Song-Selective Neurons in the Songbird Brain:
Synaptic Mechanisms and Functional Roles

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1. Introduction

Birdsong is learned and maintained via auditory experience, a process requiring interactions between auditory and song motor areas. For this reason the report that simple acoustic stimuli could evoke auditory activity in the the song "motor" nucleus HVC of anesthetized zebra finches (Katz and Gurney, 1981) caused considerable excitement in the birdsong community and initiated a flurry of research activity which continues to this day. Subsequently, McCasland and Konishi (1981) reported that auditory activity could be evoked in the HVC of awake canaries by playing back a recorded version of the Bird's Own Song (BOS). A more extensive subsequent characterization of auditory selectivity of HVC neurons, conducted by Margoliash (1983), showed that HVC neurons in the anesthetized white-crowned sparrow were song-selective, firing vigorously to forward but not reverse BOS playback, and in some cases only responded to specific note combinations in the BOS.

These remarkable findings and the relative ease of analyzing synaptic connectivity in the songbird's brain make song-selective HVC neurons extremely attractive candidates for addressing mechanisms for the generation of stimulus-specific sensory responses (**see Theunissen, this volume**). They are of additional interest to the neuroethologist because of their established role in song recognition (Brenowitz, 1991; Del Negro et al., 1998; MacDougall-Shackleton et al., 1998; Gentner et al., 2000; Halle et al., 2002; Halle et al., 2003) and because these neurons could mediate auditory-vocal interactions important to vocal mimicry.

In this chapter, we address three important questions about song selectivity. Where in the brain does song-selectivity originate? What are the synaptic mechanisms underlying the remarkably selective auditory responses recorded in HVC? What forms of experience- auditory, vocal motor, tutor song or auditory feedback- shape BOS-selective responses in HVC and other parts of the song system? In describing answers to these questions, we focus on the analysis of auditory selectivity in the anesthetized bird, an approach that provides superior recording stability and minimizes potential confounds due to changes in the animal's state of attention or arousal. These studies have provided an increasingly detailed picture of where BOS-selective responses arise in the brain, the synaptic mechanisms that contribute to BOS-selective responses in HVC, and the role

that auditory experience plays in shaping song-selective responses in HVC and other parts of the song system.

Despite the advantages to recording in an anesthetized preparation, answers to some questions of great functional relevance can only be sought in awake, freely behaving songbirds. The issue of the potential functional significance of auditory activity in HVC hinges on the degree to which HVC auditory activity is manifested in the awake bird. Here we discuss results from studies using chronic recording methods in awake songbirds, including those we have undertaken in the swamp sparrow. These studies reveal that some of the HVC neurons that project to a basal ganglia pathway important to song learning and perception display robust and highly selective auditory activity in the waking animal.

2.   What is BOS-selectivity?

BOS-selectivity is defined as a stronger neuronal response to forward playback of the BOS than either to temporally altered versions of the BOS or to conspecific songs. Such selectivity can be relative, in which both the BOS and the non-BOS stimuli evoke firing rate increases, or absolute, such that only the BOS evokes a response. Figure 1 depicts auditory responses recorded from different song system neurons exhibiting relative and absolute BOS-selectivity. Neurons that display absolute song-selectivity afford especially compelling examples of an auditory "grandmother neuron," a hypothetical cell so selective that it would respond only in the presence of one's grandmother (Marr, 1982; Gross, 2002). Many neurons in the song system of the anesthetized songbird exhibit relative BOS-selectivity (Volman, 1996; Doupe, 1997; Livingston and Mooney, 1997; Theunissen and Doupe, 1998; Mooney, 2000; Rosen and Mooney, 2000; Grace et al., 2003), and a sizable minority appears to respond exclusively to the BOS. When neurons respond to only a single song, they are referred to as song-specific neurons (Margoliash, 1983).

PLACE FIGURE 1 NEAR HERE.

The fact that BOS-selective neurons respond much more strongly to forward over reverse BOS indicates that they must be sensitive to temporal features of song, because these two stimuli have equivalent spectral content but contrasting local and global temporal features. That many BOS-selective neurons actually discriminate longer timescale features of the song is indicated by their tendency to respond more strongly to the BOS than to artificial versions of the BOS in which the note or syllable order has been reversed (Figure 1B) (Lewicki and Arthur, 1996; Volman, 1996; Doupe, 1997). Indeed, some song-specific neurons have been shown to respond in an all-or-none fashion to specific note or syllable combinations naturally present in the BOS, a feature requiring temporal integration over many tens to hundreds of milliseconds. From a functional standpoint, such long timescale integration is well suited to detect the variations in syntax that distinguish the BOS from other similar conspecific songs and to reinforce global aspects of the learned song, namely the syllable sequence. Additionally, neurons sensitive to specific harmonic combinations have been detected in songbirds with spectrally complex songs, such as the zebra finch (Margoliash and Fortune, 1992). These various findings underscore that BOS-selective neurons are sensitive to complex temporal and spectral features of the song and thus are well suited for characterizing the neuronal mechanisms that detect complex learned vocal sequences, including human speech.

As with other studies addressing sensory coding of natural stimuli in other systems, studies designed to assess auditory selectivity in HVC and other areas in the songbird brain have employed both natural stimuli, including the BOS and the songs of other conspecifics, and synthetic acoustical stimuli (Theunissen and Doupe, 1998; Mooney, 2000). However, regardless of which of these two approaches is used to identify them, a far greater proportion of BOS-selective neurons are found within the song system than in primary and secondary regions of the avian auditory telencephalon (Lewicki and Arthur, 1996; Theunissen and Doupe, 1998; Janata and Margoliash, 1999; Grace et al., 2003; Amin et al., 2004; Theunissen et al., 2004). Indeed, single unit recordings made from Field L and HVC in individual zebra finches show that the proportion of neurons displaying sensitivity to forward over reverse BOS playback, as

well as note and syllable order, increases from Field L to HVC (Lewicki and Arthur, 1996; Janata and Margoliash, 1999).

3.   Where in the brain does song-selectivity arise?

As noted above, the highly selective auditory responses of HVC neurons stand in stark contrast to the relatively non-selective auditory responses exhibited by neurons in Field L, the primary auditory telencephalon of the bird and a likely source of either direct or indirect auditory drive to HVC (Lewicki and Arthur, 1996; Janata and Margoliash, 1999). These differences reinforce the impression that auditory neurons in HVC are predominantly concerned with processing song-related information and that BOS-selectivity is largely the result of neuronal computations performed above Field L, most likely in HVC. Important issues to resolve are the exact path via which auditory information flows from Field L to HVC and the extent to which BOS-selectivity arises in structures interposed between these two areas.

Our understanding of the specific pathway that links Field L to HVC remains imprecise. An early idea was that Field L formed a direct (i.e. monosynaptic) connection with HVC. However, studies where injections of anterograde tracers were made into Field L resulted in little or no terminal label in HVC itself, but did heavily label the "shelf," a region just ventral to HVC into which a small number of HVC neurons extend dendrites (Kelley and Nottebohm, 1979; Vates et al., 1996; Benton et al., 1998). Indeed, the apparent route via which much or all auditory information reaches HVC is more complex and may include additional processing steps between Field L and HVC. A compelling clue in support of this indirect auditory route was the detection of BOS-selective auditory responses in NIf, a sensorimotor nucleus that provides dense axonal terminations in HVC (Vates et al., 1996; Janata and Margoliash, 1999). This finding lends strong support to the idea that HVC is not the site where BOS-selective responses originate, although full confirmation of this idea rested on an analysis of functional connectivity between NIf and HVC (Cardin and Schmidt, 2004; Coleman and Mooney, 2004).

4.   HVC filtering of auditory input: methodological challenges

Experimental analyses of the functional interactions between HVC and its putative auditory afferents face significant challenges. The first is the high degree of anatomical convergence of axonal inputs onto HVC, including those originating from auditory as well as non-auditory areas (Nottebohm et al., 1982; Fortune and Margoliash, 1995; Foster and Bottjer, 1998; Shea and Margoliash, 2003). A second is the necessary distinction between anatomical and functional connectivity. Thus although HVC receives input from many areas, some may play a more or less important role in driving HVC's auditory activity or may differ in the types of auditory information they provide to HVC. A third is that HVC contains, in addition to interneurons, projection neurons (PNs) innervating respectively either the song premotor nucleus RA (HVC_{RA} neurons) or the basal ganglia homologue Area X (HVC_X neurons) (Mooney, 2000). Because extracellular recordings made in HVC are biased towards sampling from interneurons, characterizing the activity of identified HVC projection neurons requires intracellular methods or extracellular methods applied in conjunction with antidromic stimulation (Mooney, 2000; Hahnloser et al., 2002; Rauske et al., 2003). Ultimately, pinpointing the origins of BOS-selectivity and establishing the exact nature of any auditory transformations in HVC requires techniques that can assess functional connectivity between neurons in different areas, probe the synaptic mechanisms that underlie highly selective suprathreshold patterns of activity, and track cellular identity. These requirements are best met with in vivo intracellular recording methods. Using these methods, we and others have made several observations that together establish that NIf provides BOS-selective input to HVC. First, reversible pharmacological silencing of NIf activity abolishes much if not all of the spontaneous and auditory activity in HVC, supporting the idea that HVC derives much or all of its auditory drive from NIf (Cardin and Schmidt, 2004; Coleman and Mooney, 2004). Second, dual electrode recordings coupled with spike-triggered averaging methods show that action potentials in NIf neurons slightly precede membrane depolarizations in HVC neurons, consistent with a monosynaptic excitatory linkage between NIf and HVC neurons (Coleman and Mooney, 2004). Third, we have used intracellular recordings from NIf neurons projecting to HVC (NIf_{HVC}) and HVC projection neurons (i.e., HVC inputs and outputs) to compare song selectivity before and after HVC processing. These studies reveal that the relative bias to

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the BOS versus other acoustic stimuli is established in NIf, but that an absolute bias to the BOS (i.e., song-specific responses) arises in HVC (Figure 1) (Coleman and Mooney, 2004). More specifically, a direct quantitative comparison of BOS-selectivity shows that NIf$_{HVC}$ neurons and HVC PNs are equally BOS-selective (Coleman and Mooney, 2004). However, NIf$_{HVC}$ neurons fire to most non-BOS stimuli, including conspecific songs and white noise bursts, and fire in a sustained fashion to the BOS (Coleman and Mooney, 2004), whereas HVC PNs fire little or not at all to non-BOS stimuli, and fire in a highly phasic manner to the BOS (Figure 1) (Mooney, 2000; Coleman and Mooney, 2004). In fact, some HVC PNs fire only during a very narrow time window during the song motif, and thus can be said to be "temporally sparse" in their firing patterns. These findings indicate that BOS-selectivity is generated prior to HVC and also characterize more precisely how auditory activity in HVC differs from that in its major auditory afferent, NIf. These differences indicate that circuit interactions between NIf and HVC, or within HVC itself, alter the temporal pattern of BOS-evoked activity.

5.   What are the synaptic mechanisms underlying BOS-selective responses in HVC?

A variety of observations point to circuit interactions within HVC as the mechanism underlying temporally sparse, song specific firing patterns of HVC PNs. First, HVC$_{RA}$ and HVC$_X$ neurons show similarly sparse patterns of BOS-evoked action potential activity, but differ in their underlying subthreshold response patterns: HVC$_{RA}$ neurons show sustained depolarizing subthreshold responses, whereas HVC$_X$ neurons show a complex mixture of hyperpolarizing and depolarizing responses (Mooney, 2000), similar to those reported by Lewicki (1996) for certain note-combination sensitive HVC neurons (Lewicki, 1996). Second, dual recordings and spike-triggered averaging methods reveal that NIf neurons make functionally excitatory connections with both PN types and interneurons in HVC (Coleman and Mooney, 2004). This pattern of excitatory connectivity from NIf to HVC suggests that the contrasting subthreshold responses of different HVC PN types to BOS playback are not simply due to differences in extrinsic input; instead, BOS-evoked hyperpolarizing responses in HVC$_X$ cells most likely arise due to interactions with inhibitory neurons local to HVC (Mooney, 2000; Mooney and

Prather, 2005; Rosen and Mooney, 2006). Third, in direct support of this idea, the firing patterns of interneurons in HVC are sustained throughout BOS playback and closely correlate with the BOS-evoked membrane hyperpolarizations of HVC_X cells (Mooney, 2000), and these interneurons are immunopositive for parvalbumin (PV) (Kawaguchi et al., 1987; Kawaguchi, 1993; Mooney and Prather, 2005; Wild et al., 2005), , a calcium-binding protein expressed at high levels in inhibitory interneurons in other systems. Fourth, intracellular recordings made in brain slices from synaptically coupled cell pairs show that these PV+ interneurons make inhibitory synapses on HVC_X cells and that both HVC_{RA} and HVC_X cells make excitatory synapses onto these PV+ interneurons (Mooney and Prather, 2005). These results suggest a model where BOS-selective inputs from NIf provide monosynaptic excitation to all three HVC cell types, and ultimately drive feedforward and feedback inhibition mediated by the HVC network to generate BOS-evoked hyperpolarizing response in HVC_X cells.

This model predicts that inactivating the local HVC circuit should cause the BOS-evoked hyperpolarizations in HVC_X neurons to disappear, whereas their depolarizing responses should persist. To more directly measure whether and how HVC transforms its extrinsic auditory input, we compared the auditory-evoked subthreshold activity of HVC neurons with the local circuit either "on" or "off." We made this comparison by intracellularly recording the auditory-evoked synaptic activity of individual HVC neurons before and after we pharmacologically silenced the local circuit (Rosen and Mooney, 2006). Synaptic responses of both HVC PN types remained BOS-selective even when the local HVC circuit was inactivated, confirming that HVC receives an extrinsic source of BOS-selective input. However, with the local circuit inactive, the subthreshold response patterns of HVC_X neurons were rendered purely depolarizing and closely resembled the response patterns of HVC_{RA} neurons recorded in the same bird. In contrast, the shape of the subthreshold response patterns of HVC_{RA} neurons changed very little with local circuit inactivation. This result supports the idea that the differences in subthreshold response patterns of the two HVC PN types is due to selective targeting of HVC_X cells by inhibitory interneurons.

Though useful, these local circuit inactivation experiments silenced action potential activity in all HVC neurons, and hence could not address whether or how

inhibition shapes BOS-evoked firing patterns of HVC_X cells. For example, it would be useful to know whether inhibition renders the suprathreshold responses of HVC_X cells highly phasic, or whether the excitatory inputs by themselves can evoke highly phasic firing. Furthermore, it remains unclear whether inhibitory and excitatory inputs onto HVC_X cells are effectively balanced, with both being recruited most strongly by the BOS, or whether non-BOS stimuli preferentially recruit inhibition, masking excitatory responses to non-BOS stimuli and ultimately resulting in greater stimulus specificity in HVC_X cells. Another important question is whether inhibition helps to regulate the temporal precision of BOS-evoked firing in HVC_X cells? Finally, one idea is that syllable combination-sensitive responses in song-specific HVC neurons might arise when hyperpolarizing responses evoked by the first note de-inactivates a low-threshold calcium current, "priming" the cell to fire a burst of action potentials to a second note that when played in isolation evokes only a subthreshold depolarization (Lewicki, 1996). If blocking inhibition onto HVC cells diminished combination-sensitive action potential bursts, this would lend support to this priming model.

To address these issues, a method was needed that could remove some of the inhibitory input onto the cell while leaving its action potential machinery intact. Furthermore, it would be best if this method deprived only the impaled cell of its inhibitory synaptic input, to avoid runaway excitation triggered by silencing the entire inhibitory network. Fortunately, substantial in vitro work from David Perkel's group had established the inhibitory repertoire of HVC_X cells, which are targeted by a wide variety of potent inhibitory inputs, including those that activate ionotropic chloride currents and G-protein coupled inward rectifying potassium currents (i.e., GIRKs) (Dutar et al., 1998; Schmidt and Perkel, 1998; Dutar et al., 1999; Dutar et al., 2000). These different forms of inhibition can be disrupted at the intracellular, single cell level either by chloride loading the cell or by dialyzing it with compounds that block G-protein signaling.

When we disrupted either ionotropic chloride currents or GIRK signaling in individual HVC_X cells, BOS-playback evoked more sustained firing patterns, qualitatively resembling those seen in NIf_{HVC} neurons and HVC interneurons (Rosen and Mooney, 2003). Therefore, interactions between excitatory and inhibitory inputs are necessary to generate highly phasic firing patterns in HVC_X cells. Blocking inhibition

also unmasked excitatory responses to non-BOS stimuli, but these responses were strongly graded, suggesting that inhibition and excitation onto HVC_X cells are balanced. This behavior may reflect feedforward inhibitory architecture from NIf to HVC, which would be expected to activate HVC interneurons most strongly in response to the BOS. This synaptic behavior, wherein the preferred stimulus as defined by excitatory responsiveness also activates the strongest inhibitory response, resembles the balanced excitatory and inhibitory synaptic interactions described in the primary auditory cortex of the rat (Wehr and Zador, 2003), suggestive of common mechanisms underlying auditory selectivity in higher level sensory and sensorimotor areas of the vertebrate. The variability of BOS-evoked spiking in HVC_X cells also increased when inhibition was disrupted, indicating that inhibition is important to regulating precise spike timing in these neurons. Finally, some of the phasic excitatory responses that were initially preceded by membrane hyperpolarizations increased when hyperpolarizing inhibition was blocked, whereas others were diminished, lending partial support to the priming model of combination sensitivity.

We know considerably less about the mechanisms that contribute to the temporally sparse patterns of BOS-evoked firing in HVC_{RA} neurons, although they appear to differ from those operating in HVC_X cells. As mentioned earlier, HVC_{RA} neurons are unlike HVC_X cells in that they display sustained depolarizing membrane potential responses in response to BOS playback, even though these sustained depolarizations drive only temporally sparse patterns of firing (Mooney, 2000). The depolarizing responses are not simply a mix of EPSPs and depolarizing IPSPs, because tonically depolarizing an HVC_{RA} neuron causes it to fire in a sustained fashion to BOS playback (Mooney, 2000). Furthermore, inactivating the local HVC circuit has little effect on the shape of the BOS-evoked depolarizing response, suggesting that HVC_{RA} neurons are targeted less heavily than HVC_X neurons by local inhibition (Rosen and Mooney, 2006). However, BOS-evoked hyperpolarizations can be detected when the cell is depolarized substantially above action potential threshold (Mooney, unpublished observations), suggesting that these cells do receive some, albeit weak, inhibition, consistent with the known synaptic connections between interneurons and HVC_{RA} cells (Mooney and Prather, 2005). The relatively weak BOS-evoked inhibition in HVC_{RA} cells

suggests that the sparse action potential output of these neurons evoked by song playback may involve mechanisms that do not depend heavily on inhibition. These mechanisms could include postsynaptic thresholding, perhaps mediated via the precise regulation of the cell's resting membrane potential or the weight of its excitatory synaptic inputs, or cooperative interactions between excitatory inputs.

In summary, in vivo intracellular recordings have revealed many interesting features of the synaptic mechanisms underlying BOS-selective firing patterns in HVC, especially in HVC_X cells. These include the finding that BOS-evoked activity in interneurons drives inhibition in HVC_X cells, transforming tonic patterns of synaptic excitation from NIf into a highly precise and phasic output. In addition to such suppressive inhibitory interactions, inhibition onto HVC_X cells can also augment BOS-evoked excitatory peaks through a priming mechanism, providing a mechanism for combination sensitivity. These various inhibitory interactions help generate auditory activity in HVC_X cells that is highly precise in its timing and more exclusively responsive to a single stimulus, namely the BOS. A fascinating feature of this process is that remarkably selective sensory-evoked responses are generated in HVC, an area traditionally associated with motor aspects of singing. Indeed, this sharpening of stimulus specificity in HVC may reflect motor-driven effects on sensory processing, as has been described for the refinement of whisker representations in the rodent motor and somatosensory cortices (Kleinfeld et al., 2002; Polley et al., 2004). The generation of such temporally precise and stimulus-specific responses in HVC may be especially important for matching auditory to motor representations of song, a process that could facilitate vocal mimicry and communication.

6. Other sources of auditory input to HVC

In addition to Nif, HVC receives input from at least three other areas that display auditory activity: Uva, a thalamic nucleus afferent to both Nif and HVC, (Nottebohm et al., 1982; Wild, 1994); CM the secondary auditory telencephalic region CM (Vates et al., 1996; Vates et al., 1997) and the anterior telencephalic nucleus mMAN (Nottebohm et al., 1982; Foster and Bottjer, 2001). The first two of these receive input from identified

auditory structures, while mMAN is thought to receive its auditory drive indirectly from HVC, and may function as a recurrent auditory pathway (Vates et al., 1997).

Anatomical pathway tracing studies we have undertaken with Martin Wild show that Uva receives ascending input from the ventral part of the lateral lemniscus, which in turn receives monosynaptic input from the cochlear nucleus (Coleman et al, in revision). Thus, the auditory pathway through Uva to NIf and HVC comprises relatively few synapses, providing a potentially short-latency source of auditory input to the song system. Extracellular recordings we made in Uva found that many neurons respond in a robust fashion to BOS playback (Coleman et al., in revision). However, although most Uva neurons are non-selective, a small fraction shows highly selective responses to BOS, suggesting that Uva could transmit selective as well as non-selective auditory information to NIf and HVC. Whether Uva actually contributes to auditory activity in HVC remains less certain: in the urethane-anesthetized zebra finch, reversibly inactivating Uva has no discernible effect on HVC auditory activity, even though low frequency electrical stimulation in Uva elicits excitatory postsynaptic potentials (EPSPs) in HVC (Coleman et al., in revision). One possibility is that Uva's functional interactions with HVC are suppressed by anesthetic, and/or high levels of Uva activity are necessary to influence HVC responsiveness, a conclusion consistent with the finding that high frequency electrical stimulation in Uva can transiently suppress spontaneous and auditory activity in HVC (Coleman et al., in revision). Alternately, Uva's functional interactions with HVC may be more purely modulatory in nature, a view supported by the observation that Uva neurons can be excited by visual and tactile stimuli as well as auditory stimuli (Williams and Vicario, 1993; Wild, 1994). Perhaps such integrative properties may enable Uva to respond to environmental stimuli that either favor or discourage singing.

Another source of auditory input to HVC is the secondary auditory telencephalic region CM (Bauer et al., in preparation), an area implicated in experience-dependent auditory plasticity (Gentner and Margoliash, 2003). CM axons make sparse terminations in HVC as well as NIf which are functionally important to auditory activity in the song system: reversibly inactivating CM strongly suppresses auditory activity in both NIf and HVC, although it does not suppress spontaneous levels of activity in either area (Bauer et al., in preparation). Both BOS-selective and non-selective cell types can be found in CM,

and both types appear to make functional connections with NIf. Because the auditory selectivity of many CM neurons can be strongly modified by operant conditioning (Gentner and Margoliash, 2003), CM may provide NIf and HVC with input that has been shaped by auditory experience, including song-related feedback or even experience of the tutor song. Thus, an important goal of future studies will be to understand whether HVC receives input from the same neurons in CM have been shown to exhibit learning-dependent changes in auditory selectivity.

The projections from Uva and CM may convey different types of auditory information to HVC. The projection from CM likely provides highly processed auditory information to the song system: CM is densely interconnected with primary and secondary regions of the auditory telencephalon, including Field L and NCM (Vates et al., 1996), and all three areas contain neurons with complex response properties (Theunissen et al., 2000; Sen et al., 2001; Grace et al., 2003; Amin et al., 2004; Theunissen et al., 2004). NCM is an important site of experience-dependent auditory plasticity, and some NCM neurons are selective for the tutor song (Chew et al., 1995; Chew et al., 1996; Bolhuis et al., 2000; Bolhuis et al., 2001; Terpstra et al., 2004). Thus the connections that NCM makes with CM may provide a potential route for tutor-selective information to enter the song system. In contrast, Uva relays mostly non-selective auditory information directly from the lower levels of the auditory brainstem to the song system, and this information may serve more of a modulatory role. Although the functional significance of this convergence of auditory information onto HVC is unknown, one possibility is that salient auditory or visual cues in the animal's environment activate Uva, which in turn modulates auditory information relayed to HVC through experience-dependent perceptual filters in CM and NIf. Another possibility is that Uva provides a short latency pathway for conveying auditory as well as proprioceptive feedback to HVC, which is then compared to experience-dependent auditory representations (i.e., auditory memories) transmitted through NCM, CM and NIf to HVC.

7. Future directions and challenges to assessing functional networks prior to NIf

Continued work is needed to fully divine the origins of BOS-selectivity. Although it has often been assumed that BOS-selectivity is generated in the song system, BOS-selective neurons can be found in different proportions throughout the auditory telencephalon. This distributed organization raises the possibility that interconnected subsets of selective cells in the auditory telencephalon form a segregated channel that routes BOS-selective information to the song system. Therefore, a major goal of future analyses is to assess functional connectivity in auditory areas presynaptic to NIf, such as CM, NCM and Field L, a goal which is likely to be more challenging to accomplish than the analysis of functional connectivity in the song system. Whereas NIf and HVC are each spatially compact, synaptically interact with each other in a largely feedforward manner, and contain neuronal populations relatively homogeneous in their auditory selectivity, the primary and secondary regions of avian auditory telencephalon are vast regions, characterized by highly reciprocal interconnections (Vates et al., 1996) and populated by a wide variety of selective and non-selective neurons (Theunissen et al., 2000; Sen et al., 2001; Grace et al., 2003; Amin et al., 2004; Theunissen et al., 2004). As a result, some circuit-analysis techniques that worked effectively in the song system, such as reversible inactivation, may be less useful in assessing functional connectivity of different regions in the auditory telencephalon. Similarly, the distributed and possibly sparse pattern of synaptic connectivity between these different auditory regions will likely make the assessment of functional connections between their constituent neurons more challenging. Despite these challenges, an important goal will be to carefully analyze how secondary auditory regions implicated in tutor song imprinting and adult forms of experience-dependent auditory plasticity communicate with sensorimotor areas necessary to the learning and maintenance of song.

8.   The role of experience in shaping BOS-selectivity in the song system

Because the bird's song is learned, BOS-selectivity must at a fundamental level reflect the effects of experience. But what forms of experience actually contribute to the development of BOS-selectivity? Do BOS-selective neurons constitute an auditory memory of self-produced songs, or do they also encode memories of the tutor song? Do they encode persistent auditory memories of any kind, or merely track the current

auditory feedback? Furthermore, because HVC and other song nuclei display song motor as well as auditory activity, is BOS-selectivity purely a reflection of the auditory feedback experienced by the bird when it sings, or is it a product of interactions between the motor and auditory systems?

Much of what we know about the role of experience in shaping song selectivity comes from studies of neurons in the song nucleus LMAN, the output of an anterior forebrain pathway (AFP) necessary to juvenile and adult forms of audition-dependent vocal plasticity (Bottjer et al., 1984; Doupe, 1997; Livingston and Mooney, 1997; Kittelberger and Mooney, 1999; White et al., 1999; Rosen and Mooney, 2000; Boettiger and Doupe, 2001; Livingston and Mooney, 2001). Because HVC_X neurons are the putative source of auditory and singing-related activity in the AFP, experience-dependent effects on LMAN auditory selectivity are likely to reflect changes in selectivity initiated in HVC. For the purposes of this chapter, we will focus on some of the common themes that have emerged from studies of experiential factors that contribute to song-selectivity in both HVC and LMAN. (For a review of the Anterior Forebrain Pathway **see Brainard, this volume**).

To a great degree, developmental studies point away from the tutor song and towards auditory feedback or auditory-vocal interactions as the major determinant of song-selectivity. First, in contrast to what would be expected for a persistent tutor song memory, the vast majority of BOS-selective neurons in the HVC and LMAN of adult birds respond more strongly to the BOS than to the tutor song (Margoliash and Konishi, 1985; Volman, 1993; Solis and Doupe, 1997, 1999; Nick and Konishi, 2005b). Second, recordings made in the HVC and LMAN of anesthetized juvenile songbirds reveal that song-selectivity is only manifested after the bird begins to sing; prior to this time, auditory responses in HVC and LMAN are typically weaker and non-selective, despite substantial auditory experience of the tutor song (Volman, 1993; Solis and Doupe, 1997). These studies also reveal that even in juvenile birds singing plastic song, the majority of HVC and LMAN neurons are selective for the BOS rather than the tutor song. Although there is one report that some HVC neurons in the awake juvenile zebra finch are tutor song-selective (Nick and Konishi, 2005b), this same study found that by early adulthood, most HVC neurons are BOS-selective. These findings suggest that BOS-selective

neurons in the adult do not represent a persistent auditory memory of the tutor, and raise the possibility that the tutor song excites HVC and LMAN neurons in the juvenile because it resembles the BOS in its acoustical structure.

A potential confound to this conclusion is that it is difficult to assess the fidelity of tutor song memory independently of the juvenile's vocal imitation of the tutor. That is, incomplete copying of the tutor song may reflect a deficit in the auditory memory of the tutor song or inaccurate song motor learning. In the former case, BOS-selectivity could reflect a flawed auditory memory of the tutor, rather than a neuronal correlate of the bird's song performance (Solis and Doupe, 1999). One way to test this idea is to artificially maximize the acoustical distance between the bird's own song and the possibly imperfect tutor song memory. In juvenile birds sustaining severe spectral degradation of the song produced by syringeal denervation, Solis and Doupe (1999) found that most LMAN neurons develop a strong selectivity for the distorted BOS over the tutor song by the mid-point of sensorimotor learning (~ PHD 65). Nonetheless, some neurons responded equally well to the distorted BOS and to the tutor song, raising the possibility that they encoded different BOS and tutor song features. However, the features in the distorted BOS and the tutor song that evoked responses were not characterized and were not necessarily those judged to be dissimilar in the two songs. Thus, it remains plausible that selectivity in LMAN is shaped by the bird's experience of its own song and dual-selective neurons in "dysphonic" birds respond to features common to the BOS and tutor song.

Another idea is that song-selective neurons are initially influenced by experience of the tutor, but that this early experience is overwritten by feedback as the bird sings its own song. According to this model, tutor-selectivity is replaced by BOS-selectivity, which then serves as a permanent referent that in the adult serves to maintain a stable vocal output (**see Woolley, this volume**). Is there any evidence that songs learned early in development leave a lasting imprint on the auditory responses of song system neurons? One way to answer this question is to allow juvenile birds to sequentially learn from two different tutors, then assess whether neuronal selectivity for the early renditions of the BOS, or the tutor model from which it was copied, is maintained following copying from the second tutor. Experiments using this approach show that, at least in LMAN,

selectivity does develop in juvenile zebra finches for the initially learned song and the model from which it was copied (Sugiyama and Mooney, 2004). However, intracellular recordings made in adult birds that were tutored sequentially show that all synaptic as well as suprathreshold responsiveness to early versions of the BOS and the first tutor song is lost after the bird copies from the second tutor (Sugiyama and Mooney, 2004). This indicates that song-selective neurons in LMAN track the current learned repertoire, rather than providing a persistent memory of early auditory experience either of the tutor or discarded versions of the BOS.

A remaining possibility is that, following crystallization, BOS-selectivity does become an indelible feature of the song system, perhaps serving to maintain a stable song. If this model is correct, then selective responses for the crystallized song should be maintained even in the face of long term exposure to distorted feedback. To test this idea, we unilaterally severed the vocal nerve in adult zebra finches singing crystallized songs, then measured auditory selectivity at different postoperative times (Roy and Mooney, in revision). Over the course of one to two weeks, neurons in LMAN, as well as NIf and HVC, could be detected that were selective for the distorted BOS over the pre-nerve-cut, crystallized song. Thus, there is little evidence that BOS-selective neurons furnish a permanent auditory memory against which the bird's song is maintained. Instead, even following song crystallization, song-selectivity can shift in the AFP to track the most current renditions of the bird's current vocal performance, regardless of how well they may match either the crystallized BOS or the tutor song.

Although these various observations reinforce the idea that experience of one's own vocalizations is the major factor influencing song-selectivity, they do not fully distinguish whether the effects of singing experience are purely feedback-driven, or instead arise through auditory-motor interactions. Both HVC and LMAN exhibit song motor related activity (McCasland, 1987; Hessler and Doupe, 1999; Solis et al., 2000; Hahnloser et al., 2002) and although auditory responses in both areas can be evoked by song playback (Margoliash and Konishi, 1985; Volman, 1993; Solis and Doupe, 1997, 1999; Nick and Konishi, 2005b), there is little evidence that these responses can be recruited by auditory feedback. Indeed, at least in the zebra finch, the singing-related activity of HVC_X or LMAN neurons is not acutely altered by distorted auditory feedback

(Leonardo, 2004; Kozhevnikov and Fee, in press), suggesting that activity recorded in these areas during singing is predominantly motor-related. An important related observation is that auditory responses of LMAN neurons are depressed in adult birds following chronic experience of distorted feedback (Solis and Doupe, 2000). Such depression of auditory-evoked activity would not be expected if auditory responses were influenced solely by auditory feedback and instead may indicate that responsiveness is determined by the quality of the match between actual and expected feedback. The source of such an expected feedback signal is unknown in the song system, but in other systems, estimates of expected sensory feedback are computed using corollary discharge from the relevant motor system (Sawtell et al., 2005; Poulet and Hedwig, 2006). A better understanding of this issue in the songbird AFP will require resolving the auditory- and motor-related properties of HVC_X neurons, because these neurons are the major source of auditory and motor-related drive to the AFP.

9. Auditory representations of song in HVC neurons of awake birds

Although one might assume that the auditory selectivity of neurons in HVC is shaped at least in part by auditory experience, investigation of the activity of HVC neurons in waking zebra finches has revealed only little or no auditory activity (Nick and Konishi, 2001; Rauske et al., 2003; Cardin and Schmidt, 2004; Nick and Konishi, 2005a), with the small amount of detectable activity apparently restricted to only a subset of HVC interneurons (Rauske et al., 2003). In contrast, robust auditory responses have been detected in HVC of awake birds of other species, although these studies relied on multiunit recording methods, and thus the identity of the responsive cells remained uncertain (McCasland and Konishi, 1981; Nealen and Schmidt, 2002; Nealen and Schmidt, 2006). If only interneurons are responsive in the HVC of the awake songbird, then it is unclear how auditory activity could propagate from HVC to other brain areas and ultimately affect behavior. Furthermore, the observed differences in the state-dependence of HVC auditory responsiveness observed across species raise questions as to whether zebra finches are more the rule or the exception in this regard.

To further explore to what extent HVC neurons exhibit auditory activity in the waking state and to investigate the auditory representations of song in the different

classes of neurons in HVC, we chose to record from swamp sparrows (Prather et al., in revision). Two primary considerations influenced our choice of swamp sparrows as the subjects for these experiments. First, the repertoire of an individual male swamp sparrow consists of a few song types, with each song type comprising ten to twenty repetitions of a single, multi-note syllable (Marler and Pickert, 1984). The presence of multiple song types allows the investigator to ask whether there are subpopulations of neurons dedicated to single song types. Second, the highly stereotyped trilled structure of swamp sparrow song is advantageous because a single song bout yields many samples of the repeated vocal unit that defines the song type. Together, these features make swamp sparrows ideal subjects in which to address questions whether auditory responses are present and how distinct song types are represented in HVC during wakefulness.

We used a lightweight microdrive (Fee and Leonardo, 2001) to record auditory activity in the HVC of the freely behaving male swamp sparrow. A previous study showed that HVC neurons are responsive to auditory stimuli in the urethane-anesthetized swamp sparrow, as in the zebra finch, and are highly selective for individual song types in the bird's repertoire (Mooney et al., 2001). Thus we first asked whether HVC neurons in the awake sparrow respond to auditory stimuli. Following methods developed by Michale Fee's group, we used antidromic stimulation methods to identify different cell types in HVC (Hahnloser et al., 2006). Song playback experiments revealed that interneurons and HVC_X neurons display robust auditory activity in the awake sparrow, while HVC_{RA} neurons lacked auditory activity altogether (Figure 2). Further, we found that HVC_X cells, but not interneurons, display remarkably selective auditory activity, responding to only a single song type in the bird's repertoire (Prather et al., in revision). Furthermore, these song type specific responses of HVC_X cells involve temporally precise firing patterns with typically one action potential per syllable (Figure 2), similar to that seen in anesthetized birds (Mooney et al., 2001). These results indicate that highly selective auditory representations are manifested in one class of projection neuron in HVC of the awake swamp sparrow, and thus have the potential to influence behavior. Indeed, the AFP has been implicated in song perception (Scharff et al., 1998) and thus it is likely that these auditory HVC_X neurons are important to this role.

We also found that different HVC$_X$ cells respond to playback of different song types, suggesting that the bird's full repertoire is represented across the entire population of HVC$_X$ cells. The contrast in the amount and selectivity of auditory responses that can be observed in the HVC of the awake swamp sparrow and zebra finch are remarkable, and may reflect species differences in the function of song. Swamp sparrows are largely solitary, highly territorial birds that broadcast their songs over long distances to attract females and in response to hearing the songs of conspecific males (Ehrlich et al., 1988). In contrast, zebra finches are colonial nomads that sing at close range to attract females, rather than to defend territory from neighboring males (Zann, 1996). Perhaps the robust auditory activity that can be detected in the HVC of the awake swamp sparrow reflects behavioral demands on territorial birds to be ever-ready to detect and respond to the songs of neighboring conspecifics. Alternatively, auditory activity in HVC neurons of awake swamp sparrows but not zebra finches may reflect some difference in the functionality of HVC associated with learning and maintenance of a more extensive song repertoire, as waking auditory responses are also observed in HVC of other species that express song repertoires, rather than a single song, like the zebra finch (McCasland and Konishi, 1981; Nealen and Schmidt, 2002; Nealen and Schmidt, 2006).

10.   Conclusions and Future Directions

The analysis of functional connectivity in the song system reveals that HVC receives at least three sources of auditory input, including NIf, CM and Uva. Of these, at least some of the input from NIf and CM provides BOS-selective information, whereas Uva provides a non-selective auditory input and may also convey other sensory information. Uva receives its auditory input from the brainstem, whereas NIf and CM receive their major auditory input from primary and secondary regions of the auditory telencephalon. Thus, HVC receives both relatively direct and non-selective auditory input via Uva and more indirect but more selective input from CM and NIf. Although NIf is a dominant source of auditory input to HVC in the anesthetized bird, we still know very little about how NIf, CM and Uva contribute to HVC auditory activity in the awake

bird, and whether these inputs are differentially regulated with changing behavioral state or over development. We also know very little about the connectivity in primary and secondary regions of the auditory telencephalon, and the precise nature of the connections these areas make with NIf and HVC. What are the earliest stages in the auditory system where BOS-selectivity can be detected? Is there a dedicated pathway for conveying song-selective information to the song system, possibly involving the minority of highly selective cells that can be detected in primary auditory regions, or does song-selectivity develop only in penultimate stages of processing, within or immediately presynaptic to NIf?

Because song is a learned behavior, BOS-selectivity must in some sense reflect the bird's auditory and/or vocal experience. Forms of auditory experience that may be important to BOS-selectivity include the tutor song and auditory feedback. The current view is that BOS-selectivity reflects the bird's experience of its own song, because BOS-selectivity in HVC and the AFP can continue to shift following crystallization, at least when birds are made to sing chronically distorted songs. However, whether this shift in selectivity is driven solely by auditory feedback or by changing patterns of song-motor related corollary discharge transmitted from HVC to the AFP remains uncertain. The finding in zebra finches that the singing-related activity of HVC_X cells, as well as those in LMAN, is unaffected by distorted auditory feedback suggests that motor-related activity may play a prominent role in shaping BOS selectivity in the AFP. More generally, the apparent insensitivity of HVC_X and LMAN neurons in the zebra finch to acute distortions in auditory feedback raises the fundamental question of where singing-related auditory feedback is registered in the brain. Thus, an important goal of future research will be to better characterize the singing-related activity of HVC neurons, as well as their inputs from NIf, CM and Uva, in order to identify potential conduits for auditory feedback. Furthermore, our present observation that HVC_X neurons in the awake swamp sparrow respond selectively to playback of certain song types in the bird's repertoire raises the possibility that the auditory properties of these neurons are also activated by singing-related feedback. If so, this would suggest that HVC_X neurons function in different capacities in different songbird species.

Although NIf neurons projecting to HVC and the projection neurons of HVC are equally BOS-selective, NIf projection neurons fire to most non-BOS stimuli, including conspecific songs and white noise bursts, and fire in a sustained fashion to the BOS, whereas HVC PNs fire little or not at all to non-BOS stimuli, and fire in a highly phasic manner to the BOS. Thus, HVC is the site where song-specific and temporally sparse and precise auditory representations of the BOS become dominant. The emergence of this representation involves local circuit interactions in HVC, including inhibitory shaping and priming in HVC_X cells. What could be the functional significance of generating a temporally sparse, highly selective and precise pattern of BOS-evoked activity in HVC_X cells? One idea is that this transformation places the auditory representation of the BOS in the same temporally sparse and precise framework as the motor code used to produce it, which could establish an exact sensorimotor correspondence that facilitates mimicry and perception of communication gestures.

Figure Captions

Figure 1 (figure size: full page)

Examples of relative auditory selectivity for the bird's own song (BOS) in NIf neurons that innervate HVC (NIf$_{HVC}$; panel A) and absolute auditory selectivity for the BOS in HVC$_X$ neurons (panel B).

(**A**) Comparison of song-evoked multiunit NIf activity with single-unit activity in NIf$_{HVC}$ neurons recorded from urethane-anesthetized zebra finches. Stimuli presented are the bird's own song (BOS), the reversed BOS (REV), individual syllables of the BOS each played forward but assembled in the reversed order of the natural BOS (BOS-RO), and conspecific song (CON). Relative selectivity is evident in the strong response to the BOS and the weaker responses to BOS-RO, CON and REV. Bottom panel, oscillogram of song stimuli; third panel, response of a NIf$_{HVC}$ neuron to a single playback of each song stimulus. Second panel, PTSH of the action potential response of this NIf$_{HVC}$ neuron to 20 iterations of each song presentation. Top panel, PSTH of the multiunit responses to 20 iterations of each song presentation; data taken from the same region of NIf and in the same bird as the corresponding single-unit data. Adapted from Figure 11 in Coleman and Mooney (2004). (**B**) Comparison of single-unit activity in HVC$_X$ neurons evoked by different song stimuli. Absolute selectivity is evident in the strong response to the BOS, the weaker response to BOS-RO, and a lack of suprathreshold response to other stimuli (REV, CON). Bottom panel, stimulus oscillogram; middle panel, median-filtered averaged intracellular membrane potential record taken from 20 repetitions of each stimulus in an HVC$_X$ neuron; top panel, histogram of action potentials evoked by 20 repetitions of each stimulus in the same HVC$_X$ neuron as the corresponding middle panel.

Figure 2 (figure size: half page)

Auditory responses in identified HVC neurons of awake, unrestrained swamp sparrows.

The strength and selectivity of auditory responses vary across the three classes of HVC neurons. Responses in HVC$_X$ neurons (left column) are reliable and highly phasic, and are evoked by only a single song type in the bird's repertoire. In contrast, HVC$_{INT}$ cells

(middle column) show tonic responses to both song types in the bird's repertoire. HVC_{RA} neurons (right column) had no auditory response to any stimulus and were almost entirely inactive in the absence of stimulus presentation (< 0.05 Hz). Top panel, example of extracellular recordings from individual neurons of each class, as identified using antidromic stimulation methods (not shown). Middle histogram and associated oscillogram, single-unit response of the neuron to 10 repetitions of one song type in the bird's repertoire (10 ms bin size in all histograms). Bottom histogram and associated oscillogram, single-unit response of the same neuron to 10 repetitions of a second song type in the bird's repertoire.

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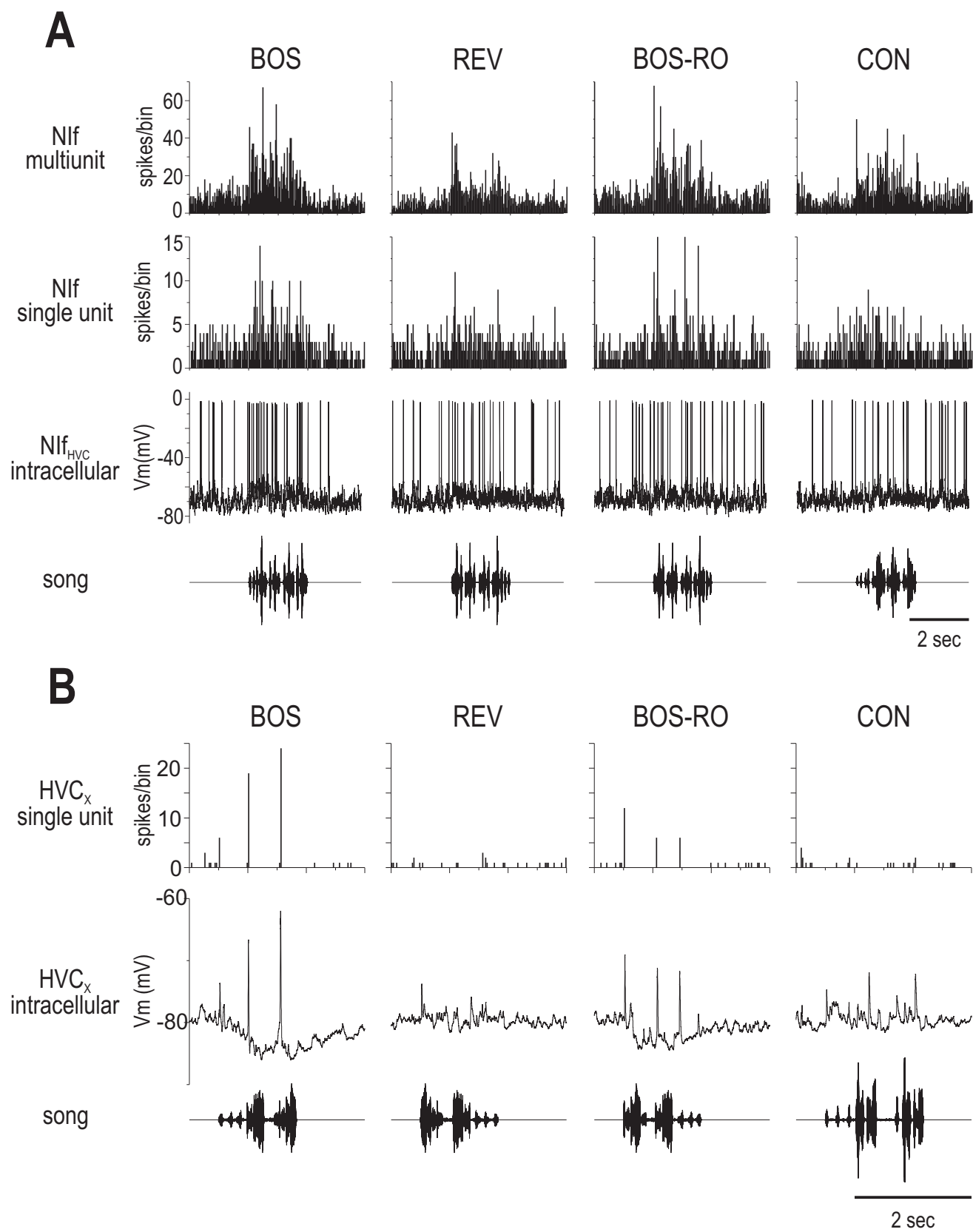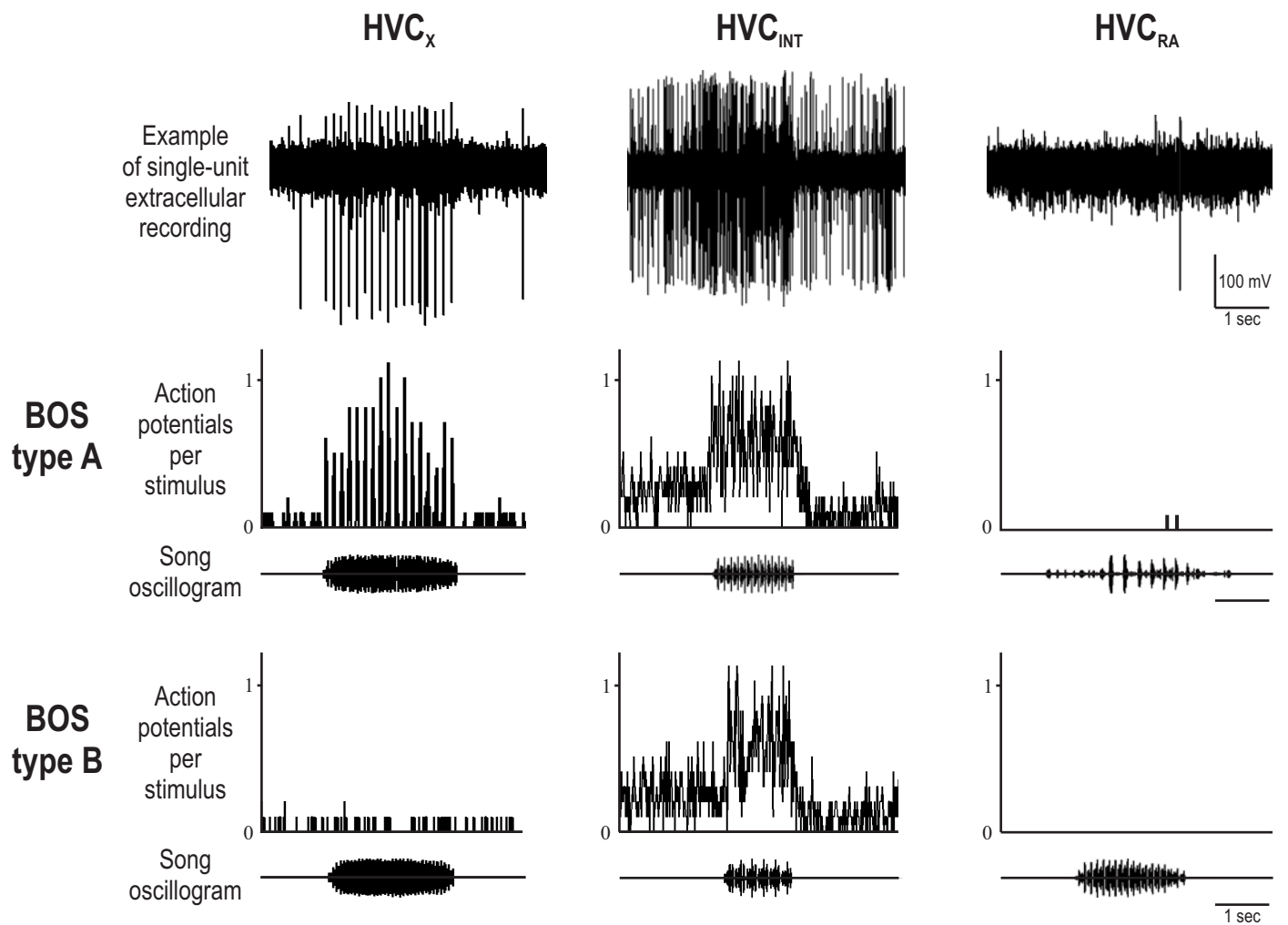Zann R (1996) The Zebra Finch: A Synthesis of Field and Laboratory Studies: Oxford University Press.

Figure 1
Prather and Mooney

Figure 2
Prather and Mooney