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TWO NEURAL STREAMS, ONE VOICE: PATHWAYS FOR THEME AND VARIATION IN THE SONGBIRD BRAIN

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Abstract—Birdsong offers a unique model system to understand how a developing brain – once given a set of purely acoustic targets – teaches itself the vocal-tract gestures necessary to imitate those sounds. Like human infants, to juvenile male zebra finches (*Taeniopygia guttata*) falls the burden of initiating the vocal-motor learning of adult sounds. In both species, adult caregivers provide only a set of sounds to be imitated, with little or no information about the vocal-tract gestures used to produce the sounds. Here, we focus on the central control of birdsong and review the recent discovery that zebra finch song is under dual premotor control. Distinct forebrain pathways for structured (theme) and unstructured (variation) singing not only raise new questions about mechanisms of sensory-motor integration, but also provide a fascinating new research opportunity. A cortical locus for a motor memory of the learned song is now firmly established, meaning that anatomical, physiological, and computational approaches are poised to reveal the neural mechanisms used by the brain to compose the songs of birds. © 2014 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: vocal learning, sensory-motor integration, motor memory, premotor cortex, basal ganglia.

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Abbreviations: AFP, anterior forebrain pathway; GTE, gesture trajectory extrema; LMAN, lateral magnocellular nucleus of the anterior nidopallium; NIf, nucleus interface; RA, robust nucleus of the arcopallium; Uva, nucleus uvaeformis; VMP, vocal motor pathway.

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INTRODUCTION

Among various forms of developmentally-regulated learning, birdsong most resembles human speech learning. Similar to human infants, juvenile male zebra finches learn to imitate a paternal vocal pattern in a two-phase process that proceeds with little or no requirement for external reinforcement. The initial ‘sensory’ phase involves the formation of an auditory memory of the paternal vocal pattern. Notably, the memory contains only the product of the paternal vocal behavior – the acoustic structure and sequence of vocal sounds. As with human speech there is minimal transmission of information about how to produce the sounds.

In zebra finches, the subsequent ‘sensory-motor’ stage of learning overlaps the initial sensory stage. Sensory-motor learning begins with highly unstructured singing (termed ‘subsinging’) that resembles the vocal babbling of human infants (see Fig. 1A). As the name implies, sensory-motor learning requires sensory feedback of the juvenile bird’s own vocalizations for song to be learned. Of critical importance is auditory feedback, which references the auditory memory of paternal song acquired during sensory learning (Price, 1979; Funabiki and Konishi, 2003). Interestingly, the variable structure of subsinging appears to be a purposeful exploration of the dynamic range of the vocal organ (Ölveczky et al., 2005; Aronov et al., 2008; Thompson et al., 2011), and perhaps provides a period of associative learning where relationships between different vocal gestures and the sounds those gestures produce are discovered. Subsinging is followed by plastic song, characterized by the emergence of a spectrally-pluripotent class of syllables (protosyllables) that progressively differentiate in a piecemeal fashion into facsimiles of the syllables and syllable sequences present in the paternal song pattern (Tchernichovski et al., 2001; Miller et al., 2010; Ravbar

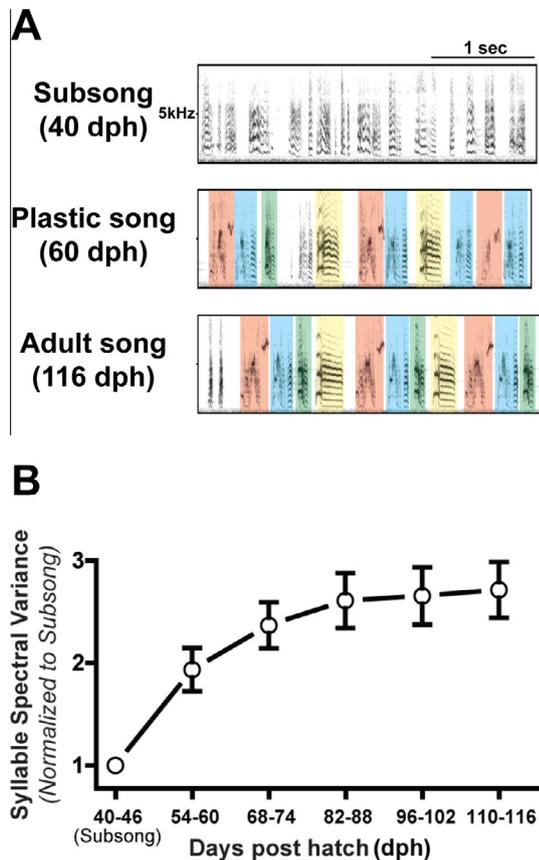


Fig. 1. (A) Frequency spectrograms from 40, 60, and 116 days post-hatch (dph) show the emergence of structured song by a developing male zebra finch. Sensory-motor learning begins with subsong, characterized by unstructured singing that is reminiscent of the babbling of human infants. Individual syllable types (colored transparencies) emerge during plastic song. Adult song is characterized by a fixed repertoire of song syllables that are produced in a canonical sequence – a facsimile of the paternal song pattern heard earlier in life. (B) Quantification of normative vocal development ($N = 9$ birds, mean \pm S.E.M.). Relative to undifferentiated subsong syllables, the complexity of syllable spectral structure rises dramatically as birds enter plastic song and plateaus as the adult form is reached. Syllable spectral variance is a composite of variance values for pitch, pitch goodness and entropy, normalized to subsong and averaged. Data are replotted from Elliott et al. (2014) and replicate original findings by Tchernichovski et al. (2001).

et al., 2012; Lipkind et al., 2013 and Fig. 1B). As male zebra finches reach adulthood (90–120 days post-hatch) the song pattern consolidates into a motor memory that is subsequently referenced and produced throughout adult life as a behavioral marker of a bird's paternal lineage.

THE PREMOTOR CONTROL OF BIRDSONG

The juvenile learning and adult production of birdsong is controlled by a bilateral forebrain network that is remarkable in its anatomical isolation and singular purpose (Fig. 2A). The nodes of this behavioral 'intranet' are distributed throughout avian cortex, basal ganglia, and thalamus. Moreover, with the exception of modulatory (aminergic) and sensory inputs and motor output, these nodes are connected primarily with one

another. Consequently, the learned vocalizations of passerine birds are the product of neural activity in an anatomically private forebrain network that drives a dynamic and quantitatively rich behavior (Fig. 2B, C). The one-to-one correspondence between a forebrain neural network and the complex learned behavior it controls makes the songbird vocal control network a unique experimental platform for elucidating the neural mechanisms of vertebrate learning and memory.

A series of classic circuit-breaking studies (Nottebohm et al., 1976; Bottjer et al., 1984; Simpson and Vicario, 1990; Scharff and Nottebohm, 1991) revealed that the vocal control network contains at least two functionally distinct pathways, termed the vocal motor pathway (VMP) and the anterior forebrain pathway (AFP). The cortical premotor region HVC (proper name, not an acronym) contributes to both pathways and plays a central role in juvenile learning and adult production of song. One population of HVC neurons projects directly to vocal-motor cortex (RA, the robust nucleus of the arcopallium) while a second population projects to the avian basal ganglia (Area X). Both pathways converge at RA (see Fig. 2A) where individual RA neurons integrate synaptic input from HVC and LMAN (the lateral magnocellular nucleus of the anterior nidopallium, Mooney and Konishi 1991; Stark and Perkel, 1999). However, synapse number shifts during the course of vocal learning, favoring LMAN over HVC at the start of sensory-motor learning ($\sim 9:1$), then favoring HVC over LMAN as the adult song emerges ($> 2:1$, Herrmann and Arnold, 1991).

While the necessity of the VMP for production of adult song came quickly into focus (Nottebohm et al., 1976; Simpson and Vicario, 1990), developing a clear understanding of the premotor function of the AFP proved somewhat more complex. Disconnecting the output nucleus of the AFP (bilateral ablation of LMAN, see Fig. 2A) produces distinctly different vocal effects, depending on whether a bird is a learning juvenile or a mature adult. LMAN ablation/inactivation during the period of juvenile learning curtails vocal development (Bottjer et al., 1984; Scharff and Nottebohm, 1991; Olveczky et al., 2005; Elliott et al., 2014) whereas LMAN ablation in adulthood initially appeared to be without effect on the structure of adult song (Bottjer et al., 1984; Scharff and Nottebohm, 1991). Experimental evidence that the AFP might make a premotor contribution to adult song arose first in the work of Jarvis and Nottebohm (1997), who demonstrated singing-driven immediate-early gene (IEG) expression in LMAN and Area X of adult birds. Later, Kao et al. (2005) demonstrated that stimulation of LMAN during adult singing induced concurrent shifts in syllable pitch. Subsequent studies demonstrated that the AFP actively contributes a subtle dispersion to the spectral and temporal attributes of adult song syllables produced by the VMP (Kao and Brainard, 2006; Thompson et al., 2011). That is, adult song becomes more structured and less variable following LMAN ablation (see example in Fig. 3). The earlier assessment that LMAN ablation is without effect on adult song is understandable – detecting subtle shifts in the dispersion of

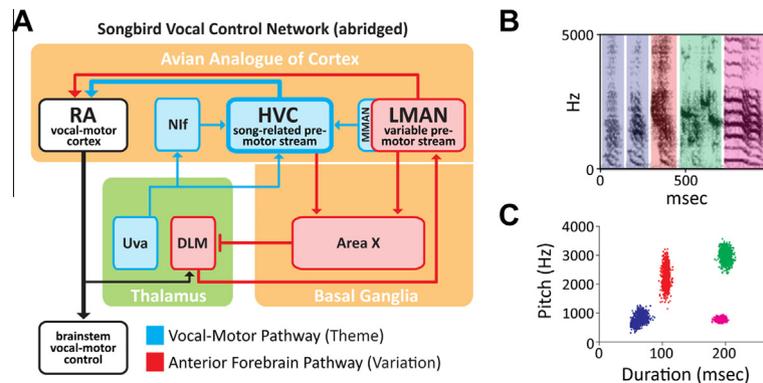


Fig. 2. (A) Sagittal schematic of the dual premotor architecture of the songbird vocal control network. The vocal motor pathway (VMP, blue) and the anterior forebrain pathway (AFP, red) generate contrasting premotor streams (theme and variation) that shape vocal output. (B) Frequency spectrogram of an adult zebra finch song, shown here as an ordered sequence of 4 distinct vocal gestures (syllables); each syllable is indicated by a different color. (C) Measurement of syllables across a day of singing. The scatter plot shows the duration and pitch of all syllables produced during a day of singing; each data point is one instance of a syllable and color coding of syllable clusters matches the spectrogram in (B). The clustering demonstrates the consistent “theme” of repeated syllables and the spread of each individual cluster represents the “variation” that is apparent between repetitions of each syllable across the day of singing.

adult song syllables required the development of automated song recording and objective syllable measurement technologies that allow capture and measurement of the acoustic structure and sequence of the large corpus of song syllables that adult male zebra finches produce each day (Tchernichovski et al., 2000; Wu et al., 2008; Daou et al., 2012).

The realization that AFP premotor activity contributes spectral and temporal variation to the structure of adult song led to the possibility that AFP activity might be responsible for the variable structure of juvenile subsong (Fig. 1A). Indeed, the ~9:1 ratio of LMAN:HVC synapses in juvenile RA (Herrmann and Arnold, 1991) would allow AFP premotor activity to effectively determine the structure of subsong. By ablating or inactivating HVC in juvenile birds that were just entering the phase of sensory-motor learning, Aronov et al. (2008) demonstrated that AFP premotor activity alone largely accounts for the variable acoustic structure of juvenile subsong (see also Öveczky et al., 2011; Thompson et al., 2011; Goldberg and Fee, 2011; Goldberg and Fee, 2012). Moreover, Aronov et al. (2008) showed that adult birds continue to sing following HVC ablation, but the acoustic structure of their songs resembles subsong (see also Chen et al., 2014). Thus, an overall picture emerges that the premotor architecture of birdsong involves two pathways (VMP and AFP) and that the two pathways vary in their contribution to vocal behavior as a function of song development.

EXPERIMENTAL TESTS OF DUAL PREMOTOR CONTROL IN ADULT BIRDS

As described above, singing by adult birds persists following ablation/inactivation of either HVC or LMAN, although the form of the singing differs depending on which region is targeted. Highly variable singing follows HVC ablation and highly structured singing follows LMAN ablation. If each premotor stream can function in

the absence of the other, how is activity in the two premotor pathways normally integrated during production of the adult song pattern? The simplest hypothesis comes from anatomy – the >2:1 ratio of HVC:LMAN synapses in adult RA (Herrmann and Arnold, 1991) logically favors song-related HVC activity over variable LMAN activity. One approach to testing this hypothesis is to experimentally alter the integration of the two premotor streams at RA by reducing (not eliminating) HVC input to RA, which can be accomplished by small, ~10% by volume bilateral microlesions of HVC (Thompson and Johnson, 2007; Thompson et al., 2007). Focal HVC damage should therefore ‘unmask’ LMAN premotor activity in adult birds.

Does use of focal HVC damage to unmask the influence of LMAN premotor activity produce the expected effect on adult song? Yes, following HVC microlesions, adult birds lose their preoperative vocal patterns and sing in a highly unstructured manner reminiscent of normal juvenile birds singing subsong (see subsong example in Fig. 1A and Thompson and Johnson, 2007; Thompson et al., 2007). However, the vocal control network proves unexpectedly resilient to focal damage as birds subsequently recover their structured preoperative song patterns within 1 week.

To test whether the initially unstructured singing of adult birds with focal HVC damage is indeed the result of ‘unmasked’ LMAN premotor activity, HVC microlesions were made in adult birds that had previously received bilateral LMAN ablation. In the absence of LMAN, focal HVC damage no longer disrupts preoperative vocal patterns (Thompson and Johnson, 2007). Similarly, if bilateral LMAN ablation follows HVC microlesions by one day, birds show a sudden recovery of their preoperative vocal patterns (Thompson et al., 2007). Thus, focal HVC damage produces unstructured singing only in the presence of the variable premotor activity of LMAN.

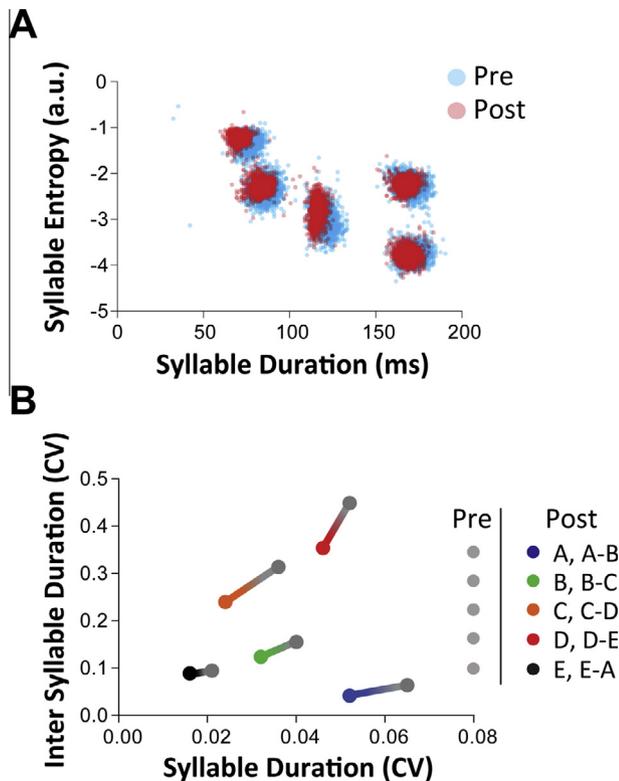


Fig. 3. Disconnection of AFP premotor activity ('Variation', see Fig. 2A) decreases statistical dispersion in the spectral and temporal structure of song. (A) A duration \times entropy scatterplot shows 5 syllable clusters from a day of singing before (pre) and after (post) bilateral LMAN ablation in an adult male zebra finch. The decrease in cluster size following LMAN ablation shows the subtle dispersion normally introduced by AFP premotor activity. Entropy is a measure of the non-harmonic structure of sound (Tchernichovski et al. 2000), thus syllables become less 'noisy' following LMAN ablation. (B) The same bird as in (A), but with measures of the variability (CV, coefficient of variation) of song temporal structure (syllable duration and inter-syllable duration). Shifts toward the lower-left corner of the plot indicate that the temporal structure of song becomes less variable following LMAN ablation.

AUDITORY FEEDBACK BALANCES THE GAIN OF THE DUAL PREMOTOR STREAMS

While the above findings are consistent with a purely anatomical explanation (reducing HVC premotor input to RA unmasks LMAN premotor activity), the \sim 1-week recovery of the preoperative song following focal HVC damage does not conform to this explanation. The time course of recovery is too rapid for a wholesale restoration of HVC input to RA via neurogenesis (Kim et al., 1999; Scharff et al., 2000), meaning that the vocal control network can adjust the relative gain of the dual premotor streams in a highly plastic manner. One approach to gain insight into this process is to ask whether auditory feedback is necessary for vocal recovery following focal HVC damage. In other words, does the vocal control network require auditory feedback of unstructured singing in order to adjust integration of the dual premotor streams to favor HVC premotor activity? Birds received bilateral HVC microlesions combined with deafening (via bilateral cochlea removal) to answer this

question. Such birds are unable to recover their preoperative songs, and persist in unstructured singing (Thompson et al., 2007; Stauffer et al., 2012). This finding suggests a surprising new role for auditory feedback – that of balancing the relative gain of the dual premotor streams to favor production of the structured song pattern.

Although the underlying mechanisms through which auditory feedback might balance the dual premotor streams are unknown, it seems likely to involve a strengthening of HVC input to RA, a weakening of LMAN input to RA, or a combination of both. This view is supported by findings from a related approach, where depriving adult birds of auditory feedback produces a gradual (weeks/months) loss of structured singing. Here, bilateral LMAN ablation is found to reverse the initial deafening-induced deterioration of the song pattern (Nordeen and Nordeen, 2010). The clear implication is that the loss of auditory feedback weakens the structured premotor activity of HVC and/or strengthens the variable premotor activity of LMAN, triggering the onset of unstructured singing in deafened adults. As with focal damage to HVC, prior ablation of LMAN protects against deafening-induced deterioration of adult song (Brainard and Doupe, 2000; Scott et al., 2007).

In addition to auditory regulation of the balance between dual premotor streams, social stimuli exert a similar effect on the premotor architecture of song. Adult male zebra finches produce their songs in at least two social contexts – in relative or complete social isolation (termed 'undirected song') and in response to the presentation of an adult female (termed 'directed song'). Directed song is more highly structured than undirected song (Kao et al., 2005; Kao and Brainard, 2006), and takes on the acoustic character of undirected singing by LMAN-ablated birds. Moreover, the decrease in spectral and temporal variation is known to be related to a reduction in AFP premotor activity (Jarvis et al., 1998; Hessler and Doupe, 1999; Kao et al., 2008; Stepanek and Doupe, 2010). Thus, stimuli associated with adult female birds are capable of influencing the acoustic structure of adult male song by suppressing AFP activity.

To this point we have emphasized the premotor architecture of birdsong. A complete picture of the zebra finch vocal control network would include information about the auditory memory of a bird's own song and the auditory memory of the paternal song acquired during the initial 'sensory' phase of learning. Although a full discussion of this work is beyond the scope of this review, it is worth noting that HVC and LMAN also contain an auditory memory of a bird's own song – revealed in anesthetized or sleeping birds by neural responses to playback of a bird's own song (Margoliash and Konishi, 1985; Dave and Margoliash, 2000; Giret et al., 2014). However, during singing, HVC and LMAN switch into a 'motor only' mode, where neurons no longer show responsiveness to auditory stimuli (Leonardo, 2004; Kozhevnikov and Fee, 2007; Giret et al., 2014; Hamaguchi et al., 2014). It is also worth noting that HVC activity is necessary for juveniles to encode an auditory memory of paternal song (Roberts et al., 2012), and

that AFP activity is necessary for birds to alter the spectral structure of their vocal output in a goal-directed fashion (Tumer and Brainard, 2007; Andalman and Fee, 2009; Warren et al., 2011; Charlesworth et al., 2012; Ali et al., 2013).

POPULATION CODING OF THE THEME IS AXIAL

As alluded to earlier, the necessity of HVC activity for the production of structured adult song (the theme) was evident from the classic works of (Nottebohm et al., 1976; Simpson and Vicario, 1990). However, whether song-structured premotor activity was generated within, or was relayed by, HVC remained an open question until recently. HVC receives afferent input from thalamic and multiple cortical sources (Fig. 2A), a circuit anatomy suggestive of the possibility that HVC may simply be a relay for song-structured activity arising at upstream nodes in the network. Indeed, it was known since the work of McCasland (1987) that Nif (nucleus interface of the nidopallium) activity precedes HVC activity during the production of song. However, by using a micro Peltier device to selectively control the temperature of HVC or RA in singing adult zebra finches, Long and Fee (2008) demonstrated that song-structured activity is generated within zebra finch HVC. Mild cooling or heating of HVC produced a uniform slowing or speeding of song (with little effect on the spectral structure of song), whereas cooling or heating of RA failed to alter the temporal or spectral structure of the song. These data suggested that zebra finch HVC encodes the motor timing necessary to shape

the acoustic trajectory of song and that it does so across a single timescale.

However, subsequent experimentation using micro Peltier cooling of zebra finch HVC revealed evidence for a second timing mechanism – when respiration is measured in addition to song, most inspiratory pulses respond to cooling in a non-uniform fashion (Andalman et al., 2011). Similarly, HVC cooling in another songbird species (canary) initially slows songs (as it does in the zebra finch), but further decreases in temperature alter the pattern of respiration and progressively ‘break’ individual canary syllables into two or more syllable segments (Goldin et al., 2013). This finding again suggests the presence of a second timing mechanism for respiratory control that interacts with, but does not necessarily reside within, HVC. It is presently unclear whether the syllable ‘breaking’ observed by Goldin et al. (2013) represents a species difference in the neural control of song (perhaps related to the fact that canary songs contain many more syllables and lack the rigid syntax of zebra finch songs) or whether the temperature decrease employed by Long and Fee (2008) and Andalman et al. (2011) was insufficient to produce respiratory ‘breaking’ of syllables. While future experiments will be necessary to resolve this question, at this point we can say that HVC encodes the motor timing necessary to shape the acoustic trajectories of individual syllables – Andalman et al. (2011), Long and Fee (2008), and Goldin et al. (2013) all agree on this point. However, this timing mechanism appears to interact with a second respiratory timing mechanism that can influence the initiation and termination of individual syllables.

Located at the dorsal surface of avian cortex, HVC is comprised of three subpopulations of neurons – excitatory RA-projecting neurons (HVC_{RA}), excitatory Area X-projecting neurons (HVC_X), and inhibitory interneurons (HVC_{INT}) – arranged in a superficially isomorphic mosaic. Fig. 4 shows an example of the mosaic distribution of HVC_X neurons as viewed from a dorsal perspective (the other two populations are distributed similarly). While subtle regional differences in cytoarchitecture are observed (Fortune and Margoliash, 1995; Wild et al., 2005) there is little to suggest a spatial or topographic organization that might underlie the precise temporal organization of song. Additionally, the auditory responses to song playback observed in anesthetized birds do not differ throughout the spatial extent of HVC (Margoliash, 1986; Margoliash et al., 1994; Sutter and Margoliash, 1994).

The largely mosaic anatomy of HVC combined with the spatial uniformity of auditory responses to song playback suggest that a motor memory for song might be encoded in a diffuse or highly distributed network of cells. However, the results of the temperature manipulations described above (Long and Fee, 2008; Andalman et al., 2011; Goldin et al., 2013) suggest a highly organized architecture, where individual song elements are generated by feed-forward activation across chains of HVC neurons (Hahnloser et al., 2002; Fee et al., 2004; Li and Greenside, 2006; Jin et al., 2007; Long et al., 2010). To elucidate the network organization of a motor memory in singing birds, small ~10% by

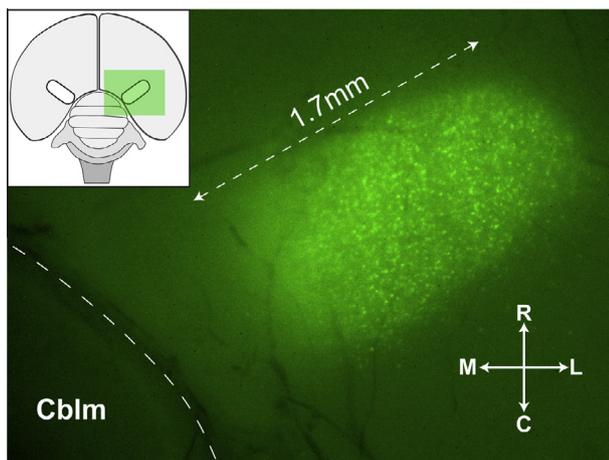


Fig. 4. Dorsal view of the right hemisphere of a male zebra finch brain shows the superficial location of HVC. As shown in the inset (upper left), the dorsal surface of the cerebellum (Cblm) is at lower left and the curved dashed line indicates the medial edge of the right hemisphere. HVC neurons that project to Area X were back-filled by injection of a fluorescent dye (DiO) into Area X. The mosaic distribution of these neurons throughout HVC is evident from this dorsal view. The distribution of HVC neurons that project to RA and HVC interneurons is similarly mosaic. However, recent evidence indicates that HVC neurons are anatomically and physiologically connected primarily within the rostro-caudal axis (Stauffer et al., 2012) and that song-related neural activity traverses the rostro-caudal axis of HVC during singing (Poole et al., 2012).

volume bilateral HVC microlesions were combined with singing-driven IEG expression to index HVC neural activity (Stauffer et al., 2012). If zebra finch song is encoded by a highly distributed network of cells, focal damage located anywhere within HVC should produce symmetrical reductions in singing-driven IEG labeling throughout the spatial extent of HVC.

While a dramatic decrease in singing-driven IEG labeling is observed following HVC microlesions, this decrease is axially organized. A distinctive swath of reduced IEG labeling extends in the rostral and/or caudal direction from the position of HVC microlesions. In contrast, robust IEG labeling is observed medial and/or lateral to the position of the HVC microlesions. Thus, the population coding of song in HVC appears to involve rostro-caudally oriented swaths of neurons that have limited medio-lateral connectivity. This view has been confirmed anatomically – focal injections of retrograde tract-tracing dyes into HVC produce cell labeling that extends primarily in the rostral and/or caudal direction from the injection site (see also Nottebohm et al., 1982), and physiologically – *in vitro* stimulation and recording experiments using horizontal slices of HVC reveal robust evoked activity only when the recording electrode is placed within the rostro-caudal axis of the stimulating electrode (Stauffer et al., 2012). The *in vitro* stimulation and recording data also suggest that the rostro-caudal swaths of interconnected HVC neurons are no more than ~400 μm wide. Evoked activity falls rapidly once the recording electrode is moved more than 200 μm off the rostro-caudal axis (in either direction) of the stimulating electrode. However, to what extent these rostro-caudal swaths overlap or are interleaved across the medio-lateral axis of HVC is presently unknown.

Recent work expands on the rostro-caudal axial organization of a motor memory for song in zebra finch HVC. For example, if the bulk of neural activity traverses the rostro-caudal axis of HVC during singing, a rostro-caudal transection (dividing HVC into medial and lateral halves) should have little effect on song. Poole et al. (2012) observed this predicted non-effect following rostro-caudal knife-cuts of HVC, even in deafened birds that are no longer receiving auditory feedback. Similarly, if the bulk of neural activity traverses the rostro-caudal axis of HVC during singing, electrode arrays placed in HVC should detect an organized pattern of rostro-caudal activity. Day et al. (2013) placed multi-electrode arrays in HVC of anesthetized birds and observed rostral-to-caudal waves of spontaneous neural activity. Together, these data provide the strongest evidence to date that population coding of a motor memory for zebra finch song is organized across the rostro-caudal axis of HVC.

COMPUTATIONAL APPROACHES TO COMPOSITIONAL TECHNIQUE

How might rostro-caudally oriented swaths of interconnected HVC neurons compose a motor memory for structured adult song? A multi-swath arrangement suggests that song will not be encoded in a unitary

fashion, but rather in something like the piecemeal (syllable by syllable) fashion that it is learned (Ravbar et al., 2012; Lipkind et al., 2013). Two other lines of evidence also suggest this will be the case. The first is that different elements of the song pattern are distributed across left and right HVC (Ashmore et al., 2008; Long and Fee, 2008; Wang et al., 2008), meaning that it is unlikely that a single swath in left or right HVC encodes the entire pattern. The second is that the song elements present within each HVC appear to be encoded more than once, possibly encoded in parallel across two or more rostro-caudal swaths. Parallel encoding is necessitated by the observation that birds with bilateral focal (microlesion) damage at various locations within HVC can still produce their pre-operative song pattern (Thompson and Johnson, 2007; Thompson et al., 2007; Stauffer et al., 2012). Thus, it appears likely that rostro-caudal swaths of HVC neurons encode individual elements of the song pattern (e.g., individual syllables, or the transitions from one syllable to the next) and that some swaths mirror the encoding of others.

The three subpopulations of HVC neurons that comprise each swath show distinctive profiles of activity during singing. Excitatory HVC_{RA} neurons are depolarized during song and show clocklike ~10-ms bursts of activity that recur at a single, specific temporal location within the 0.5–1s songs of adult male zebra finches (Hahnloser et al., 2002; Long et al., 2010). In contrast, inhibitory HVC_{INT} neurons show relatively tonic activation during singing, with bursting and suppression of activity at several distinct temporal locations within each song (Hahnloser et al., 2002; Kozhevnikov and Fee, 2007; Long et al., 2010; Amador et al., 2013). Excitatory HVC_X neurons are hyperpolarized during song and generate discrete bursts at one to several distinct temporal locations within each song (Kozhevnikov and Fee, 2007; Long et al., 2010; Hamaguchi et al., 2014). Because HVC_{RA} neurons provide the premotor output of HVC, any role played by HVC_{INT} and HVC_X neurons in the motor encoding of song must occur via mono- or polysynaptic connectivity with HVC_{RA} neurons. Here, the work of Mooney and Prather (2005) suggests excitatory connectivity between HVC_{RA} neurons, and bidirectional connectivity between HVC_{RA} and HVC_{INT} neurons, between HVC_X and HVC_{INT} neurons, and between HVC_{RA} and HVC_X neurons. Few connected HVC_{INT} neurons were observed, but this may be because the search strategy favored pairs containing HVC_X neurons. Given this possible sampling bias, excitatory connectivity between HVC_X neurons is notably absent. Thus, a wide variety of mono- and poly-synaptic interactions could occur between HVC neurons. One challenge is to develop a circuit model of these interactions that can produce patterns of activity that reflect those observed *in vivo*. That is, to define the circuit that is the encoded song.

Two general approaches – temporal and gestural – have been used to model the encoding of song within HVC. Based on the distinctive, clock-like physiology of HVC_{RA} neurons it has been hypothesized that these neurons generate a continuous, feed-forward sequence of activity over time (Hahnloser et al., 2002; Fee et al., 2004; Long et al., 2010). That is, at each moment in the

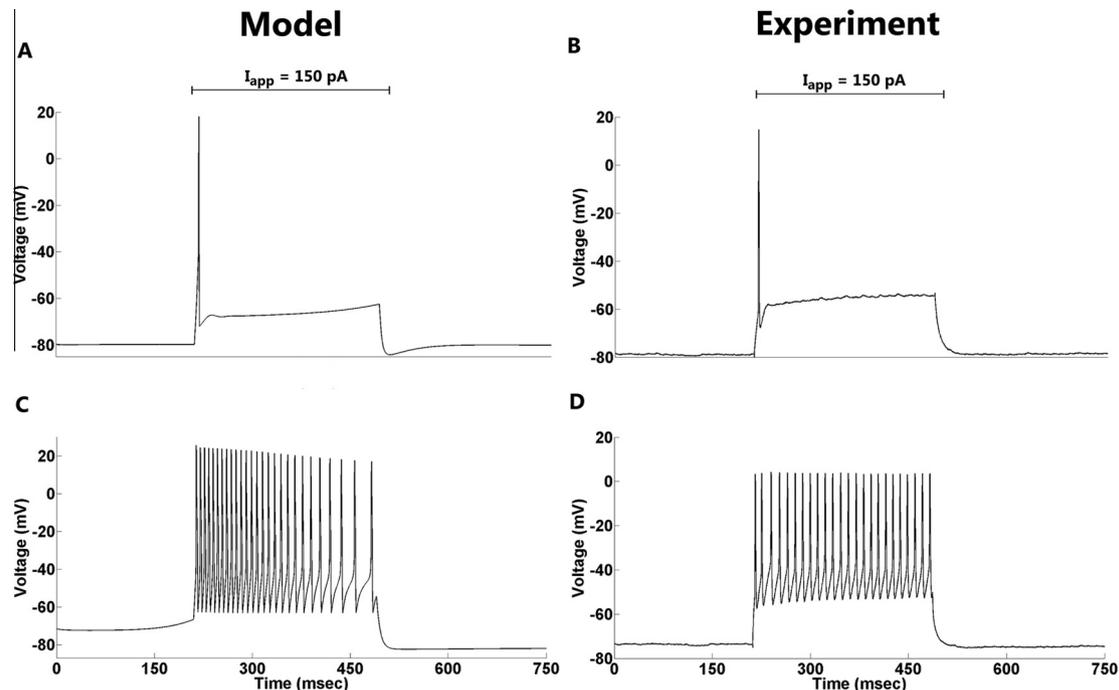


Fig. 5. Computer simulations with a biophysical model (A) can be calibrated to the activity of HVC neurons (B, an HVC_{RA} neuron stimulated with 150 pA of depolarizing current) and used to generate predictions (C, with SK current removed) that can be tested (D, with SK current blocked by apamin) to understand the roles played by the different ionic currents. Reproduced from [Daou et al. \(2013\)](#).

song, there is a small ensemble of HVC_{RA} neurons active only at that time, and each ensemble transiently activates a subset of RA neurons determined by the synaptic connections of HVC_{RA} neurons in RA ([Leonardo and Fee, 2005](#)). In one form of this ‘temporal encoding’ approach, HVC_{RA} neurons alone play the role of the conductor of the song with little or no role played by HVC_X and HVC_{INT} populations ([Li and Greenside, 2006](#); [Jin et al., 2007](#); [Long et al., 2010](#)). Other forms of temporal encoding require synaptic interaction between HVC_{RA} and HVC_{INT} populations ([Drew and Abbott, 2003](#); [Weber and Hahnloser, 2007](#); [Jin, 2009](#); [Gibb et al., 2009a](#)), while another incorporates brainstem feedback that provides inter-hemispheric coordination of song and activation and sequencing of ensembles of syllable-specific HVC_{RA} neurons and HVC_{INT} ([Gibb et al., 2009b](#)). In contrast, [Amador et al. \(2013\)](#) have proposed a ‘gesture encoding’ model that posits that all classes of HVC neurons modulate their activity in relation to the timing of vocal-motor gestures. In this report, HVC projection neurons appear to preferentially burst at gesture trajectory extrema (GTE), while HVC_{INT} neurons tend to show local minima in their activity at GTE ([Amador et al., 2013](#)). In other words, the bursting of HVC projection neurons and the modulation of HVC_{INT} activity appears to correlate with particular configurations of the vocal-motor apparatus. Although the sequential firing across the population of HVC projection neurons unfolds in an ordered fashion, vocal gestures (and not time) are explicitly represented. A notable feature of the ‘gesture encoding’ model is that it does not distinguish between the activity of HVC_{RA} and HVC_X neurons ([Amador et al., 2013](#)).

To explore potential polysynaptic configurations that might underlie the motor encoding of song, we developed the first biophysical (ionic current-based) computational models of the three populations of HVC neurons ([Daou et al., 2013](#)). Biophysical models add to integrate and fire models by specifying the recipe of ionic currents needed to replicate the unique physiology of each HVC cell type, thus incorporating how the intrinsic properties of each cell type process synaptic inputs to produce a distinct output. This is particularly important for circuits that require precisely timed activity (arguably, the song network fits into this category). For example, the brain stem circuit believed to analyze interaural time differences appears to require cells that contain a low-threshold K⁺ current. Without this biophysical element, the circuit cannot process inputs with the required temporal precision required to accurately locate a sound source ([Reyes et al., 1996](#); [Grau-Serrat et al., 2003](#); [Meng et al., 2012](#)).

Moreover, with biophysical models the ion channel activity in each population type can be manipulated *in silico* to make predictions that can be tested *in vitro* and *in vivo* using ion channel pharmacology. For example, [Fig. 5](#) shows how biophysical modeling can be used to identify ion channel activity that controls the distinctive bursting behavior of HVC_{RA} neurons. Here, a model (panel A) was calibrated to match the behavior of an HVC_{RA} neuron in a brain slice under application of a 150-pA depolarizing current (panel B). In both cases, a single spike is produced, followed by a voltage plateau. The model suggests that the cessation of spiking following the first spike is due to a strong

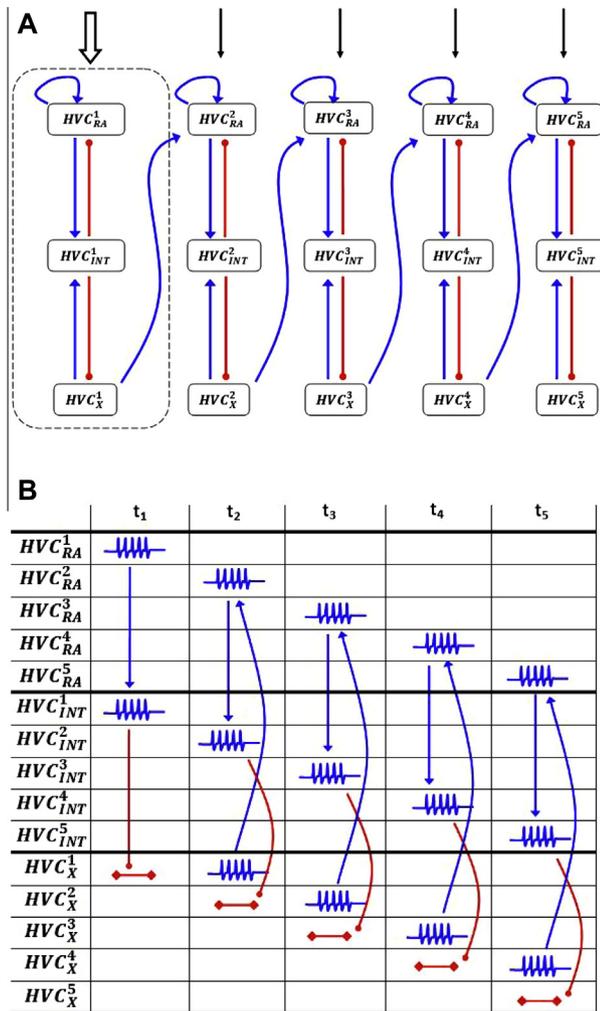


Fig. 6. (A) Models of single biophysical neurons can be synaptically coupled to interrogate the emergent properties of neuronal networks in the HVC. Blue arrows indicate excitatory connections and red arrows indicate inhibitory connections. This architecture illustrates five interconnected microcircuits, the first of which is indicated with the dashed box. (B) The output of the model network is illustrated at five different time points. Blue spiking patterns indicate electrical bursts, while red horizontal lines indicate silent cells.

small-conductance Ca^{2+} -activated K^+ current (i.e., an SK current). This leads to the prediction that if the SK current is eliminated *in silico* ($g_{\text{SK}} = 0$ in the model), then spiking should continue throughout the duration of the depolarizing current pulse (panel C). This prediction was then tested *in vitro*, using the SK channel blocker apamin, and, as predicted, the HVC_{RA} neuron spiked continuously during the depolarization (panel D).

As the above example illustrates, biophysical models provide a useful tool for designing pharmacological experiments that illuminate the ionic currents underlying the electrical activity of HVC neurons. Once validated, biophysical models can also be combined with ion channel pharmacology in singing birds to investigate possible network configurations, or predict the role that particular patterns of neural activity play in encoding the song. For example, the data shown in Fig. 5 suggest that apamin could be used to vary the burst duration of

HVC_{RA} neurons and thereby explore the role that the burst duration of HVC_{RA} neurons plays in the network activity of HVC. However, a first step is to assemble an exploratory network architecture that simulates the characteristic activity patterns of HVC_{RA} , HVC_{X} and HVC_{INT} neurons in singing birds. This is illustrated in Fig. 6A, where the three types of HVC neurons are synaptically connected into five microcircuits, simulating the activity of five rostro-caudal swaths of interconnected HVC neurons. This network architecture takes into account both temporal and gestural modeling approaches. First, in agreement with a ‘temporal encoding’ approach, the network architecture exhibits a feed-forward mechanism that controls the circuit dynamics (e.g., Li and Greenside, 2006; Jin et al., 2007; Jin, 2009; Gibb et al., 2009a; Long, et al., 2010). However, instead of a feed-forward chain of HVC neurons orchestrating the song, the network architecture contains chains of microcircuits and sequential activity propagates from one microcircuit to another rather than from one neuron to another. Second, in agreement with a ‘gesture encoding’ model, sequential activity involves all classes of HVC neurons (Amador et al., 2013). Inhibition exerted by HVC_{INT} onto HVC_{X} neurons is the main ingredient for transferring sequential activity forward in time via rebound firing. The network architecture lacks the brainstem feedback proposed by Gibb et al. (2009b) and therefore does not explicitly address inter-hemispheric coordination (Ashmore et al., 2008; Long and Fee, 2008; Wang et al., 2008) or include a mechanism to vary the sequencing of individual syllables.

The first microcircuit shown in Fig. 6A is enclosed in a dashed box. Note that each neuron in the microcircuit is a mean field representation of a small population of neurons of that type. The types of connections are based on those discovered by Mooney and Prather (2005). Spatially, the connectivity is highly local and limited to neighboring microcircuits, similar to the observed connectivity of HVC (Stauffer et al., 2012). Moreover, each microcircuit receives tonic (un-patterned) excitatory input from upstream nuclei (Nif, Uva (nucleus uvaeformis), and MMAN (medial magnocellular nucleus of the anterior nidopallium), see Fig. 2A), but the first microcircuit receives the strongest afferent input (downward arrows in the diagram). Of the three afferent nuclei, only ablation of Uva severely disrupts production of adult song (Foster and Bottjer, 2001; Cardin et al., 2005; Coleman and Vu, 2005) and the model therefore assumes that Uva input is the main excitatory input for HVC activity (Coleman et al., 2007).

The output of the network is illustrated in Fig. 6B at five successive time points. During the first time point the HVC_{RA} neurons in the first microcircuit fire due to afferent input and the self-excitation (HVC_{RA} neurons synapse onto other HVC_{RA} neurons of the same microcircuit). This is illustrated in Fig. 6B as a burst of spikes (in blue) at time point t_1 . This activity is passed on to the interneurons of the same microcircuit, causing them to fire (burst of interneuron activity at time point t_1). The HVC_{X} neurons of the microcircuit are suppressed, since they receive inhibitory input from the

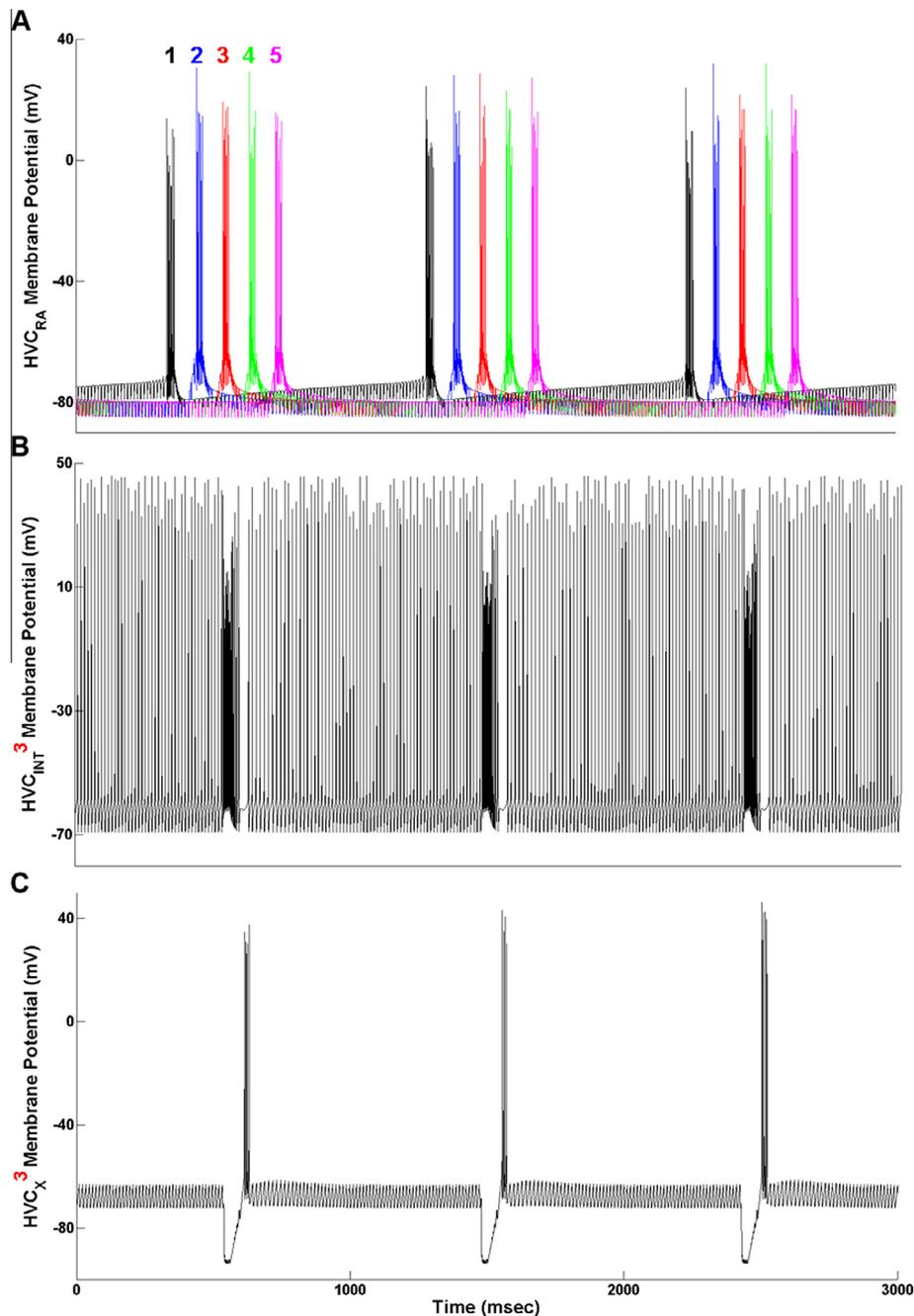


Fig. 7. Activity patterns for the network architecture shown in Fig. 6A. (A) Sequential bursting of HVC_{RA} neurons of the five microcircuits within the network (labeled with numbers over the first epoch) showing three epochs of activity. (B) Firing pattern of a sample interneuron in the network (HVC_{INT}³) over the course of the three epochs showing one burst per epoch as a result of the excitation from HVC_{RA}³. (C) An HVC_X neuron (HVC_X³) displays hyperpolarization followed by rebound bursting once per epoch. It exhibits rebound bursting due to release from synaptic inhibition from HVC_{INT}³, which is crucial for the network activity pattern, and which was observed in intracellular recordings in Daou et al. (2013).

interneurons (red horizontal line at time point t_1 , indicating no spiking). The HVC_{RA} neurons eventually stop firing, due to the buildup of slowly-activating K⁺ currents (SK current and a Na⁺-activated K⁺ current). When spiking stops in these neurons, it also stops in the interneurons, allowing the HVC_X neurons to escape from inhibition and produce a rebound burst of spikes (blue spiking activity at time point t_2). This activity is propagated to the HVC_{RA} neurons of the second microcircuit, which in

turn begin to fire (blue spiking activity of HVC_{RA} neurons in microcircuit 2 at time point t_2). The process continues in this way down the series of microcircuits in a feed-forward manner.

The output of the model simulation for this network is shown in Fig. 7. The top panel shows the activity of the HVC_{RA} neurons from each of the five microcircuits superimposed (color coded). The HVC_{RA} neurons in successive microcircuits produce short bursts of activity

in succession. After a quiescent period, the activity in the network begins again. Three epochs of patterned activity are shown. Fig. 7B shows the firing pattern of a sample interneuron from the third microcircuit in the network (HVC_{INT}^3) over the course of the three epochs, exhibiting bursts of activity as a result of the excitation from HVC_{RA}^3 intermixed with tonic spiking. Fig. 7C shows the firing pattern of a sample HVC_X neuron (HVC_X^3), which displays a burst occurring once per epoch. These activity patterns have similarities to those observed in recordings from the singing bird. For example, each model HVC_{RA} neuron bursts exactly once per epoch and always at the same point in the epoch, replicating the pattern recorded in singing birds (Hahnloser et al., 2002; Kozhevnikov and Fee, 2007; Long et al., 2010). However, while each model HVC_{INT} fires in a relatively tonic fashion with a single burst and suppression of activity per epoch, HVC_{INT} neurons in singing birds show tonic firing with several bursts and suppressions of activity per song (Hahnloser et al., 2002; Kozhevnikov and Fee, 2007; Long et al., 2010; Amador et al., 2013). Each model HVC_X neuron exhibits brief hyperpolarization followed by bursting that occurs at the same point in each epoch, whereas HVC_X neurons in singing birds show tonic hyperpolarization and more commonly show more than one burst of activity per song (Kozhevnikov and Fee, 2007; Long et al., 2010; Hamaguchi et al., 2014) although single bursts have been observed (Kozhevnikov and Fee, 2007).

Incorporation of more interconnections between microcircuits, rather than just the one that is shown in Fig. 6A, can yield more HVC_{INT} bursts and more HVC_X bursts per epoch (data not shown) while still remaining truthful to the known connectivity between all three types of HVC neurons (Mooney and Prather, 2005). We emphasize that our purpose here is exploratory and we are not advocating for a particular model – certainly, our exploratory model does not rule out architectures that are limited to connectivity between HVC_{RA} neurons, or between HVC_{RA} neurons and HVC_{INT} , such as Drew and Abbott (2003), Li and Greenside (2006), Jin et al. (2007), Weber and Hahnloser (2007), Gibb et al. (2009a,b), Jin (2009), and Long et al. (2010). However, our model suggests a general architecture that could begin to account for the activity of all three types of HVC neurons during singing, and it illustrates how computational models can be used to generate alternate explanations for experimental data, as well as to make novel predictions that can be tested experimentally.

For example, the network architecture presented in Fig. 6A makes a clear prediction – it predicts that HVC_X neurons play a key role in song production. This prediction appears to conflict with existing data that birds can still produce their structured adult song following selective ablation of HVC_X neurons (Scharff et al., 2000). Indeed, the Scharff et al. (2000) result is typically cited as a reason to exclude HVC_X neurons from model architectures. However, in the Scharff et al. (2000) study only ~60% of identified HVC_X neurons were ablated, while ~40% remained. Is it possible for a network model that relies on HVC_X activity to account for these data? To check this, we reduced the synaptic weight from

HVC_X neurons by 60% and indeed the sequence of HVC_{RA} neuron activity was disrupted. However, the sequential bursting of HVC_{RA} neurons was restored when the tonic drive to the microcircuits was increased in magnitude. Thus, while complete ablation of HVC_X neurons would cause irreparable damage to the activity produced by this network, a physiologically-plausible modification of synaptic strength allows the network to compensate for a partial reduction in the number of HVC_X neurons. The power of numerical simulations with model neurons is that one can investigate the spiking patterns produced by many coupling topologies, exploring the range of possibilities *a priori* and generating testable hypotheses. Successful predictions will constrain and select from the range of possible network configurations, creating a cycle of modeling and experimentation that will lead to identification of the key anatomical and physiological parameters necessary to replicate neural function.

In summary, we have described how a general framework for the anatomy of a motor memory for birdsong has been worked out – rostral-caudal swaths of interconnected HVC neurons, each presumed to encode a selected element of the song pattern. Moreover, electrophysiological data have revealed the characteristic activity of each HVC cell type during singing. Computational modeling is beginning to unite these well-established anatomical and electrophysiological features of HVC and successful simulations should lead to the discovery of the compositional technique used by the brain to encode the songs of birds. In turn, this knowledge will provide unique insight into the neural and population mechanisms that encode and consolidate our own human speech patterns.

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