Developmental modulation of the temporal relationship between brain and behavior

Running head: Development of a brain-behavior relationship

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Aspects of this study have previously appeared in abstract form (Aoki and Nick 2006; Crandall and Nick 2006; Nick 2005).
SUMMARY

Humans and songbirds shape learned vocalizations during a sensorimotor sensitive period or ‘babbling’ phase. The brain mechanisms that underlie the shaping of vocalizations by sensory feedback are not known. We examined song behavior and brain activity in zebra finches during singing as they actively shaped their song toward a tutor model. We now show that the temporal relationship of behavior and activity in the premotor area HVC changes with the development of song behavior. During sensorimotor learning, HVC bursting activity both preceded and followed learned vocalizations by hundreds of milliseconds. Correspondingly, the duration of bursts that occurred during ongoing song motif behavior was prolonged in juveniles, as compared to adults, and was inversely correlated with song maturation. Multi-electrode single unit recording in juveniles revealed that single fast-spiking neurons were active both before and after vocalization. These same neurons responded to auditory stimuli. Collectively, these data indicate that a key aspect of sensory critical periods, prolonged bursting, also applies to sensorimotor development. In addition, prolonged motor discharge and sensory input coincide in single neurons of the developing song system, providing the necessary cellular elements for sensorimotor shaping through activity-dependent mechanisms.

Key Words: development, electrophysiology, birdsong, learning, memory, vocal
The brain mechanisms of sensorimotor integration and motor skill learning are not well understood. However, the birdsong model has begun to illuminate fundamental principles of forebrain control of behavior (Perkel 2004). The birdsong system has the necessary characteristics to enable a systematic investigation of the neural bases of sensorimotor learning. Song production requires precise coordination of muscles in the vocal organ and respiratory system (Goller and Cooper 2004; Suthers and Zollinger 2004), and these coordinated patterns are learned using sensory feedback (Konishi 1965). In addition, the song behavior depends on a series of anatomically and functionally distinct brain areas (Nottebohm et al. 1976) and, importantly for plasticity studies, matures over several months (Immelmann 1969), enabling day-by-day assessment and comparison of neural and behavioral changes.

The pallial (cortical) song area HVC resides at the interface of sensory and motor pathways and projects directly to a cortico-basal-ganglia-thalamocortical loop (the Anterior Forebrain Pathway) that enables song plasticity (Bottjer et al. 1984; Brainard and Doupe 2000; Williams and Mehta 1999). Thus, HVC is anatomically well-positioned to have a role in sensorimotor integration and learning. HVC both controls learned song (Ashmore et al. 2005; Nottebohm 2004; Vu et al. 1994) and receives auditory information (Katz and Gurney 1981; Margoliash 1983; McCasland and Konishi 1981; Mooney 2000; Theunissen and Doupe 1998). Moreover, HVC exhibits patterned activity that is locked to song production (Fee et al. 2004; McCasland 1987), implicating this area in the control of song timing. Recent studies indicate that HVC contains a pattern generator (Mooney and Prather 2005; Solis and Perkel 2005), suggesting that temporal aspects of song are stored in the HVC circuitry. Although these and other data identify HVC as a crucial player in song production, its role in song plasticity and learning is not clear. No recordings of HVC activity in singing finches in the process of song learning have ever been published.

As with human speech, song learning is divided into two phases: the sensory phase during which the songbird memorizes a tutor song (explicit memory) and the sensorimotor phase during which he then uses comparison of auditory feedback and the memorized tutor song template to shape his song (implicit memory) (Bolhuis and Gahr 2006; Konishi 1965). During the sensorimotor phase, HVC serially recalibrates its response to playback of the bird’s own song. During sleep (Nick and Konishi 2005a) and anesthesia (Volman 1993), HVC always responds most to the current version of the bird’s own song, even when it is little more than repeated begging calls. During waking in the sensorimotor phase, HVC responds preferentially to the memorized song of an adult male tutor (Nick and Konishi 2005b). Collectively, these data show that HVC contains or receives signals that code for both the current version of the bird’s own song and the tutor song memory. Further, they indicate that HVC activity has a functional and dynamic role in song learning. However, changes in HVC activity during song learning have not been investigated.

To understand the neural signals that drive song learning during development, we employed long-term population and single unit (multi-electrode) recording techniques to...
examine HVC activity in juvenile finches as they actively shaped their song toward the mature adult form.

Methods

Subjects

For chronic population recording experiments, 35 juvenile (< 90 days) and 18 adult (> 200 days) male zebra finches (Taeniopygia guttata) were surgically implanted. Of these, 14 juveniles had high quality recordings (root mean square premotor signal:noise ≥ 2) and 5 sang while in the sensorimotor phase (earliest ages recorded for each juvenile: 62, 65, 66, 72, 72 days). Of the adults, 6 had high quality recordings and 3 sang. The low song yield is presumably due to our strict requirement for undirected song (emitted in the absence of a female). All recordings were made in the absence of a female. Undirected song is thought to reflect song practice (Jarvis et al. 1998) and is thus most relevant to learning studies. One finch implanted as a juvenile was not recorded singing until adulthood (Red-87, age 141 days) and was therefore used in the adult group (total adult N = 4; age recorded for other three adults: >200 days). All juvenile and all but three adult finches were reared in our facility on a 12:12 light cycle. Three adult birds were obtained from Magnolia Bird Farm (Anaheim, CA). None of the finches used in the population recording experiments were ever exposed to auditory playback.

For the multi-electrode (triode) experiments, 88 juvenile (< 70 days) finches were implanted. Of these, 6 juvenile finches (age 53 – 62 days) vocalized (undirected) before deterioration of the recording. All juveniles used in the triode experiments were reared in an acoustic chamber that also contained their parents and same clutch siblings until day 45 and then isolated in an acoustic chamber thereafter. All procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Chronic physiological recording

For population recordings, finches were implanted with a set of recording electrodes: 1 or 2 50-µm nichrome-formvar electrodes in HVC (1.1 – 1.8 MΩ, AM Systems, Carlsborg, WA), a 50-µm nichrome-formvar electrode adjacent to HVC for use as a reference electrode (1.1 – 1.8 MΩ), a ground electrode (Relative to lambda: 5-6 mm lateral, 6 mm anterior. Depth: 0; inserted between the skull and the dura) and an electroencephalogram electrode (Relative to lambda: 2-3 mm lateral, 7-8 mm anterior. Depth: 0; inserted between the skull and the dura). The headstage and recording environment were previously described (Nick and Konishi 2001; Schmidt and Konishi 1998).

For increased stability and recording quality in chronic population recordings, we (1) modified the implant such that the recording electrodes extended rigidly from the connector in the implantable orientation, which decreased wire handling and accidental stripping; (2) scored the juvenile one-layer skull with a scalpel blade and made tiny holes in the top layer of the more mature two-layer skull to increase adherence of the dental
cement; (3) did not plate the recording electrode wires and reference, with the aim of increasing tissue adherence; and (4) gave the finch an anti-inflammatory (Metacam, Boehringer Ingelheim, Germany) and antibiotic (Baytril, Bayer, Germany) in lactated ringers immediately following surgery and antibiotic in lactated ringers for three days after surgery.

For multi-electrode single unit recordings, the electrode configuration was: a gold-plated triode in HVC (wire diameter 12- or 18-µm, AM Systems or Kanthal, Palm Coast, FL), a gold-plated reference electrode with the same resistance as a single wire of the triode adjacent to HVC (≤ 1 MΩ), and a ground wire placed as above. Two of the triode implants also consisted of a titanium microdrive and bipolar stimulating electrodes placed in or near Area X and the robust nucleus of the arcopallium (RA).

Finches were recorded 24-hr per day. Food and water were available ad libitum. All data were acquired with custom-written LabView software (Datafleet, Minneapolis, MN) at a sampling frequency of 44.1 kHz. During recording, a light-weight operational amplifier was attached to the bird and connected to a mercury commutator via a flexible cable. HVC neural activity was amplified and filtered 300 – 10,000 Hz. Song behavior was high-pass in-line filtered at 100-Hz (Shure, Niles, IL) and recorded with a microphone (Earthworks, Milford, NH). Localization of electrodes to HVC was confirmed with premotor activity in all cases and cresyl-violet histology in 7 of 13 finches.

**Sound playback (multi-electrode single unit only)**

Behaviorally-relevant and artificial sounds were played back to 4 of 6 birds implanted with multi-electrodes. Stimuli consisted of noise (3 or 15 sec), silence, the Bird’s Own Song (only available for two birds), the tutor song, and two conspecific songs from two different adult males that were not the song tutor. All stimuli were delivered in random order during every trial set. The onset interstimulus interval of stimuli was 20 sec. Trials during which movement or vocalization occurred during the playback stimulus were discarded. The method of detection of movement and vocalization has been previously described (Nick and Konishi 2005b).

**Behavioral analysis**

The Sound Analysis Pro (Tchernichovski et al. 2000) program was used to compare 25 randomly selected motifs from each day to 25 randomly selected motifs from the most mature day available to obtain percent similarity values. Feature calculation was tuned to zebra finch sounds (default). Sound Analysis Pro was also used to cluster syllables based on the following features: syllable duration, mean pitch, mean entropy, mean frequency modulation, and mean goodness of pitch (see the Sound Analysis Pro manual for specific details, available at the website http://ofer.sci.ccny.cuny.edu/html/sound_analysis.html). After clustering, the longest duration syllable was analyzed for entropy variance across development.
Chronic Population Analysis

All data were analyzed with custom-written Matlab functions (by T.A.N.). Initial analysis consisted of the sorting of sound data. Unlike vocalizations, movement noise tends to be highly variable at time intervals < 20 msec, which enabled the detection and elimination of most movement noise. Sound data were further sorted according to temporal properties. Preliminary songs were defined as sounds lasting \( \geq 500 \) msec with time gaps of no more than 20 msec.

To examine prolonged bursting associated with vocalizations, we analyzed HVC activity that occurred within 5 sec of vocalization. Ongoing activity was measured during 2-sec silent periods that occurred between vocalizations. 20 randomly-selected silent periods were measured per day. 3 days were analyzed per electrode using root mean square (RMS), which is a measure of the power of the neural population recording. These data were compared using a two-tailed Student’s t-test.

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

We focused our analysis on action potentials (instead of, for example, low frequency potentials) and behaviorally relevant vocalizations (instead of, for example, movements) by band-pass filtering our electrode and vocal data prior to low-pass filtering at 50 Hz to obtain an amplitude envelope. Thus, amplitude envelopes of the canonical motif and all preliminary songs (~4 terabytes of data) were constructed by band-pass filtering the sound recording at 1 – 8 kHz and then low pass filtering the rectified waveform at 50 Hz (all digital filters were Fixed Impulse Response Hamming). Amplitude envelopes of the canonical motif and preliminary songs were cross-correlated. Sharp peaks in the cross-correlation revealed the onset of a motif that matched the canonical motif (Supp. Video 1). Behavioral song motifs and corresponding neural activity were then excised and saved for further analysis.

To examine patterns of population spiking activity, amplitude envelopes of rectified neural activity during motifs were created by band-pass filtering 300 – 6000 Hz and then low-pass filtering at 50 Hz. The line plots of amplitude envelopes shown in Figs. 2 and 3 were normalized to the maximum. Peaks in activity (bursts) were identified as continuous periods above the mean of neural motif activity from the same day. The findings that we report were not dependent on a specific threshold (mean), since they held if median or mean + standard deviation were used instead (Supp. Fig. 1). For assessment of differences in activity peak duration and rate between groups, the mean of the youngest juvenile and adult recordings were compared using a Wilcoxon rank sum test (\( \alpha \)).
Each electrode contributed one datum per comparison. Comparison of activity across age within the same finch was achieved using an ANOVA ($\alpha = 0.05$). All data points are means and all errors are standard errors of the mean. Although experience and not age is probably the strongest predictor of song system maturity, age is correlated with experience and presents a more quantifiable parameter.

**Chronic Single Unit Analysis**

Waveforms for clustering were found by filtering the triode data (band-pass 300 - 5000 Hz), finding all spikes above the threshold (mean + 2 x standard deviation; calculated independently for each channel) that did not occur during a movement artifact and saving the spike time (relative to the entire cluster session), waveform (16-bit; ~0.36 msec), vocalization, and movement data for each data file such that they could be retrieved later, across trials, by a custom loading function. A subset of Matlab functions taken from the MClust suite (by A.D. Redish) was used to obtain the clustering parameters (Energy, Derivative of Energy, First Principle Component) across all three wire electrodes (giving a total of 9 parameters in all cases but one stereotrode (two wires), from which 6 parameters were available), to feed these parameters to the clustering algorithm KlustaKwik (by K. Harris), and to assess the quality of clusters using the L-ratio (a measure of the amount of noise or contamination near a cluster; ‘good’ quality $< 0.05$) and Isolation Distance (a measure of how far a given cluster is from the noise distribution; ‘good’ quality $> 16$) metrics (Schmitzer-Torbert et al. 2005). For display and spike width analysis, a matrix of 48-bit waveforms was created with the same indices as the 16-bit waveform matrix used for clustering.

Clustering was performed either on a Dell PC with a 64-bit processor running Matlab in 64-bit mode with a Fedora Linux operating system or on a Sun V880 provided by the Supercomputing Institute at the University of Minnesota (Matlab and KlustaKwik codes were parallelized for the workstation by S. Zhang). Clusters that were not well separated (L-ratio $< 0.05$ and Isolation Distance $>16$) were discarded. In pilot studies, manual manipulation of clusters after KlustaKwik sorting and selection based on L-Ration and Isolation Distance did not further increase cluster quality (i.e., decrease L-ratio and increase Isolation Distance). Thus, clusters were not manually manipulated after KlustaKwik sorting, but checked by an observer who was blind to all aspects of the clusters except energy (Note that timing of spikes within clusters is of primary interest and thus most likely to be subject to experimenter bias). A given cluster was discarded if it overlapped by more than ~1/2 of its area with noise (all spikes in clusters that did not have acceptable L-Ratios or Isolation Distances) in a 2-dimensional energy plot and in a 2-dimensional principle component 1 plot.

Spike width was measured from negative to positive peak and at 25% positive peak amplitude, per a previous study in adults (Rauske et al. 2003). Baseline spike rate was measured in 500 msec bins that contained no vocalizations or playback stimuli (number of bins: 60 – 148). Premotor and postmotor spike rates were measured during 50-msec before or after the vocalization, respectively. Responses to an array of auditory
stimuli were compared using a one-way ANOVA with a post-hoc Tukey-Kramer ($\alpha = 0.05$). An ‘Auditory’ neuron responded significantly above baseline to at least one of the auditory stimuli 250 – 750 msec after stimulus onset.
Results

Vocal-associated activity in HVC changes with development

We investigated the development of the brain-behavior relationship during the sensorimotor phase, when auditory feedback and a memory of the tutor song are used to sculpt song production (Konishi 1965). Unlike activity in the adult HVC, which is tightly correlated with song behavior (Fig. 1B), juvenile HVC activity both preceded and followed vocalizations by up to several seconds (Fig. 1A; Supp. Video 2). To quantify the apparent prolonged bursting before and after vocalizations, we analyzed the RMS of 2-sec of ongoing activity during silent periods that occurred within 5 sec of vocalization. We found that HVC near-vocalization activity in juveniles was significantly greater than that of adults (RMS μV; Juvenile (N = 18 days): 26.31 ± 2.25; Adult (N = 18): 15.38 ± 1.72; p < 0.0005, t-test). These data suggest that a substantial amount of ongoing activity in the juvenile HVC is not directly involved in vocal production and, thus, may have a role in vocal learning.

Activity bursts during learned motifs are longer in juveniles

To investigate the role of developmental modulation of HVC activity patterns in song plasticity, we examined HVC activity during ongoing learned vocal behavior (singing). We were able to maintain stable population recordings over several weeks, which allowed us to obtain detailed maps of the changes in neural activity and vocalizations during song development. We precisely excised behavioral and neural song motifs (highly stereotyped sequences of learned sound that are concatenated to produce songs) from all vocalizations that each finch produced during the recording period. Motifs were excised from multiple days for each animal by cross-correlating the amplitude envelope of a “canonical” (Williams 2004) behavioral motif with all available song data across development (see Supp. Video 1). For each finch, an experienced observer selected a single relatively mature behavioral motif. This canonical motif was used to extract motifs from all recording days, enabling the precise alignment of song motifs and corresponding neural activity by an unbiased computer program. The canonical motifs of all of our adults were longer in duration than all of the canonical motifs of our juveniles (note the duration of motifs in Fig. 2A and B). This is consistent with previous studies that demonstrated the emergence of longer sequences of different syllables with song learning (Arnold 1975; Tchernichovski et al. 2001).

We found that HVC neural activity during the act of singing was different in juveniles compared to adults. In Figure 2, panels (A, juvenile) and (B, adult) each show, from the top, (1) a sonogram for the selected canonical motif to show the spectral properties of the song behavior, (2) amplitude envelopes of the canonical motif and a subset of extracted motifs to emphasize that the motif extraction relied on the matching of amplitude envelopes of the two sounds, (3) a three-dimensional plot of the behavioral motif vocalization amplitudes produced on the same day (plotted from top to bottom in the order that they were produced) to illustrate the precision of the behavioral alignment, (4) amplitude envelopes for the neural activity accompanying the motifs shown in 2,
above, to show the neural activity associated with well-aligned motifs and to illustrate the threshold (mean, yellow line) above which we considered continuous neural activity a ‘burst’, and (5) a three-dimensional plot of the associated neural activity that occurred during the behavioral motifs shown in 3, above. The columns of activity that can be seen in the bottom panels of both Figs. 2A and 2B emphasize that (1) our motif identification technique is temporally precise and (2) the electrodes were stable.

For illustration purposes only, two bursts are indicated by green bars in Fig. 2 A and 2 B above the 3-dimensional HVC activity amplitude plots. The HVC activity bursts (peaks above the mean (yellow line in the HVC activity amplitude line drawing)) during juvenile motifs (Fig. 2 A) were longer in duration than those of adults (Fig. 2 B). In addition, the rate of bursts was higher in adults. Statistics on group data revealed that both burst duration and rate were significantly different between adults and juveniles (Fig. 2 C-D; p < 0.02).

We were initially concerned that the observed differences between adults and juveniles might be due to time since surgical implantation and not a real developmental effect. To address this issue, we compared adults and juveniles that had been implanted the same number of days. Controlling for implant days, we found that burst duration and rate were significantly different between adults and juveniles (Days implanted: 6, Adult (bird Blk161): Duration: 23.1 ± 0.4 msec, Rate: 22.2 ± 0.2 Hz, N = 116 motifs; Juvenile (Bu70): Duration: 24.7 ± 0.3 msec, Rate: 20.3 ± 0.2 Hz, N = 216 motifs; Days implanted: 7, Adult (Blk161): Duration: 20.7 ± 0.4 msec, Rate: 24.1 ± 0.4 Hz, N = 103; Juvenile (Bu81): Duration: 28.2 ± 1.2 msec, Rate: 17.3 ± 0.7 Hz, N = 41; for all p < 0.0001 t-test).

HVC vocal activity is sculpted daily during song development

The experiments above revealed that HVC bursting activity changes between the sensorimotor phase and adulthood. To determine the time course of these changes, we made longitudinal recordings of both brain activity and behavior in the same animals throughout the sensorimotor phase. We found that, similar to song itself, HVC activity matures over many days (Fig. 3). An abrupt crystallization of HVC activity was not observed, but instead motif burst duration and rate were shaped daily and plateaued at adult values at the approximate time of song crystallization (~ 90 days). For timing comparisons relative to behavior, Figure 3 A shows the sonogram of the canonical motif used to extract song motifs over both behavioral and neural activity columns below. The behavior extracted on each day and corresponding neural activity are shown side-by-side in Fig. 3 B-C (and Supp. Fig. 2). Consistent with adult-juvenile comparisons, longitudinal recordings from the same finch reveal that the duration of activity bursts decreased and their rate increased as song learning proceeded (Fig. 3 D-E). Comparison across all days revealed a transition from long bursts at a low rate to short bursts at a higher rate at approximately 80-85 days post-hatching in this animal. The interactions between age and burst duration and between age and burst rate were significant (ANOVA: Duration: F(24,267353) = 63.8, p < 0.0001; Rate: F(24,15825) = 92.2, p < 0.0001). By using a canonical motif to extract the data, we sampled only the most mature vocalizations. The fact that developmental changes in bursting activity are clear in spite
of this bias indicates that examination of less mature vocalizations may reveal an even stronger effect. In addition, the finding that initially long continuous bursts appear to split into narrower bursts during development may provide a neural correlate of behavioral chunking (Williams 2004).

Transitions in HVC activity correlate with behavioral changes

Both HVC bursting activity and singing behavior change during development (see above). To determine the relationship between brain activity and behavior, we compared song behavior and HVC burst duration on a day-by-day basis. Individual song syllables were tracked during development using the computer program Sound Analysis Pro (Tchernichovski et al. 2000), which enables characterization and clustering of individual syllables. For each day, syllables were extracted from 50 randomly selected motifs. The entropy variance feature of a single syllable decreased during vocal learning (Fig. 4 a, red line, bird Blue-70), as previously reported (Deregnaucourt et al. 2005). This change in the vocalization was paralleled by a decrease in HVC burst duration during learned motifs (Fig. 4 A, gray line). The correlation between the changes in HVC bursting activity and song behavior was significant and fairly strong (Fig. 4 B). Depending on the syllable, entropy variance may increase or decrease with development (Deregnaucourt et al. 2005). Analysis of entropy variance data from another juvenile finch (shown in Supp. Fig. 3) indicated that HVC burst duration correlated with entropy variance regardless of the direction of developmental change of this syllable feature. Entropy variance only measures a single syllable, whereas our burst data are from the entire motif. Thus, we also analyzed motif maturation by computing the percent similarity of 25 randomly selected motifs from each day to 25 randomly selected motifs from the most mature day recorded. As with syllable entropy variance, song maturity and HVC burst duration were correlated (Supp. Fig. 4). Collectively, these data indicate that the time course of the shortening of HVC bursts parallels the maturation of song behavior.

A subset of single HVC neurons are both pre- and postmotor

The findings that HVC activity outlasts vocalizations and that burst duration during learned motif production is longer in juveniles than in adults suggest the potential for temporal overlap of motor command and sensory feedback. However, the data are from a neural population. It is possible that different subtypes of HVC neuron are active during premotor and postmotor time periods. Were this the case, cellular integration of sensory and motor activities would not occur in these neurons. To resolve this issue, we recorded stably from single units within the HVC of vocalizing juveniles using multi-electrode techniques (triode; stereotrode in one case).

All HVC neuronal subtypes respond to auditory stimuli under anesthesia (Mooney 2000). However, auditory feedback during singing can only occur during the wake state (because singing can only occur in the wake state) and most HVC neurons do not respond to auditory stimuli in the wake state (Rauske et al. 2003). The auditory responsiveness of HVC neurons during actual singing behavior is completely unknown. In addition, postmotor activity that follows vocalizations, such as that described above, has not been
investigated. Further, auditory responses and motor associated activity have not been systematically compared in any song area. To understand sensorimotor integration in the song system, we compared premotor, postmotor, and auditory activity in stably-recorded and rigorously identified HVC single units in juveniles that were actively vocalizing in the sensorimotor phase of song learning.

Using established spike-sorting techniques and software (Harris et al. 2000), we found that single HVC neurons fired both before and after vocalization (Figs. 5 and 6; Table I). Figure 5 shows a sample of the simultaneous sonogram (Fig. 5 A), raw triode recording (Fig. 5 B), and reference recording (Fig. 5 C; deflections of the reference recording reveal movement). The activity of a single unit (Fig. 5 D, red) was identified using 9 clustering parameters, 3 of which are shown (Fig. 5 E, red). Single wire single unit recording traditionally uses one clustering parameter (amplitude). For added rigor, we subjected all multi-electrode data to strict criteria previously determined to characterize a reliable or ‘good’ spike cluster (Schmitzer-Torbert et al. 2005) (i.e., the cluster is more likely to contain the activity of only a single neuron and all of the activity of that single neuron). The mean waveforms for each channel and 50 randomly selected clustered spikes are shown (Fig. 5 F). We found that single neurons within HVC were active both before and after the vocalization (Fig. 5 D).

**Pre/postmotor neurons respond to auditory stimuli and belong to a putative subclass of interneurons**

If the neurons that fired both before and after vocalizations (pre/postmotor neurons) described above integrate sensory and motor information, then they must also respond to sensory stimuli during waking. To test this hypothesis, we played back a variety of auditory stimuli to juvenile finches and recorded responses of single HVC neurons. Based on spike width, we found the three extracellularly characterized physiological subtypes of HVC neurons (Rauske et al. 2003): projection neurons, fast-spiking interneurons, and slower-spiking interneurons (examples shown in Fig. 6; Table I).

All putative interneurons were active prior to vocalizations (premotor) (Table I, Fig. 6, red and green cells). A subset of putative interneurons also fired after vocalization. The spike widths of these neurons that fired after vocalizations were not different from those of premotor-only neurons. Neurons with longer duration action potentials (putative projection neurons) (Rauske et al. 2003) did not increase their firing rate immediately prior to vocalization. One of these neurons was inhibited by auditory stimuli (Table I; Fig. 6, blue cell). Antidromic stimulation identified this cell as Area X-projecting (D. Cygnar & T.A.N., unpublished results). Previous work has shown that some RA-projecting neurons fire immediately prior to calls (Hahnloser et al. 2002). However, it did not appear from the Hahnloser study that all RA-projecting neurons fired immediately prior to the beginning of vocalizations. Thus, except for the identified Area X-projecting neuron, the putative projection neurons that we recorded may have been either Area X- or RA-projecting (Table I, ‘Other’).
All pre/postmotor neurons were putative interneurons (based on spike width comparisons with a previous study (Rauske et al. 2003)). Pre/postmotor neurons were also auditory (e.g., Figure 5, green unit, Supp. Video 3). Based on spike rate, they were phasically activated by all auditory stimuli (Supp. Figs. 5 - 6). These neurons fired phasically to all auditory stimuli for approximately 250 msec. To assess stimulus preference, we examined spike rate after the phasic burst (250 – 700 msec after stimulus start). Overall, these neurons were activated most by tutor song (Fig. 6 J), although the response to tutor song was not significantly greater than that to other conspecific songs. To assess whether the response was experience dependent, we played back songs from two adult male zebra finches that were not the song tutor. Consistent with biased responding to auditory stimuli resembling the tutor song, response to Conspecific Song 2 (Fig. 6 J; 80% similar to tutor song as computed by the Sound Analysis Pro software) (Tchernichovski et al. 2000) trended to be greater than that to Conspecific Song 1 (51% similar).

These data indicate that a putative subclass of HVC interneuron (1) fires prior to vocalization (forms part of the motor command network), (2) fires after vocalization (exhibits the temporal dynamics necessary for sensorimotor integration), and (3) responds to auditory stimulation (receives auditory inputs). These are required characteristics for sensorimotor integration in this system. Interestingly, the identification of premotor neurons that respond phasically to sounds suggests a role for the introductory notes that occur before singing (Williams 2004): they may be used to “boot up” the song system pattern generator by driving it with autogenous sounds.

Discussion

Understanding song development requires the measurement of neural motor activity during singing. However, previous studies of the developing song system have focused either on auditory responses (Nick and Konishi 2005a, b; Solis and Doupe 2000; Volman 1993) or on brain areas that are not necessary for song production (Olveczky et al. 2005). We have employed extremely stable recording techniques to assess the development of activity in the song control nucleus HVC. Recording neural population activity over several weeks has revealed that activity bursts during learned song behavior decrease in duration and increase in rate with song learning. In addition, multi-electrode recording combined with spike sorting techniques have enabled reliable identification and systematic analysis of the auditory and motor activities of single neurons over several hours. We have found that the same neurons are premotor and auditory. Further, these premotor neurons exhibit prolonged bursting that outlasts the vocalization. This ‘postmotor’ bursting has not been described previously and may offer a key to the mechanisms underlying song learning.

Prolonged bursting has also been recorded in developing sensory systems prior to periods of extreme environmental sensitivity, such as the critical period for ocular dominance plasticity (Fagiolini and Hensch 2000). Our data indicate that a subpopulation of HVC neurons exhibit prolonged bursts that outlast the vocalization in the plastic
These data suggest that prolonged bursting may have a role in the environmental shaping of neural circuits, be they sensory or sensorimotor. Prolonged bursting in HVC occurs before, during, and after learned vocalizations in juveniles. Whether prolonged bursting is also associated with unlearned vocalizations is an important question for future study.

It is possible that the prolonged bursts after vocalizations result from prolonged sensory feedback. Since sensory feedback in the adult takes less than 100 msec (Sakata and Brainard 2006; Troyer and Doupe 2000) and some observed prolonged bursts were much longer (over 1 sec), the sensory feedback would have to be delayed considerably. In addition, sensory feedback cannot explain the prolonged activity that occurred before the start of the vocalization that we observed. A more parsimonious hypothesis is that the prolonged bursting reflects the activity of the HVC pattern generator (Mooney and Prather 2005; Solis and Perkel 2005). Early in the sensorimotor phase, the pattern generator or its synaptic targets may not effectively engage or coordinate the muscles of the respiratory system and vocal organ, resulting in the observed disconnect between HVC activity and song behavior.

Is there a function for the prolonged activity? There are three main hypotheses that are not mutually exclusive: (1) The prolonged activity may be necessary for the development of individual neurons within HVC (e.g., HVC neurons may require activity-dependent release of trophic factors from their synaptic targets to develop appropriately (for example, see Nick and Ribera 2000)). (2) The prolonged bursting may be required for the appropriate synaptic connectivity of neural circuits in the song system (e.g., prolonged inhibition of Area X-projecting neurons may be necessary to release a downstream nucleus from inhibition and thus enable vocal experimentation (Kao et al. 2005; Olveczky et al. 2005)). (3) Sensorimotor integration may not be possible without the prolonged bursting (Fig. 7). Sensorimotor integration and learning may require the temporal coincidence of activity in presynaptic and postsynaptic neurons, which underlies plasticity in many systems (Bi and Poo 2001; Kandel 2001; Spitzer 2004). As the distance and number of synapses from the periphery increases, as in the vertebrate forebrain, the coincidence of activity in sensory and motor processing streams becomes more difficult to achieve due to significant synaptic delays between related motor and sensory activity. We propose that, to enable plasticity, the time gap between motor command and sensory feedback (Troyer and Doupe 2000) is bridged by ongoing activity in the motor control system during sensorimotor learning (Fig. 7 A), but not during adulthood (or to a lesser degree), when the capacity for plasticity is decreased (Fig. 7 B). Further, we hypothesize that prolonged bursting activity is primarily related to motor command, but is modulated by sensory feedback. According to these hypotheses, prolonged motor activity allows postsynaptic potentials from sensory feedback pathways to bias spike-timing dependent plasticity and shape the motor pattern. The weak preference for tutor song expressed by the pre/postmotor neurons makes sense in younger finches that will utter sounds that only remotely resemble the tutor song, since the best of initially poor imitations must be reinforced and shaped toward the mature song (Immelmann 1969). In addition, the thousands of motif repetitions during development
combined with Hebbian activity-dependent plasticity would amplify even the smallest of synaptic biases, making a larger effect potentially detrimental to plasticity.

Three key predictions of the sensorimotor integration hypothesis described above are supported by data presented in this manuscript: (1) HVC activity during learned behavior is less precise during vocal plasticity, consistent with prolonged activity in the motor network; (2) single neurons that fire after vocalization in juveniles also fire before, consistent with their hypothesized role in motor planning; and (3) these same pre/postmotor neurons respond to auditory stimuli during waking, revealing a cellular site of convergence of sensory and motor signals.

We have identified single HVC neurons that appear to integrate sensory and motor information in the sensorimotor phase of vocal learning. The next key steps in understanding the role of HVC in song learning require assessment of the sensory response of HVC neurons to auditory feedback during the behavioral act of singing, characterization of the effects of these putative interneurons on HVC neuronal ensembles, and perturbation of inhibitory pathways within HVC and examination of behavioral plasticity.

References


Table I. Properties of the spikes of single neurons in vocalizing juvenile zebra finches.

<table>
<thead>
<tr>
<th></th>
<th>Peak-to-peak Spike width (msec)</th>
<th>25% Positive peak Spike width (msec)</th>
<th>ISI (msec)</th>
<th>Baseline Spike Rate (Hz)</th>
<th>Premotor Spike Rate (Hz)</th>
<th>Postmotor Spike Rate (Hz)</th>
<th>Auditory Response</th>
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<td>Pre/Postmotor</td>
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<td>0.30 ± 0.00</td>
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<td>0.08 ± 0.00</td>
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<td>Premotor only</td>
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<td>0.17 ± 0.00</td>
<td>557.8 ± 6.2</td>
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“Pre/Postmotor” cells had significant activity above baseline both before and after vocalization (50 msec window). “Premotor” cells only had significant activity above baseline in the premotor time window. “Other” cells had no pre- or postmotor activity above baseline. Auditory neurons had a significant response above or below baseline to at least one auditory stimulus. Sound playback data were available for 4 of 6 finches (Not Available; NA).
**Figure Legends**

**Figure 1.** Vocal-associated activity is developmentally modulated in the song system. In the HVC of juvenile zebra finches, activity can begin hundreds of milliseconds to seconds before vocalization and continue seconds after (A; bird Blue-81, age 67 days). HVC activity in adults is tightly locked to the vocalization (B; bird Black-161, age >200 days). In (A) and (B), the sonogram of vocal behavior is overlaid with the HVC multi-unit recording to facilitate comparison of the timing of activity. For comparison of pre- and postmotor activity, each inset shows amplitude envelopes of electrode (black) and vocal (red) data in an expanded time scale (total frame 1.8 sec). The dotted line in the inset indicates baseline activity. The gray shading indicates the timing of the vocalization. Scale bar: 100 µV for (A), 75 µV for (B); 1 sec.

**Figure 2.** Brain activity that drives learned vocal behavior changes during development. In juveniles, the HVC neural activity during behavioral song motifs (A; example finch Blue-46, age 65 days) is longer in duration and lower in frequency than that of adults (B; example finch Black-161, age >200 days). In (A) and (B), all five graphs are temporally aligned. From the top: (1) sonogram of the canonical behavioral song motif used to identify developing motifs through cross-correlation; (2) amplitude envelopes of the canonical behavioral motif (red) and all behavioral motifs that fell within one standard deviation of the mean amplitude envelope for that day (black); (3) A 3-dimensional plot of the behavioral vocalization amplitude during the first 116 motifs with x-axis time, y-axis motif number, and z-axis amplitude. (4) mean amplitude envelope of HVC population neural activity (red) and of all motifs shown in 2 (black) (The dashed yellow line indicates the mean of all motif activity for that day.); (5) A 3-dimensional plot of the HVC neural activity associated with the first 116 motifs with x-axis time, y-axis motif number, and z-axis amplitude. For illustration purposes only, Green bars indicate bursts. White indicates the highest amplitude, black the lowest. For ease of comparison across birds, all data in A-B were limited to the maximum number of motifs of either bird (adult in b, N = 116). Scale bar: 100 msec. (C-D) Comparison of bursts (peaks above mean; two each are indicated in A and B by green bars) across HVC activity motifs reveals that the mean duration of HVC activity bursts decreases, whereas the mean rate of bursts increases with development (Juvenile finches (62 – 72 days): N = 5 birds, 6 electrodes; Adult finches (141 - >200 days): N = 4 birds, 6 electrodes; p < 0.02, Wilcoxon). These statistics were obtained motif-by-motif and thus do not depend on the temporal alignment of motifs across any time period.

**Figure 3.** Premotor activity during learned vocal behavior is developmentally regulated. Continuous recording throughout the sensorimotor phase reveals the development of neural activity underlying learned song motif behavior (data shown are from a single bird, Blue-70, age 72 – 111, N = 22,527 motifs). (A) Sonogram of canonical motif used to retrieve song motifs across development is aligned with 3-dimensional plots below for temporal comparisons. (B-C) LEFT, TOP: Amplitude envelopes of the canonical motif (red) and all motifs that fell within one standard deviation of the mean amplitude envelope for that day (black); LEFT, BOTTOM: A 3-dimensional plot of the amplitude envelopes of the first 116 vocalizations for the given day with x-axis time, y-axis motif number, and z-axis amplitude. White indicates the highest amplitude, black the lowest. RIGHT, TOP: Mean amplitude envelopes of HVC population activity (red) and of all motifs shown in top left (black) (The dashed yellow line indicates the mean of all motif activity for that day.); RIGHT, BOTTOM: Similar to lower left, but amplitude
envelopes of HVC neural population activity are shown. Scale bar: 100 msec. (D-E) Data across all 37 days of recording reveals a shift in neural bursting activity with regard to duration and rate at the end of the sensorimotor phase.

**Figure 4.** Changes in HVC bursting activity correlate with song features. (A) As previously reported, a descriptive feature of a single learned syllable, entropy variance, decreased with song learning (Deregnaucourt et al. 2005). The developmental timeline of changes in entropy variance (red line) matched corresponding changes in HVC bursting activity during learned motifs (gray dotted line) in a developing finch. The insets show representative sonograms of the analyzed syllable at three different ages. Entire frame of each inset x: 250 msec, y: 0.5 – 8 kHz. (B) The correlation between entropy variance and HVC motif burst duration was significant for this finch (Pearson r = 0.61; p < 0.0001).

**Figure 5.** Single unit recordings reveal that the same neurons are pre- and postmotor during singing in the sensorimotor phase. (A) Sonogram of juvenile vocalizations is temporally aligned with data below. Red color indicates increased amplitude. All data are from bird Orange-273, age 60 days, implanted 3 days. (B) Raw HVC multi-electrode voltage traces (each of three electrode recordings shown separately). (C) The reference electrode recording confirms that recorded activity was neural (not movement) and that it emanated from HVC and not the reference area. Scale bar: 100 µV, 1 sec. (D) Spikes of a single neuron as computed by spike sorting techniques. (This spike cluster was well-separated: L-ratio = 0.0125; Isolation distance = 60.3). Gray bars indicate timing of the vocalizations. (E) A 3-dimensional plot of 3 of the 9 parameters used to cluster the spikes shown in (D). The black dots were not part of the well-separated cluster, whereas the red dots were included. The large cloud of black dots consists of spikes and noise that were just above the inclusion threshold. (F) Mean (red) and 50 randomly-selected (black) waveforms of spikes contained in the red cluster in (E). Electrodes shown in (B) top-to-bottom are here left-to-right. The reference is on the far right. Scale bar: 100 µV, 1 msec.

**Figure 6.** Juvenile HVC neurons that are active before and after vocalizations also respond to playback of auditory stimuli. (A) Sonogram of a 15-s time segment of recording chamber sounds during song playback and vocalization. The sonogram is temporally aligned to the clustered spikes below. (B) Activity of three single units identified by spike sorting techniques. Red: putative fast-spiking interneuron. Green: putative slower-spiking interneuron. Blue: putative projection neuron. Note that the green neuron fires during song playback, before call vocalizations, and after calls. Color codes indicate the same single units throughout this figure. (C-E) Mean (in color) and 50 randomly-selected (black) waveforms of single units shown in B. For each panel, left and middle waveforms are recordings from a stereotrode. Right waveform is the reference. Note that although in some cases the overall waveform is complicated by a second spike, only the first 16 bits of these 48-bit waveforms were used for clustering. Scale bar: 100 µV, 1 msec. (F-H) Motor-associated activity of each of the three units. The red unit was premotor only, the green unit was both pre- and postmotor, and the blue unit did not change its firing pattern before or after vocalizations. (I-K) Auditory responses of the single units shown above. (* p < 0.05 versus silence, + p < 0.05 versus tutor, ANOVA with post-hoc Tukey-Kramer).
**Figure 7.** The finding that, during learning, activity in a premotor area outlasts the behavior suggests a simple model to overcome the problem of sensory-motor delays: During song learning (A), but not in adulthood (B), motor command activity lasts long enough to enable overlap of ongoing motor activity and sensory feedback.

**Supplementary Figure 1.** Choice of threshold for measurement of bursts during motifs does not affect the conclusion. The data shown in Figures 2 and 3 used the mean of each day’s activity as the threshold. Similar results are seen with the use of median (*p < 0.03, Wilcoxon) or mean + standard deviation (p = 0.06) as the threshold.

**Supplementary Figure 2.** Premotor activity during learned vocal behavior is developmentally regulated. This is an expanded view of the data shown in Fig. 3A-C. Continuous recording throughout the sensorimotor phase reveals the development of neural activity underlying learned song behavior (Blue-70, age 72 – 111, N = 22,527 motifs). (A) Sonogram of canonical motif used to retrieve song motifs across development. (B-E) LEFT: A 3-dimensional plot of the vocalization amplitude during the first 200 motifs with x-axis time, y-axis motif number, and z-axis amplitude. For ease of comparison, the number of motifs shown was limited to 200. RIGHT: A 3-dimensional plot of the neural activity associated with the first 200 motifs with x-axis time, y-axis motif number, and z-axis amplitude. White indicates the highest amplitude, black the lowest. Neural activity (right) is horizontally aligned with corresponding behavioral motifs (left). Comparison across selected days shows the previously described tempo increase of motif behavior as song learning progressed (Arnold 1975; Zann 1996). As would be expected, corresponding temporal compression of HVC activity also occurs. Scale bar: 100 msec.

**Supplementary Figure 3.** HVC bursting activity correlates with song maturation. Correlations between HVC motif burst duration and entropy variance of the longest duration song syllable are plotted for multiple days and two electrodes from bird Blue 81 (68 – 83 days). (A-B) Consistent with overall adult-juvenile comparisons, the mean duration of HVC bursts during motifs decreased with age in two electrodes. (C) Unlike the syllable from bird Blue 70 (Fig. 4), the entropy variance increased with development. (D) The burst duration during motif production was correlated with entropy variance for electrode 1 (Pearson r = -0.76, p = 0.011) and electrode 2 (Pearson r = -0.56, p = 0.076, not significant). Although the direction of change of the syllable feature was in the opposite direction to Blue 70, the feature was still well correlated with HVC activity changes.

**Supplementary Figure 4.** Changes in HVC bursting activity correlate with song maturation. (A) Similarity of motifs produced throughout development (25/day, randomly selected) compared to mature motifs from day 111 (25 from day 111, randomly selected). Song maturity increased with age during the sensorimotor phase, as previously reported (Immelmann 1969; Tchernichovski et al. 2001). (B) The correlation between song maturity and HVC motif burst duration was significant (Pearson r = -0.52; p = 0.0009).
**Supplementary Figure 5.** Peristimulus time histograms of each of the three units shown in Figure 6. Color codes correspond to those in Fig. 6. Histogram shows the sum of events in 1-msec bins over 40 trials. All auditory stimuli induced an increase in activity in the green unit (middle). The red unit (top) showed no response, whereas the blue unit (bottom) was inhibited by sound playback. Conspecific Song 1 was 51% similar to the tutor song, whereas Conspecific Song 2 was 80% similar to the Tutor Song.

**Supplementary Figure 6.** Phasic responses of the cells shown in Figure 6 to auditory stimuli measured during the first 250 msec after stimulus onset. Color codes correspond to Fig. 6. (* p < 0.05 versus silence, ANOVA with post-hoc Tukey-Kramer).
Supplementary Video 1. Simplified moving schematic of the method used to precisely extract song motifs (stereotyped learned sequences of sound).

Sonogram (TOP), filtered vocal amplitude recording (MIDDLE, black), filtered ‘canonical’ motif used to extract all motifs from this finch (MIDDLE, red), and cross-correlogram (BOTTOM) are shown temporally aligned. There are peaks in the cross-correlation when the canonical motif matches the pattern of the recorded sound. Audio on for best results.

Supplementary Video 2. Population recordings reveal striking differences between adult and juvenile vocal-associated activity.

Sonograms (TOP) and population recordings (BOTTOM) are shown temporally aligned. A recording from adult is followed by recordings from juveniles. All animals sang during the recording. Audio on for best results.

Supplementary Video 3. Activity of a single unit recorded in the HVC of an awake juvenile during vocalization and playback.

Sonogram (TOP) and the activity of a single unit identified with multi-electrode recording and spike sorting (BOTTOM) are shown temporally aligned. Note that the same unit fires during playback and before and after vocalizations. Audio on for best results.
A. Juvenile: Motor and sensory activity coincide

Motor Command

Behavior

Sensory Feedback

B. Adult: Motor and sensory activity do NOT coincide

Time
A. Threshold: Median

B. Threshold: Mean + STD
A. 

Percent Similarity to Mature Motif

Days Post-Hatching

B. 

Percent Similarity to Mature Motif

Motif Burst Duration (msec)