2009; Shibata, 2007; Smith et al., 2011). This finding indicates that deafening reduces myelinated axonal connections in the auditory cortex and, hence, likely alters synaptic connectivity in the auditory cortex. In summary, although prior studies have not yet resolved synaptic level consequences of deafening in sensorimotor areas important to vocal output, previous studies in both humans and animal models provide ample evidence that hearing loss alters synaptic transmission in auditory areas, including the auditory cortex.

The current study is the first to demonstrate that deafening-induced changes in synaptic transmission in the central auditory system propagate into and alter synaptic connectivity within sensorimotor areas important to the learning and maintenance of vocalizations, providing a framework to understand how changes in auditory feedback

drive changes in vocal output. The observation that deafening alters the dendritic

spines, excitability, and spontaneous activity of HVC_X neurons indicates that the effects

of deafening on auditory areas are translated into changes in synaptic inputs onto and

the neural output of sensorimotor neurons, and more specifically, sensorimotor neurons

that project into a striatal pathway important to song learning. These results are

especially interesting in light of the finding that damage to the basal ganglia in adult

humans can cause speech deficits, including changes in prosody, articulation,

comprehension and production (Damasio et al., 1982). Thus, the finding that deafening

drives changes to dendritic spines in HVC_X neurons prior to the onset of song

degradation suggests that altered auditory feedback permeates quickly into the song

sensorimotor network, and the effects of hearing loss in humans on sensorimotor

neurons that control speech learning and production, including corticostriatal circuits,

may likewise be relatively rapid.

How do the effects of deafening on HVC dendritic spines compare to the effects

of sensory deprivation on spine dynamics in other sensory domains? Studies in the rat

somatosensory system have shown that whisker trimming decreases the stability of

dendritic spines, by driving the loss of spines that were previously stable and

stabilization of newly-formed spines in barrel cortex (Holtmaat et al., 2006; Trachtenberg

et al., 2002). Similarly, focal lesions of the retina cause a dramatic decrease in levels of

spine stability in the visual cortex, leading to an almost complete replacement of

dendritic spines in the deafferented region of cortex (Keck et al., 2008). I found that

deafening weakened synaptic inputs and decreased spine stability in HVC_X neurons,

suggesting that decreased spine stability leading to synaptic reorganization may be a

general effect of sensory deprivation on higher level sensory and sensorimotor brain

regions. Interestingly, whisker trimming and visual deprivation have both been shown

to induce structural reorganization of inhibitory cortical circuits (Marik et al., 2010; Chen

et al., 2011), which can precede reorganization of excitatory circuits (Marik et al., 2010).

Thus, an important goal will be to test the possibility that changes in inhibitory inputs

onto HVC_X neurons may accompany the observed changes in excitatory inputs onto this

cell type following deafening.

Although the effects of deafening on HVC dendritic spines mirror those seen in

cortical neurons during other forms of sensory deprivation, two lines of evidence

support the idea that the synaptic reorganization observed in the current study is driven

by perturbation of singing-related auditory feedback and not merely by auditory

deafferentation. First, I observed cell type-specific effects of deafening on excitatory

synaptic inputs, despite the fact that both HVC PN types receive auditory inputs from

extrinsic sources (Bauer et al., 2008; Cardin and Schmidt, 2004; Coleman and Mooney,

2004; Nottebohm et al., 1982). Because excitatory inputs to HVC PNs are thought to

carry auditory drive, removal of auditory input would be expected to affect dendritic

spines on both cell types. Thus, it is difficult to imagine how changes to dendritic spines

would fail to occur in both HVC PN types if they were simply a result of auditory deprivation. Second, I conducted a single imaging experiment in which a bird did not sing for the first week following deafening. The HVC$_X$ neuron imaged in this bird did not exhibit a decrease in spine size index or spine stability, providing further support for the idea that deafening-induced changes are linked to perturbation of singing-related auditory feedback, rather than auditory deafferentation.

The identity of the excitatory synaptic inputs to HVC$_X$ neurons that undergo reorganization following deafening is an important issue to resolve in future studies. HVC receives a number of extrinsic inputs, including those from the cortical region mMAN, the thalamic nucleus Uva, and two areas of the auditory telencephalon, NIf and CM (Bauer et al., 2008; Cardin and Schmidt, 2004; Coleman and Mooney, 2004; Foster and Bottjer, 1998; Fortune and Margoliash, 1995; Vates et al., 1996). Lesions of mMAN disrupt song learning in juveniles but do not drive song degradation in adult birds (Foster and Bottjer, 2001), indicating that this area does not convey auditory feedback information important to song maintenance to HVC. The nucleus Uva is also an unlikely candidate to convey auditory feedback information to HVC, because reversible inactivation of Uva does not affect auditory responses in HVC (Coleman et al., 2007). As a major source of auditory input to HVC, NIf is a promising candidate to convey auditory feedback information to HVC (Cardin and Schmidt, 2004; Coleman and Mooney, 2004; Fortune and Margoliash, 1995; Vates et al., 1996). However, lesions of

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NIf do not trigger song degradation in adult zebra finches (Cardin et al., 2005), and NIf is not required for song decrystallization driven by vocal nerve cut, a process that is thought to result from distorted auditory feedback (Roy and Mooney, 2009). Additionally, strong and selective auditory responses persist in HVC and LMAN following NIf lesions, suggesting that HVC may receive an alternate source of auditory information (Roy and Mooney, 2009).

A parallel source of auditory input to HVC is CM, which is active during singing and during auditory presentation of other bird's songs (Bauer et al., 2008). Moreover, CM contains neurons whose singing-related activity is sensitive in real-time to feedback perturbation (Keller and Hahnloser, 2009). Although these findings hint that CM could convey auditory feedback information to HVC, a causal role for CM in feedback-dependent song degradation remains to be characterized, and the cell type-specificity of its projections to HVC await description. In addition to extrinsic excitatory inputs, HVC$_X$ neurons receive local excitatory inputs from HVC$_{RA}$ and other HVC$_X$ neurons. However, HVC$_{RA}$ neurons also receive inputs from both HVC PN types, making it unlikely that connections intrinsic to HVC would change in a cell type-selective manner following deafening (Mooney and Prather, 2005). While the presynaptic partners to the spines that are weakened by deafening await identification, investigating the circuit basis for the cell type-selectivity of deafening-induced changes in HVC, with a particular focus on CM, is an important future direction (see Appendix A).

6.2.2 Deafening reduces the size of stable spines on HVC$_X$ neurons prior to song degradation

The finding that deafening-induced changes in HVC$_X$ spine size index occur

prior to the onset of vocal deterioration links the observed changes in HVC synaptic

inputs to perturbation of auditory feedback rather than to changes in vocal output.

Notably, the method of behavioral analysis employed here allowed me to detect changes

to song days to weeks earlier than in previous deafening studies (Brainard and Doupe,

2000; Horita et al., 2008; Lombardino and Nottebohm, 2000; Nordeen and Nordeen,

1992) and allowed the detection of subtle vocal changes in deafened birds with LMAN

lesions (in contrast to Brainard and Doupe, 2000), indicating that this analysis is highly

sensitive to early changes to song following deafening. Additionally, my finding that

significant effects of deafening on song spectral features can be detected within the first

few days following deafening across a large range of ages contrasts with previous

studies (Lombardino and Nottebohm, 2000) and further supports the idea that my

analysis is sensitive to extremely early deafening-induced changes to song.

It is notable that although I detected relatively rapid effects of deafening on song

behavior, these changes were still not instantaneous. This delayed effect of deafening on

vocal output contrasts with the real-time (<100 ms) effects on vocal output of other

changes in auditory feedback, as exhibited during Doppler-shift compensation in bats

(Schnitzler, 1973), during the Lombard effect in humans, birds, and even whales (Cynx

et al., 1998; Lane and Tranel, 1971; Lombard, 1911; Scheifele et al., 2005) and during

frequency shift compensation in humans (Elman, 1981). Alternative manipulations of

auditory experience in songbirds, such as song-targeted electrical stimulation of the

auditory thalamus (Lei and Mooney, 2010) and playback of white noise that is

contingent on song pitch (Andalman and Fee, 2009; Tumer and Brainard, 2007) have

been shown to drive faster degradation of song than deafening, with changes becoming

detectable within hours. However, it remains unclear whether these manipulations tap

into the same mechanisms that allow singing-related auditory feedback to shape vocal

output or whether they drive song degradation through an alternative mechanism, such

as learned aversion to electrical stimulation or white noise playback. Regardless of

potential differences, all of these methods exert a delayed effect on song behavior,

raising the idea that a neural integrator somewhere in the brain accumulates auditory

error information to drive changes in vocal output. Indeed, the finding that deafening

drives slow changes to dendritic spines on HVC_X neurons raises the possibility that

these cells act as slow integrators of error information, although an alternative

possibility is that the integrator lies outside of HVC_X neurons and can only slowly drive

changes in the song sensorimotor circuit.

6.2.3 Deafening-induced spine changes in HVC depend on an intact AFP

A challenge of localizing the effects of deafening in a highly recurrent

sensorimotor network such as the song system is that auditory feedback may act in a

distributed manner, and its effects on a particular sensorimotor area may be dependent

on input from other sensorimotor areas. Consistent with this possibility, I found that

deafening-induced changes to HVC$_X$ dendritic spines depend on the output of the AFP.

This result is surprising, because HVC does not receive direct input from LMAN, and

the function of possible indirect connections from LMAN to HVC remains enigmatic.

One possibility is that deafening-induced changes in LMAN output actively drive the

observed changes in synaptic inputs to HVC$_X$ neurons following deafening. In this case,

auditory feedback information would enter the AFP independently of HVC, perhaps via

the ventral tegmental area (Gale et al., 2008; Vates et al., 1996), which provides input to

Area X (Lewis et al., 1981), and changes in LMAN output would propagate back to HVC

via recurrent circuitry to drive the observed synaptic reorganization following

deafening. Thus, auditory feedback would bypass HVC and the observed changes to

HVC$_X$ dendritic spines, although driven indirectly by perturbation of auditory feedback,

would depend on the AFP. A second possibility is that LMAN output acts permissively

to enable feedback-driven changes in HVC. In this case, auditory feedback would

impinge directly on HVC, but recurrent signals from LMAN would maintain HVC in a

competent state to respond to these feedback signals. In either scenario, LMAN could

influence synaptic reorganization in HVC via the monosynaptic connection from RA to

HVC (Roberts et al., 2008), through a brainstem pathway indirectly linking RA and HVC

(Striedter and Vu, 1998), or through midbrain dopaminergic inputs to HVC (Bottjer,

1993; Soha et al., 1996).

A number of experiments could be carried out to distinguish between these two possibilities. Given that LMAN activity becomes less variable in older birds as song stereotypy increases (Kao and Brainard, 2006), one idea is that feedback perturbation drives changes to vocal output by increasing the amount of LMAN activity and thereby enhancing the ability of LMAN to drive changes in synaptic connectivity in RA Thus, one experiment would be to test whether acute increases in LMAN activity are capable of driving changes to HVC dendritic spines, and if so, whether the cell type-specificity of dendritic spine changes matches that seen following deafening. A second experiment to test whether auditory feedback information impinges directly on HVC_X neurons would be to use intracellular recordings in behaving birds (Long et al., 2010) to search for rapid changes in subthreshold inputs to HVC_X neurons following feedback perturbation. Although deafening-induced changes to HVC_X dendritic spines were observed over a few days in the current study, the detection of more rapid changes to synaptic inputs to HVC_X neurons would lend support to the idea that auditory feedback acts directly on HVC. An additional important step would be use the same technique to search for effects of feedback perturbation on synaptic inputs to LMAN and to characterize the timing of any observed changes relative to those found in HVC.

While the exact involvement of the AFP in deafening-induced synaptic reorganization in HVC awaits resolution, the current findings allow us to distinguish between two general ideas regarding the locus where auditory feedback signals act

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within song sensorimotor circuits to influence vocal output. In the first model, HVC is a

static source of song timing signals and is insensitive to auditory feedback. In the

second model, auditory feedback information accesses HVC, and feedback perturbation

drives changes in HVC that occur prior to vocal deterioration. I found partial support

for the first model, because changes to excitatory synaptic inputs on HVC$_{RA}$ neurons

were not detected following deafening. However, the finding that deafening drives

changes in synaptic inputs onto HVC$_X$ neurons indicates that this cell type is sensitive to

auditory feedback. Although deafening-induced synaptic changes depend on the

output of the AFP, indicating that a purely feedforward view of auditory feedback

processing is overly simplistic, my results refute the notion that HVC$_X$ cells are

insensitive to changes in auditory feedback. In summary, these findings implicate the

input stage to the AFP in the processing of auditory feedback.

6.2.4 Behavioral relevance of effects of deafening on HVC dendritic spines

The major finding of these experiments is that synaptic inputs to HVC$_X$ neurons

are sensitive to deafening. An important future direction will be to determine whether

changes to HVC$_X$ dendritic spines are driven by extrinsic auditory inputs, occur in

response to changes to inputs from HVC interneurons, are driven indirectly by recurrent

inputs from the AFP, or are driven by changes in some combination of these inputs.

Regardless of which inputs drive synaptic reorganization in HVC, the cell type

specificity of deafening-induced changes to dendritic spines is striking and suggests that

changes in auditory feedback ultimately cause the preferential refinement of synaptic inputs onto HVC$_X$ neurons.

A major unresolved issue is the significance of these structural changes to singing and song learning. Therefore, an important future goal will be determine whether the observed changes to HVC$_X$ dendritic spines affect the singing-related output of these cells and ultimately affect song. The sequence of structural changes to dendritic spines on HVC$_X$ neurons allows us to speculate when and how the output of these neurons might change following deafening. Deafening drives an early shrinking of stable dendritic spines on HVC$_X$ neurons followed by a delayed increase in spine turnover. Taken together, these findings raise the possibility that spine weakening must reach a critical threshold in order for increases in spine turnover to ensue. Additionally, deafening tends to increase firing frequency and significantly decreases inter-spike intervals measured in HVC$_X$ neurons, at least in anesthetized birds. A parsimonious explanation for these structural and functional changes is that, within the first few days after deafening, HVC$_X$ neurons begin broadly integrating weaker synaptic inputs, resulting in decreased precision in their action potential output. Thus, a reasonable prediction is that the singing-related output of HVC$_X$ neurons would become more variable within this same time frame.

Notably, spine turnover in HVC$_X$ neurons returns to control values at long times (~40d) post-deafening, indicating that not all of the deafening-induced changes to HVC$_X$

dendritic spines persist indefinitely. One possibility, then, is that deafening increases the variability of singing-related activity in HVC$_X$ neurons, but as spine turnover returns to control levels, that output of these neurons resettles back into to a precise and time-locked pattern. However, it remains untested whether the average strength of synaptic inputs to HVC$_X$ neurons remains smaller than controls at long times post-deafening. If this were the case, the spiking output of HVC$_X$ neurons might be maintained indefinitely in a more variable and less time-locked state during singing.

Although it is not currently possible to record from the same HVC$_X$ neuron for more than a few hours, one way to test these ideas would be to compare the precision of the singing-related activity of different HVC$_X$ neurons at different time points after deafening. Additionally, experiments using optogenetics to selectively manipulate the singing-related activity of HVC$_X$ neurons would shed light on the behavioral consequences of changes in the action potential output of this cell type. The finding that selective ablation of the majority (~85%) of HVC$_X$ neurons in adult birds does not drive song degradation (Scharff et al., 2000) raises the possibility that altered feedback affects song by modulating the singing-related activity of HVC$_X$ neurons rather than by silencing their output altogether. Thus, future optogenetic experiments should test the behavioral effects of either increasing the amount or the variability of singing-related HVC$_X$ neuron output or changing the manner in which the output of these neurons is time-locked to song.

It is surprising that dendritic spines on HVC$_{RA}$ neurons don't change following deafening, even after song has begun to severely degrade. One possibility is that HVC$_{RA}$ neurons generate an invariant timing signal that remains constant in adult zebra finches, even in the face of feedback perturbation. To address this question, future studies could measure the singing-related activity of HVC$_{RA}$ neurons at different time points after deafening. The finding that dendritic spines on HVC$_{RA}$ neurons are not altered by deafening or by ensuing song degradation suggests either that deafening-induced changes to song are driven solely by synaptic reorganization in HVC$_X$ neurons and in downstream areas of the AFP or, alternatively, that song degradation drives changes in HVC$_{RA}$ neurons that do not manifest as structural changes to dendritic spines.

While the findings of the current study implicate HVC$_X$ neurons in the processing of auditory feedback, it is unclear how the effects of deafening on HVC$_X$ dendritic spines relate to the synaptic reorganization that occurs when a juvenile bird uses auditory feedback to correct vocal errors during sensorimotor learning. Deafening removes all singing-related auditory feedback, and an assumption of this study is that this manipulation drives the production of a global error signal that eventually results in the degradation of the entire song motif. In contrast, other perturbations of auditory feedback, such as contingent playback of white noise (Andalman and Fee, 2009; Tumer and Brainard, 2007) and syllable-triggered stimulation of the auditory thalamus (Lei and Mooney, 2010) presumably increase the amount of information contained in the singing-

related auditory feedback signal. Thus, it remains unclear how neural changes driven by manipulations that either subtract or add information to the singing-related auditory signal compare to the neural changes that occur during sensorimotor learning. Despite these limitations, there is evidence that the AFP is involved in a number of distinct auditory feedback-dependent behavioral processes, including the Lombard effect (Kobayasi and Okanoya, 2003). Thus, while the exact details of synaptic reorganization that occur when auditory feedback is during sensorimotor learning may differ from the effects of deafening, it is likely that both processes depend on the same underlying neural circuit and involve synaptic reorganization in HVC$_X$ neurons.

6.3 Are tutor song information and singing-related auditory feedback compared in HVC?

In order for song learning to occur, singing-related auditory feedback must be compared with the stored representation of the tutor song, error signals must be generated when there is a mismatch between the bird's own song and the tutor song, and these error signals must be used to drive adaptive changes in song motor output. In this context, the findings of the current study are significant because they demonstrate for the first time that HVC is sensitive to both types of auditory information important for song learning. However, an important goal for future studies will be to determine whether HVC serves as a relay for tutor song information and auditory feedback, a

comparator of these two types of information and a source of error signals, or is important to the implementation of error signals to drive changes in vocal output.

What are the challenges of determining whether HVC compares tutor song information and auditory feedback to guide song learning? First, although tutor song experience drives rapid synaptic reorganization in HVC, it remains to be seen whether downstream sensorimotor areas are also affected by tutor song experience. Even if tutor song memory formation depends on the strengthening of HVC synapses, it is possible that the memory is later stabilized and consolidated in a manner that depends on sensorimotor areas downstream of HVC. In this case, comparison of the tutor song memory with auditory feedback could occur in a distributed manner in multiple locations within the song sensorimotor network. Likewise, future studies must determine which sensorimotor areas outside of HVC are sensitive to perturbation of auditory feedback and, additionally, whether any observed effects of feedback perturbation in these areas precede or follow the synaptic reorganization observed in HVC. Thus, a definitive identification of the neural comparator circuit awaits a complete characterization of the sensitivity of the entire song sensorimotor circuit to tutor song experience and auditory feedback.

To determine whether HVC might act as part of a comparator circuit, it is useful to consider whether HVC behaves as we would expect a comparator to behave. First, the comparator must have access to tutor song information and auditory feedback

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information, and HVC$_X$ neurons in particular satisfy this criterion. Second, the output of

the comparator should scale with the goodness of match between the tutor song and the

bird's vocal output. However, it remains unknown whether the comparator changes its

output depending on this goodness of match on a trial-by-trial basis, or rather, slowly

accumulates goodness of match information and only changes its output over longer

timescales. HVC$_X$ neurons do not fit the requirements for a comparator that acts in real-

time, because their action potential output is not affected by real-time perturbations of

auditory feedback (Kozhevnikov et al., 2008; Prather et al., 2008). Nonetheless, an

important goal of future studies will be to determine whether the action potential output

of HVC$_X$ neurons changes more slowly following perturbations of auditory feedback but

prior to behavioral change. Thus, although the current study has established for the first

time that HVC is sensitive to tutor song and auditory feedback, it still remains to be seen

whether HVC is part of the comparator circuit.

In summary, this study is the first to show that HVC synapses are sensitive to

tutor song experience and deafening. My findings demonstrate that tutor song

experience exerts a dramatic strengthening and stabilizing effects on synaptic inputs to

both HVC PN types, while deafening acts selectively to sculpt synaptic inputs onto

HVC$_X$ neurons, implicating this cell type in the processing of auditory feedback as well

as tutor song experience. Although HVC has been shown to be a source of precise

timing signals for song production, the findings of the current study indicate that HVC

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is a site where auditory experience drives synaptic reorganization that could ultimately drive changes in vocal output. While the specific role of HVC in the comparison of these tutor song experience and auditory feedback remains to be determined, my findings link changes in sensory experience that drive behavioral learning to synaptic reorganization in sensorimotor circuits that control behavior and thus shed light on the mechanisms through which auditory experience is used to guide vocal learning.

Appendix A: In vivo imaging of NIf axons in HVC
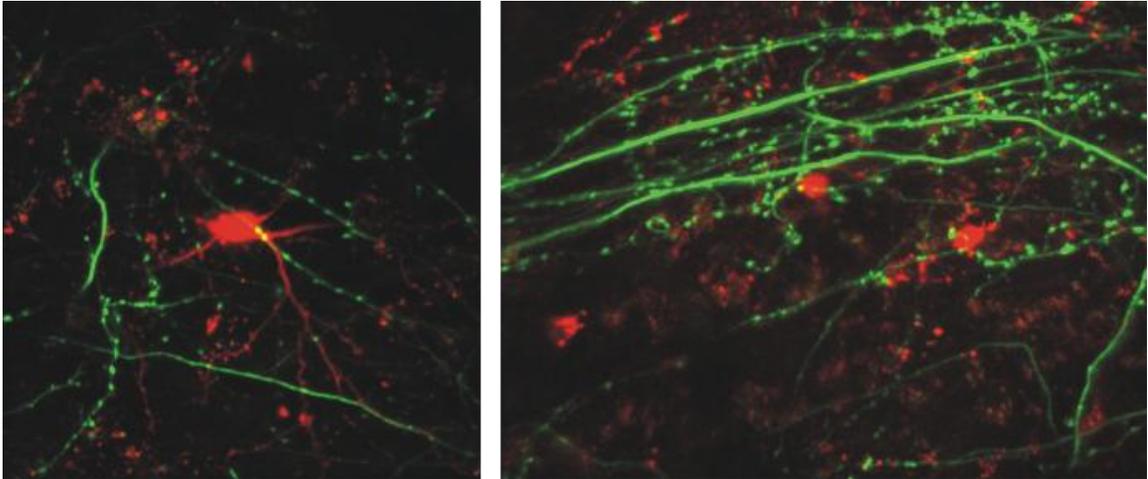
Figure 32: In vivo imaging of NIf axons in HVC. Two images are shown of in vivo labeling of NIf axons (green) seen coursing through HVC. HVC_RA neurons are labeled in both images with a red retrograde tracer.

The two images above show in vivo labeling of NIf axons (green) coursing through HVC (HVC_RA neurons are labeled in red). These preliminary images demonstrate the feasibility of imaging and tracking the structural dynamics of extrinsic auditory inputs to HVC, including those from NIf and CM, to determine whether these inputs are sensitive to tutor song experience or deafening.

Appendix B: Experimental methods

Tutoring and behavioral measurements of tutor song learning

Isolation and tutoring of juveniles: Juvenile males were removed from their home cages in the general colony around 10 dph, an age before juvenile zebra finches are able to form an auditory memory of a tutor song. These untutored juveniles were subsequently raised with adult females, which do not sing, and other untutored siblings until the beginning of the experiment. Thus, untutored birds had largely normal social and auditory experiences during development, except for the fact that they never heard the song of an adult male. Control birds were raised in our breeding colony and were given full access to their adult tutor until at least 43 dph. Following pre-tutoring experimental measurements, juveniles were provided with tutor experience for three consecutive days, either via introduction of a live tutor or via keypeck-triggered tutoring. In the case of juveniles that were tutored using keypeck-triggered tutoring, juveniles were able to trigger playback of a pre-recorded tutor song by pecking a small key placed inside their cage. Birds tutored in this manner were used to control for any potential social effects of live tutor experience on synaptic inputs onto HVC projection neurons. Following removal of the live tutor or the keypeck, birds were raised in visual isolation from other birds until they reached adulthood (120 dph).

Quantification of tutor song learning: Song behavior was recorded continuously starting at least two days before the first day of tutoring out until at least three days

post-tutoring. Thereafter, songs were recorded semi-continuously until adulthood (120

dph). Song learning was quantified using the "percentage similarity" measure in Sound

Analysis Pro to measure the similarity between the tutor song and the pupil song.

Syllable clusters in the juvenile song that were proto-syllables for the adult song motif

were retroactively identified. Briefly, the acoustic similarity between the tutor song and

the juvenile's song was calculated by measuring pitch, amplitude modulation, frequency

modulation, Weiner entropy, and goodness of pitch of the tutor and pupil songs across

10 ms time windows, omitting sections that do not account for 10 ms x 100% similarity,

followed by computing the percentage of elements that were significantly similar ($p <$

0.1) between the pupil and the tutor song across 70 ms time windows. Simply stated,

this metric reflects the percentage of elements in the pupil's song that are similar to

those in the tutor's song. I first calculated the similarity between the tutor song and the

adult (120-130 dph) song of the pupil ($N = 45$ comparisons per bird per time point.

Then, to control for any spurious resemblance between birds' pre-tutoring vocalizations

and the tutor song, I calculated the similarity between the tutor song and the song of the

pupil 1 day prior to tutor exposure.   This value was then subtracted from the similarity

score of the pupil's adult song tutor to generate a learning index for each bird (similarity

of adult song to tutor minus similarity of pre-tutoring song). To measure the onset of

changes in song following tutoring, I calculated the entropy variance, which is a

measure of song complexity and an early indicator of tutor song imitation, of identified

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proto-syllable clusters from afternoon recording sessions, starting two days prior to

tutoring and during the three days of tutoring.

Feedback perturbation and behavioral measurements of song degradation

Feedback perturbation: Male zebra finches aged 85 to 150 dph were anesthetized by

isoflurane inhalation (2%) and were deafened by bilateral removal of the cochleae.

Quantification of deafening-induced spectral changes to song: Song behavior was

recorded continuously starting at least two days before deafening until at least 1 week

post-deafening and out as long as 5 weeks post-deafening. Changes to the spectral

features of song following deafening were determined by quantifying the entropy and

entropy variance of each syllable in a bird's song. Song syllables were defined as song

elements separated by at least 15 ms of silence, and song motifs were defined as single

renditions of a group of syllables (i.e., ABCD). Thirty examples of each syllable were

measured on each day of song, and the values from two baseline days were pooled to

obtain a baseline distribution of values for each syllable. The onset of degradation was

defined as the day on which the distribution of values for either entropy or EV of any

syllable differed significantly from the baseline distribution of the measure for that

syllable (one-way ANOVA, $p < 0.05$) and was maintained significantly different on all

subsequent days measured. The sensitivity of this analysis was verified using custom-

written, semi-automated software that allowed measurements to be made of all

occurrences of a syllable on each day (Matlab, written by Kosuke Hamaguchi), and equivalent results were obtained. It should be noted that this syllable-by-syllable analysis allowed me to detect deafening-induced changes to spectral features of song earlier than any other method previously reported in the literature; I observed song degradation in 95% of the birds within the first three days of post-deafening song. This is likely because previous studies have used global measures of song spectral features rather than separately considering the trajectory of each syllable.

A cumulative entropy difference was used to determine how many motifs a bird sang prior to song degradation (Lei and Mooney, 2010). Briefly, the first syllable in each bird's song to undergo degradation was identified post hoc, and the baseline entropy or EV was calculated and subtracted from the value of each rendition of the syllable produced during the baseline day. The standard deviation of the cumulative sum of the resulting differences was calculated (CS_{stdev}) and used to determine how many post-deafening motifs were sung before the post-deafening CS of differences from the baseline mean exceeded three times the value of CS_{stdev}.

I also calculated a global index of song degradation by normalizing each syllable's entropy and entropy variance measurements to that syllable's baseline values and then pooling the normalized values for all syllables on each day of song. This global measure of song degradation was sensitive enough to detect long-term differences in deafening-induced song degradation related to age and LMAN lesion (see later section)

but was not sensitive enough to detect the earliest changes to song (within the first three days of post-deafening song) detected by the syllable-by-syllable analysis.

Quantification of deafening-induced temporal changes to song: Changes to the temporal features of song were determined by measuring the sequence consistency of the song. The most typical sequence of syllable transitions in a bird's song was defined (for example, a bird singing the song motif ABC would have four typical transitions: silence to A, A to B, B to C, and C to end), and sequence consistency was measured for 30 motifs from each day of song as (number of typical transitions/total transitions). The onset of temporal change to song was defined as the first day on which the mean sequence consistency was lower than the lower bound of the confidence interval for the mean sequence consistency as measured on the last pre-deafening day.

In vivo, longitudinal imaging of dendritic spines

Fluorescent labeling of HVC neurons: Male zebra finches were anesthetized with isoflurane inhalation (2%) and were placed in a stereotaxic apparatus. Injection target sites were located using stereotaxic coordinates and multi-unit neural recordings. A glass microelectrode attached to a Nanoject II (Drummond Scientific) was used to deliver GFP-lentivirus (expressing enhanced green fluorescent protein under the control of the Rous Sarcoma Virus LTR (FRGW)) to HVC or the neuronal retrograde tracers Fast Blue and/or Alexa-Fluor 594 conjugated dextran amines to Area X and RA (lentivirus: 32.2 nL/injection with a total injection volume of about 1 µL; tracers: 32.2 nL/injection

with a total injection volume of 64-160 nL). Lentiviral injections were performed at least

two weeks before imaging, and tracers were injected 4-7 days before imaging.

In vivo, two-photon imaging: Beginning at least 1 week prior to the first imaging

session, birds were placed on a reversed day-night cycle, to minimize effects of imaging

on their daytime singing behavior. On the first night of imaging, birds were

anesthetized with isoflurane inhalation (2%) and placed in a stereotaxic apparatus. The

scalp overlying HVC was removed, the scalp margins were sealed to the surface of the

skull using Vetbond (n-butyl cyanoacrylate), and a headpost was affixed the anterior

skull using dental acrylic. Bilateral craniotomies of 1-2 mm^2 were made in the skull

overlying HVC. The dura mater was excised, leaving the pia mater and the 60-150 μm

thick layer of neural tissue and lateral telencephalic ventricle overlying HVC intact. A

custom-cut coverslip (No. 1 thickness) was placed over the pial surface and was sealed

in with dental acrylic. Birds were placed on a custom stage under a Zeiss Laser

Scanning Two-Photon Microscope 510. Only GFP-labeled neurons located within a field

of retrogradely-labeled neurons were classified as HVC neurons and imaged. Dendritic

segments of identified HVC neurons were imaged at high resolution during the bird's

subjective night (1024 x 1024 pixels, 76 x 76 μm^2 image size, 3.2 μs/pixel, averaging 2

samples per pixel with 1 μm z-steps, focuses through a 40x/0.8NA Zeiss IR-Archoplan

immersion objective). Birds were imaged twice a night; after the imaging session

starting at time 0, birds were returned to a darkened holding cage and allowed two

sleep until re-imaging 2 hrs later (time 2). Two imaging sessions were repeated at the

same time on each subsequent night for as many nights as possible, in some cases, up to

4 weeks in total.

For the tutoring experiments, HVC neurons in 60 dph juvenile males were

imaged longitudinally, beginning the night prior to the first tutoring session. For the

auditory feedback experiments, birds ranging from 85 to 140 dph were imaged

longitudinally, beginning 1-2 nights prior to deafening.

Image analysis: Dendritic segments to be analyzed were selected and identified in

image stacks collected either 2h or 24h apart. For the tutoring experiments, all sets of

image stacks were coded and scored by researchers blind to the experimental condition.

For the auditory feedback experiments, spine stability and density measurements were

blinded. For spine size measurements, the measurements were semi-blinded; the

experimenter knew the night of imaging (night 1 scored against night 2, night 2 against

night 3, etc.) but did not know the cell type (HVC_{RA} or HVC_X), did not know the onset of

song degradation for the animal, and did not perform the final calculation (size index)

until all measurements had been made for a given cell. Standard non-parametric and

parametric statistical methods were used to detect significant differences (alpha = 0.05).

A. **Determination of projection neuron class:** Projection neuron class was

determined either by the presence of retrograde label or using the morphological

criterion of soma size. Soma area measurements were made for each cell from a

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single plane of a z-stack of images, in which the largest amount of the soma was in focus. Distributions of soma size area for the two HVC PN types were calculated by measuring soma area in a large number of in vivo-imaged HVC neurons that were double-labeled with GFP and retrograde tracer. Based on this distribution, any neuron in the current study with a soma area greater than 222 μm^2 (mean HVC_{RA} soma area plus two standard deviations) was categorized as an HVC_X neuron, and any neuron with a soma area less than 173 μm^2 (mean HVC_X soma area minus two standard deviations) was categorized as an HVC_{RA} neuron.

B. **Spine size measurements:** Spine size for each cell was examined across 24h intervals (calculated across nights). The size of a given spine at a single time point was determined by calculating the integrated optical density of the spine head; this value was then background-subtracted and normalized to the average brightness of the adjacent dendritic shaft. Change in size for a single spine across 24h (size index) was calculated as (time 24 size)/(time 0 size). These individual size index values were averaged for all of the spines measured across the time interval, yielding a single mean size index value for that cell across that single interval. For each cell, all mean size index values were normalized to the last pre-deafening mean size index measurement for that cell. Following this

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normalization procedure, measurements from multiple cells were pooled for statistical analyses.

C. **Spine stability measurements:** Spine stability for each cell was calculated as the percentage of spines that were stable over 2h intervals (calculated within night). For each cell, all spine stability measurements were normalized to the last pre-deafening stability measurement made for that cell. Data from multiple cells were pooled for statistical analyses.

D. **Spine density measurements:** Spine density for each cell was calculated as number of spines/length of dendrite. Measurements were made from the first imaging time point of each night and were compared across nights.

In vivo and in vitro sharp electrode intracellular recordings

In vivo sharp intracellular recordings: Sharp intracellular recordings were made from electrophysiologically-identified HVC neurons in diazepam-anesthetized birds. Recordings were made both with and without injection of hyperpolarizing current (referred to as hyperpolarized and spontaneous recordings, respectively). Firing frequency and inter-spike intervals were measured in the spontaneous recordings. In hyperpolarized recordings, current was injected to reduce the membrane potential of neurons to ~-85 mV, and the amplitude and frequency of depolarizing postsynaptic potential (dPSPs) were measured. Recordings were made from hearing control birds and also from a separate group of deafened birds. Recordings from deafened birds were

made 1-5d post-deafening (mean time was 3d post-deafening), well within the time

range in which structural changes to dendritic spines were observed.

In vitro sharp intracellular recordings: Sharp recordings were made from HVC neurons

in 400 μm thick sagittal sections. Trains of negative and positive current were injected

into HVC_X neurons, and the following intrinsic properties were calculated: spike

afterhyperpolarization amplitude, time-to-peak, and time-to-decay; spike duration;

inter-spike interval; latency to spike; percent sag; phasicness; resting membrane

potential; spike frequency; membrane time constant; and threshold current to spike.

Neurons were identified by their spontaneous spike rates and spike shapes (Mooney

and Prather, 2005).

LMAN lesions

LMAN lesions: The boundaries of LMAN were mapped out using multi-unit electrical

recordings, and LMAN was lesioned bilaterally by injection of 60-200 nL of 7% ibotenic

acid. Birds were allowed to recover for at least four days following LMAN lesion before

any additional manipulations were performed (imaging, deafening, etc).

Estimation of lesion extent: Lesion extent was assessed in Nissl-stained tissue sections.

Nissl-stained tissue from 2 non-injected, LMAN intact control birds (4 hemispheres

total) was used to estimate the average volume of LMAN in fixed tissue, and the volume

of LMAN remaining in LMAN lesion birds was divided by this value to estimate the

lesion extent. Additionally, injections of retrograde tracer were made into RA 4-5 days

prior to sacrificing the animals to retrogradely label any remaining RA-projecting

neurons in LMAN and to aid in the identification of remaining, non-lesioned portions of

LMAN in Nissl-stained tissue sections.

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Biography

My name is Katherine Anne Tschida. I was born in Davenport, IA, on November 28, 1983. I attended Grinnell College (Grinnell, IA) and received a B.A. in biology in May 2005. I have published the following articles: "Nitric oxide, cAMP, and the biphasic muscarinic modulation of ACh release at the lizard neuromuscular junction" and "Rapid spine stabilization and synaptic enhancement at the onset of behavioural learning." Since obtaining my bachelor's degree, I have been awarded the James B. Duke fellowship and the National Science Foundation Graduate Research fellowship.