SONGBIRD PREMOTOR NUCLEUS HVC AUDITORY RESPONSES ARE ROBUST, DYNAMIC, AND REFLECT LEARNED PREDICTIVE VALUES IN AWAKE ZEBRA FINCHES

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ABSTRACT OF THE THESIS

Songbird Premotor Nucleus HVC Auditory Responses are Robust, Dynamic, and Reflect Learned Predictive Values in Awake Zebra Finches

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Premotor and motor areas of the human brain are known to take part in various perceptual phenomena, the most significant of which is speech perception. The present study tests the involvement of a premotor vocal structure in perception of various acoustic signals, using the zebra finch (*Taeniopygia guttata*), a songbird species that provides the best-studied model organism for vocal learning. Water-deprived zebra finches were trained in a differential classical conditioning task in which CS+ stimuli predicted a water reward while CS- stimuli did not result in any outcome. When stimulus discrimination reached criterion, a set of novel auditory stimuli was passively presented in the absence of water-deprivation outside of the training apparatus ~20 hours before the neural recordings. In awake, restrained birds, electrophysiological activity in response to bird's own song (BOS), CS+, CS-, passively familiar (PasFam), and novel stimuli was recorded from multiple sites in the vocal premotor nucleus HVC and the HVC Shelf region bilaterally. Multi-unit

responses in HVC were found to be highest for BOS, followed by CS+ and Novel, which were higher than CS- stimuli. Furthermore, responses decreased in magnitude with stimulus repetition (adapted) for all stimuli except BOS. BOS-bias over Novel stimuli was found to be higher in right than in left HVC. In HVC Shelf, there was no BOS-biased activity and responses to all stimuli decreased with repeated presentation. Responsive single neurons in HVC showed the same pattern as multi-unit activity among stimulus categories, but, as a population, their responses did not adapt to stimulus repetition. Overall, the results suggest that the premotor nucleus HVC has robust and dynamic auditory responses that also reflect learned predictive values in awake zebra finches. These neural mechanisms may potentially reveal general principles that can be applied to understand the role of human speech production brain regions in recognition and discrimination of speech sounds.

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Introduction

How certain brain structures that are generally known to be responsible for planning and execution of movements also facilitate perception is an intriguing question in neuroscience. A growing body of evidence suggests that premotor and motor areas in the human brain may take part in speech perception. Acoustic speech signals vary to a great extent due to coarticulation and variability among speakers which causes ambiguities between the physical sound stimuli and the corresponding perceived speech units. It has been argued that one mechanism by which the brain resolves these ambiguities is by analyzing how the subject's own vocal motor system would produce the gestures that must underlie the incoming speech sound by auditory-to-motor mapping and use this to infer what the sound must be (Liberman & Mattingly, 1985). Although there has been some effort to prove this "motor theory of speech perception" using various neuroimaging methods, the validity and the interpretation of these studies have been strongly criticized (Hickok & Poeppel, 2004; Lotto, Hickok, & Holt, 2009). However, Broca's area, and the general inferior frontal cortex (IFC) where it resides, are repeatedly shown to contribute to syntax comprehension (Stromswold et al., 1996; Embick et al., 2000; Fiebach et al., 2005; Meltzer et al., 2010). Others studies linked IFC activity to various other linguistic capabilities such as semantic processing (Roskies et al., 2001; Whitney et al., 2010), discourse (Menenti et al., 2009), and metaphor processing (Cardillo et al., 2012). Although it is clear that premotor and motor areas of the human brain participate in speech perception, a detailed examination of the neurobiological mechanisms underlying the nature of the contribution of these motor and premotor structures in discrimination of acoustic speech signals and recognition of their predictive/associative meanings requires studies in animal models since the currently

available neuroscience methods in humans do not provide us with finely localized neural activity with millisecond-range temporal resolution.

Songbirds provide an excellent model for studying the neurobiological basis of auditory perception and learning due to their use of a set of complex learned signals in social communication. They learn their vocalizations from conspecific tutors through a process of vocal imitation with many parallels to speech acquisition (Doupe & Kuhl, 1999). The songbird auditory forebrain is highly sensitive to stimulus exposure history (Chew et al., 1996a, 1996b; Smulders & Jarvis, 2013), social context (Vignal et al., 2005), acquired predictive value (Thompson & Gentner, 2010; Jeanne et al., 2011) etc. in addition to the physical characteristics of acoustic signals. HVC (used as a proper name) is a songbird premotor nucleus that plays a major role in controlling the downstream motor areas during song production via its projections to Robust Nucleus of Arcopallium (RA), which is analogous to the mammalian vocal motor cortex (Mooney, 2009; see Figure 1 for anatomical connections). HVC also sends indirect projections to RA via corticostriatal projections to a basal ganglia homologue region, Area X. Interactions in this pathway play a central role in learning to imitate the tutor song during development.

In addition to its role in vocal motor control, HVC receives auditory information from several forebrain regions, nucleus Avalanche (Akutagawa & Konishi, 2010) and Nucleus Interface (Nif; Cardin et al., 2005), and thalamic nucleus Uvaeformis (Uva; Coleman et al., 2007). On the other hand, HVC has been traditionally believed to show little or no response to acoustic signals other than bird's own song (BOS) based on studies in anesthetized birds (Margoliash, 1986). These observations were interpreted as an indication that the role of auditory input to HVC was specifically to processes auditory

feedback from the bird's own vocalizations, which is a necessary function for vocal learning and song production (Mooney, 2000). However, neural activity in HVC in response to auditory stimuli is tightly modulated by the behavioral state of the bird (Cardin & Schmidt, 2003). While only BOS elicits high neural activity during anesthesia, sleep, and sedation, strong and variable neural responses to other conspecific vocalizations can be observed during wakefulness (Raksin et al., 2012), which suggests that HVC might also play a significant role in auditory perception in awake birds.

In an early study, Brenowitz (1991) reported that female canaries with partial lesions to HVC showed a decrease in auditory discrimination: copulation solicitation displays (CSD) were given in response to both male canary and white-crowned sparrow songs while birds with sham lesions produced CSDs only to canary songs. These findings were replicated in another study reporting that female canaries that showed CSD only to male canary songs prior to chemical HVC lesions responded indifferently to canary and greenfinch songs after the lesion (Del Negro et al., 1998). These studies suggest that HVC is important for perceptual discrimination of conspecific versus heterospecific songs in female canaries. Using an operant conditioning paradigm, Gentner and colleagues (2000) trained European starlings to discriminate an individual conspecific song from several others, by pecking one response key to play that song, while other keys played the other songs. Partial HVC lesions after successful training did not result in deteriorated discrimination performance. However, when the song-to-key associations were reversed, the size of HVC lesions was found to be positively correlated with the latency to learn the new association, which suggests that HVC may not hold the auditory memories, but is critical for acquisition of learned categories of acoustic signals. Similarly, Bengalese

finches that received HVC lesions took longer to successfully discriminate two auditory stimuli in an operant task compared to intact birds (Okanoya et al., 2001). Other studies have shown that lesions to structures in the vocal motor pathway other than HVC, i.e., anterior forebrain pathway (Scharff et al., 1998) and RA (Vicario et al., 2001), also result in perceptual deficits.

The most comprehensive electrophysiological examination of the auditory response properties of HVC was reported in a recent study by Raksin et al. (2012). In awake male zebra finches, neural responses recorded in HVC were found to be heterogeneous, with a subpopulation responding selectively to BOS and another subpopulation responding to other novel conspecific songs. A significant proportion of neurons showed activity patterns that were reliably predictable by physical parameters of the stimuli, which is a characteristic feature of the auditory processing nuclei in the midbrain and the primary auditory cortex. Thus, HVC is responsive to conspecific songs other than BOS; however the role of HVC activity in stimulus discrimination and the recognition of the learned predictive values of auditory signals remains untested.

The region immediately ventral to HVC, HVC Shelf, is defined by the projections from the subfields 1 and 3 of the primary auditory forebrain area Field L and higher-order auditory area Caudal Lateral Mesopallium (CLM, Vates et al., 1996). Although, this area was primarily thought to supply a major auditory input to HVC, anatomical and functional studies revealed that connectivity between these two structures is strikingly sparse (Mello et al., 1998). Functional analyses of auditory activity in the Shelf did not yield conclusive results (Poirier et al., 2009; Poirier et al., 2011). Thus, the exact perceptual function of the Shelf remains unclear and demands further examination.

In the light of above discussion, the goal of the current study was to investigate the auditory activity profiles of HVC in response to various categories of acoustic signals in awake, restrained zebra finches (*Taeniopygia guttata*). Birds were trained in a differential classical conditioning task in which CS+ stimuli predicted water rewards while CS- did not deliver any outcome. Furthermore, about 20 hours before the acute electrophysiological recordings, birds were passively exposed to stimuli different from the ones in the training. Single - and multi-unit responses in HVC to BOS, CS+, CS-, passively familiar, and novel stimuli were analyzed in detail. In the same birds, the auditory response properties of HVC Shelf were also investigated to enhance our understanding of its role in perception. Functional hemispheric differences in the auditory and motor brain structures of the zebra finch and other songbirds have been described, although the degree and sidedness of lateralization vary across brain regions and species (for a review, see Moorman & Nicol, 2014). Hence, possible lateral differences in HVC and the Shelf auditory responses were also probed.

Method

Subjects

Seven naïve adult (>120 days) male zebra finches (*Taeniopygia guttata*) born and raised in our aviary (12:12 light:dark, 72-78 Fº) were used in the current study. All birds lived in a colony with *ad libitum* food and water until the start of the experiment. Throughout the experiment, the subjects were housed individually in sound isolation boxes with *ad libitum* food. Access to water was manipulated as described below. All procedures were approved by the Animal Care and Use Committee of Rutgers University.

Stimuli

Two to five days prior to the start of the experiment, all birds' songs were recorded in individual sound isolation boxes using Sound Analysis Pro (SAP) software (Tchernichovski et al., 2000). Singing was elicited by introduction of a female conspecific. One sample from the recordings was selected for each bird and noise-filtered, trimmed, and sampled (44.444 kHz) in Signal software. The resulting BOS stimuli consisted of one motif. The remaining experimental stimuli were selected from our corpus of zebra finch songs amassed in years prior to the experiment to ensure that the birds in the current study had never been exposed to them before. Nine stimuli, each consisting of one motif, were selected and processed as above for BOS stimuli. The duration of experimental stimuli ranged from 564 to 1199 milliseconds. To control for nonspecific effects due to physical sound characteristics, all stimuli were pseudo-randomly assigned to stimulus categories CS+, CS-, Passive Familiar, and Novel (see below for details) for each bird so that each stimulus appeared in each category for one to three times among birds. The acoustic similarity of each stimulus category to BOS for each bird, was measured in SAP

(Tchernichovski et al., 2000), and found to be comparable across subject birds (Mean ± SD = 52.65% ± 1.29%, $F(3,18) = .16$, $p = .92$).

Behavioral Training

Birds were trained in a differential classical conditioning task in which CS+ stimuli predicted a ~10 µl water reward delivery while CS- stimuli did not result in any outcome. The apparatus included a water delivery unit hidden by a panel with a small opening through which the birds could get access to the water reward (Figure 2*A*). An infrared beam system crossed this opening to detect the birds' 'reward-checking' behavior. Above the water delivery unit was a speaker that played the experimental stimuli.

Birds were water-deprived throughout the experiment except non-training days. The behavioral training started with a 1-2 day acclimation stage in which the water reward was administered at random intervals between 30 and 60 seconds with no auditory stimulus in order to familiarize the birds with the apparatus (see Figure 2*B* for the general procedure). Initially, the panel that would eventually hide the water delivery unit was placed high so that the birds could see the water reward tube. The birds' behavior was monitored; when each bird was successfully retrieving the water rewards, the panel was lowered in stages to its final position to hide the water delivery unit. In the discrimination 1 stage, one CS+/CS- pair was introduced. Auditory stimuli were presented at random intertrial intervals between 40 and 75 seconds. Immediately following the CS+ stimulus, a ~10 µl droplet of water was delivered without any behavioral requirement, whereas CS- stimulus did not yield any outcome. Seven seconds from the stimulus onset was defined as the behavioral window in which a break in the infrared signal caused by the bird's reward checking behavior was recorded as a response. Birds were trained daily for 200-300 trials

during weekdays. Accuracy was measured as the percentage of correct responses and no responses for CS+ and CS- stimuli, respectively. When a bird reached 75% overall accuracy, it was moved to the next stage. In the discrimination 2 stage, a second CS+/CS- pair was introduced to the training in addition to the first pair. All training parameters were the same as discrimination 1. The success criterion for the discrimination 2 stage was 75% overall accuracy for both CS+ and CS- pairs separately for three consecutive days. When birds reached the criterion, they were either moved to the next stage or trained for one to three days more depending on the availability of the electrophysiological recording system. At the end of the behavioral training, all birds were given water *ad libitum*.

Surgery

The day following the end of the behavioral training, birds were anesthetized with isoflurane (1.5-2 % in oxygen), placed in a stereotaxic apparatus, and a craniotomy was made over the region of interest. A metal pin was attached anterior to this opening with dental cement to be used to fix the bird's head during the awake restrained electrophysiological recordings. The craniotomy was covered with silicon elastomer until the recordings. All birds recovered within few hours and were water-deprived starting from 24 hours after the surgery for one more day of post-surgery behavioral training (two days after the surgery) to ensure that all birds were able to perform the task successfully after surgery and before the electrophysiological recordings. This training day was identical to the discrimination 2 stage.

Passive Auditory Exposure

Immediately following the post-surgery training, birds were given water *ad libitum* and monitored to ensure that they drank freely for 90 minutes. The water delivery unit and the

panel hiding it were removed and the birds were placed back in the apparatus for passive auditory exposure. Two previously unheard stimuli (Passive Familiar, PasFam) were presented, 200 times each, in a random order at random intervals between 8 and 15 seconds. Thus, these stimuli would have been recently heard and thus familiar to the bird at the time of electrophysiological recordings (similar to the CS+ and CS- stimuli), but would lack any relation to the differential conditioning task. A higher-order auditory forebrain structure, the Caudal Medial Nidopallium (NCM), is well-known to show a neuronal memory for passively exposed stimuli that lasts more than 20 hours (Chew et al., 1996a, 1996b), so this exposure should be sufficient to make these stimuli familiar. During this auditory exposure, birds spent almost the entire time on a perch without much movement. At the end of the exposure, birds were put back in their cages and given water *ad libitum*.

Electrophysiology

Awake restrained electrophysiological recordings were conducted in a walk-in sound attenuation chamber (IAC Inc., Bronx, NY) 17:40 to 20:00 hours after the beginning of the passive auditory exposure. The bird's body was restrained in a comfortable plastic tube and its head was fixed to a stereotaxic apparatus via the pin on the skull. Two separate silicon probes (NeuroNexus Technologies, Ann Arbor, MI), one for each hemisphere, were used for recordings. Each probe had 16 recording sites (0.2-0.9 MΩ impedance) in a 4-by-4 grid layout with 200 µm inter-site distance. The probes were inserted in the brain in such a way that the 4-by-4 grid extended along anterior-posterior and dorsal-ventral axes (Figure 3). Prior to insertion, the probes were dipped into a DiI solution (10% in ethanol) and allowed to dry, producing a fine coating of particles that would be deposited in the brain and used for later histological analyses (see below for details). The dura was opened and

the probes were placed on the surface of the brain, one on each hemisphere, according to the stereotaxic coordinates of HVC; +2.4 mm lateral, +.3 to -.3 mm anterior-posterior from the deccussation of the sagittal sinus. Then, they were lowered by means of a hydraulic drive (Narishige, Tokyo, Japan) until the characteristic spontaneous bursting activity of HVC was observed at a majority of the recording sites. The BOS that would be played during the experiment and a novel conspecific song that was not included in the experimental stimuli were played back, no more than 20 times each, to validate that recording sites indicated stimulus-driven and BOS-biased activity. Following a five-minute break at the end of this search stage, the experiment started. The experimental playback set consisted of 10 stimuli; one BOS, two CS+s, two CS-s, two Passive Familiars (PasFam), and three Novels; these were played back in a shuffled order. Each stimulus was played 20 times at an inter-stimulus interval of 8 seconds. Neural recordings were high- and low-pass filtered (0.3 and 5 kHz), amplified (10,000 x), digitized (25 kHz), and saved to disk using Spike2 software (CED, Cambridge, England). During the recording, the bird was monitored via a camera placed in the sound attenuation chamber and the trials in which the bird's both eyes were closed during the stimulus presentation were coded, as index of the bird's behavioral state. The assignment of the silicon probes to two hemispheres was counterbalanced among birds to control for effects that could result from differences between the two probes.

Histology

Before insertion into the brain, the probe electrodes were dipped into a solution of DiI (10% in Ethanol; Sigma Aldrich, St. Louis, MO), a lipophilic fluorescent dye that does not diffuse in the brain and is commonly used for verification of electrode placement.

Immediately after the electrophysiological recordings, birds were deeply anesthetized by an overdose of pentobarbital and transcardially perfused with saline (0.9%, 40 ml) and paraformaldehyde (4%, 40 ml), then decapitated. Skulls were left in paraformaldehyde for 2 days of post-fixation. Then, the brains were extracted and post-fixed with paraformaldehyde for 2 more days. Sagittal sections (50 µm) were cut on a Vibratome, then collected on slides. Unstained sections were visualized under a fluorescence microscope. Cytoarchitectural markers of HVC in unstained sections can easily be visualized in dark-field microscopy (Kirn et al., 1991). Grayscale digital images of the same sections were collected (10x magnification) under 345/455 nm and 570/576 nm excitation/emission filters for anatomical markers and DiI, respectively. Two images from the same sections were pseudo-colored (blue for anatomy, red for DiI) and superimposed to create composite images. A scaled drawing of the silicon probe was used to validate the recording sites that were in the boundaries of HVC (Figure 3). Although the exact boundaries of HVC Shelf cannot be visualized with anatomical markers, previous work concluded that the terminals of the projections reaching HVC Shelf can be found up to 200-500 µm from the ventral border of HVC (Mello et al., 1998). Thus, in the present study, the region extending 200 µm from the ventral border of HVC was defined as HVC Shelf and recording sites that fell in this region were analyzed separately (Figure 3).

Data Analysis

Response Magnitude. Multiunit recordings on each channel were thresholded at two standard deviations (calculated from the whole recording) and positive threshold-crossings were marked with time-stamps in Spike 2, each representing a spike. First, the firing rate (spikes/second) during each baseline period (0.5 seconds preceding each

stimulus presentation) was calculated by dividing the sum of spikes in this period by 0.5. Then, for each recording site separately, these baseline firing rates were regressed against trial number to test for any increasing or decreasing trend in baseline firing rates across the experiment. The slopes of the regressions were not significantly different from 0 across recording sites (Mean ± SEM = -.03 ± .05, $t(83) = -.60$, $p = .55$). For further analyses, the firing rate predicted by the regression analysis for each trial was used as the baseline firing rate (FRbase). The firing rate during each stimulus presentation was calculated as spikes/second by dividing the sum of the spikes from stimulus onset to 0.1 seconds following the stimulus offset by the stimulus duration plus 0.1 seconds. The 'Response Magnitude' (RM) for each presentation of each stimulus was calculated by subtracting the predicted firing rate of baseline (FRbase) from the firing rate of stimulus (FRstim).

Linear Trend. Visual inspection of responses indicated a stimulus-specific decrease in RMs as a function of stimulus repetition for many stimuli at many recording sites. Thus, the responses to each stimulus at each recording site were modeled by a linear regression predicting RMs from trial numbers. The overall R^2 of all linear fits was 0.16 ± .15 (Mean ± SD), although this varied across stimuli (see Results). The magnitude of the response on the first trial with each stimulus was estimated as the predicted response from the regression of all 20 trials with that stimulus (First Predicted Response, FPR). In addition, the Slope of the regression was used to analyze at what rate the responses increased (positive slopes) or decreased (negative slopes) for each stimulus.

BOS-bias of responses. The degree at which each recording site demonstrated higher responses for BOS than Novel stimuli was calculated as

$$d' = \frac{2(\overline{RM}_{BOS} - \overline{RM}_{Novel})}{\sqrt{\sigma^2_{BOS} - \sigma^2_{Novel}}},$$

where $\overline{RM}$s are means and σ^2 are variances of BOS and Novel stimuli across all 20 trials. Three *d'* values were calculated for the three novel stimuli separately and their average was taken to represent a single BOS-bias measure for each recording site. Positive *d'* values denote more BOS-biased activity. Two additional *d'* values were also calculated in the same way as above, one for the first 10 and one for the last 10 trials, to analyze BOS-biases in the initial and latter parts of the experiment.

Spike Sorting. For single-unit analyses, multi-unit recordings were manually thresholded at a level that separates the spikes with largest amplitudes from the rest of the recording. Thresholded spikes were first clustered via automatic template-matching-based methods in Spike2. Then, the spike waveforms were processed through a principal components analysis (PCA) and the clusters were projected on a 3-dimensional space consisting of the three largest components of the PCA. A manual operator investigated the clusters and combined or separated them until a satisfactory separation was reached. The resulting 52 single-units had <3% (Mean ± SD = 2.029 ± .911 %) of their spikes within a 2 ms refractory period. Single-unit RMs, FPRs, and Slopes were calculated for the single-unit data as explained above for multi-unit data.

Results

A total of 84 and 31 multi-unit recordings were histologically verified to be in HVC and HVC Shelf, respectively. Spike-sorting methods isolated 52 single-units from the HVC multi-unit data. All electrophysiological analyses were conducted on multi-unit recordings unless noted. An alpha level of .05 was used to test statistical significance and was Bonferroni-corrected for multiple comparisons where needed.

Birds learn the predictive value of auditory signals rapidly

Behavioral performance in the training was analyzed by accuracies and reaction times (RT). The birds reached the criterion of 75% accuracy in 1-4 and 3-7 days for discrimination 1 and 2, respectively. However, these times cannot be directly compared because stimuli from discrimination 1 continued to be played intermixed with the new stimuli of discrimination 2 and the criterion for discrimination 2 was more severe (3 consecutive days of 75% accuracy instead of 1d). Thus, the second discrimination does not actually take longer than the first (detailed analysis below). The performance accuracy on the first and the last days of discrimination 1 was analyzed by a two-way repeated-measures ANOVA with within-subjects variables: Training Day (First, Last) and CS (Plus, Minus). Since two birds reached the success criterion on the very first day of training, these analyses were conducted on the remaining five birds. Overall, accuracy on the Last day of training was significantly higher than on the First day ($F(1,4) = 28.033$, $p = .006$, Figure 4*A*), and CS- accuracies were significantly greater than CS+ accuracies ($F(1,4) = 27.001$, $p = .007$). More importantly, the interaction between Training Day and CS was statistically significant ($F(1,4) = 164.350$, $p < .001$). Bonferroni post-hoc comparisons revealed that, while CS- accuracies did not significantly change from the First to the Last training day,

CS+ accuracies significantly increased ($p < .001$). In parallel, RTs for CS+ showed a strong trend towards decreasing from the First to the Last day of training ($t(4) = 2.914$, $p = .044$, not significant at the Bonferroni-corrected alpha level of .025, Figure 4*B*), whereas no such difference was observed for CS- ($t(4) = .111$, $p = .917$). Overall, these results indicate that successful behavioral performance in the discrimination 1 stage was driven by learning the predictive value of CS+.

To analyze how the birds reacted to the second CS+ stimulus on the first day of its introduction, performance to this stimulus was compared to the performance to the first CS+ in the first day of discrimination 1. The accuracy for CS+2 stimulus in the first day of its presentation was significantly higher than CS+1 accuracy on its first training day ($t(6) = 4.351$, $p = .005$, Figure 4*C*). Corresponding RTs were lower for CS+2 than for CS+1 stimulus, although this difference was not significant ($t(6) = 1.963$, $p = .097$, Figure 4*D*). These findings suggest that the birds learned the task dynamics - that certain stimuli lead to reward - and applied this knowledge readily to novel signals.

Although, CS+2 accuracies increased, from 73% to 80%, and RTs decreased, from 3.859 to 3.433s from the first to the last day of discrimination 2, these changes were not statistically significant. Accuracies for two CS+/CS- pairs separately were significantly higher than the 75% success criterion in the last three days of the discrimination 2 stage, as revealed by six separate one-sample t-tests (all ps $< .008$). The birds were subjected to one more day of discrimination 2 after the surgery, to ensure that the surgical procedures did not disrupt the task performance. Post-surgery test day accuracies were comparable to the last training day (all ps $> .127$, Figure 4*E*).

Response magnitudes show BOS-bias and CS+/Novel vs CS- difference

To test whether average Response Magnitudes (RM) in HVC differed between hemispheres or among stimulus categories, a two-way mixed Analysis of Variance (ANOVA) with between-subjects variable Hemisphere (Left, Right) and within-subjects variable Stimulus Category (BOS, CS+, CS-, PasFam, Novel) was conducted. The main effect of Hemisphere was not significant ($F(1,82) = .005$, $p = .946$) whereas there was a significant main effect of Stimulus Category ($F(4,328) = 79.776$, $p < .001$, Figure 5*A*). Bonferroni post-hoc tests indicated that BOS RMs were significantly higher than those of all the other stimulus categories (all *p*s < .001). Furthermore, RMs for CS- were significantly lower than for CS+ ($p = .006$) and Novel ($p < .001$). No other pairwise comparison was significant. Although the ANOVA also revealed a significant interaction between Hemisphere and Stimulus Category ($F(4,328) = 2.577$, $p = .038$, Figure 5*B*), post-hoc comparisons showed the identical pattern for both hemispheres: BOS RMs were significantly higher than RMs of all the other stimulus categories (all *p*s < .001). All the other pairwise comparisons were non-significant.

The first CS+/CS- pair was introduced earlier in the behavioral training (during discrimination 1) than the other pair, thus all birds had more exposure to CS+1 and CS-1 than to CS+2 and CS-2. The differences in RMs between CS pairs were analyzed by a two-way repeated measures ANOVA with within-subjects variables: Stimulus Category (CS+, CS-) and Pair (1, 2). The only significant main effect was that of Stimulus Category ($F(1,83) = 18.507$, $p < .001$, Figure 5*B*), indicating greater RMs for CS+ than for CS-. The main effect of Pair and the interaction between Stimulus Category and Pair were not significant ($F(1,83) = .125$, $p = 725$, and $F(1,83) = .173$, $p = .679$, respectively).

Accordingly, Bonferroni-corrected pairwise comparisons confirmed that both CS+1 and CS+2 RMs were significantly greater than both CS-1 and CS-2 RMs (all *p*s < .005), while there was no significant difference between the two CS+ or two CS- stimuli.

Responses decrease with stimulus repetition for all stimuli except BOS

Across recording sites, it was common for RMs elicited by a specific stimulus to decrease as a function of stimulus repetition, as shown for a novel stimulus in Figure 6*A*. In order to examine these response dynamics in detail, the RMs in 20 repetitions of each stimulus in each recording site were modeled by a linear regression (see Materials and Methods). To test whether First Predicted Responses (FPR; the Trial 1 response estimated from the regression) showed differences among stimulus categories and between two hemispheres, a two-way mixed ANOVA with between-subjects variable Hemisphere (Left, Right) and within-subjects variable Stimulus Category (BOS, CS+, CS-, PasFam, Novel) was conducted. The main effect of Hemisphere was not significant ($F(1,82) = .098$, $p = .756$), however there was a significant main effect of Stimulus Category ($F(4,328) = 19.635$, $p < .001$, Figure 6*B*). The results of post-hoc comparisons were exactly the same as the results for overall RMs: BOS FPRs were significantly higher than all the other stimulus categories (all *p*s < .002) and CS- FPRs were significantly smaller than both CS+ ($p < .001$) and Novel FPRs ($p = .002$). In addition, there was a significant interaction between Stimulus Category and Hemisphere ($F(4,328) = 3.146$, $p = .015$, Figure 6*B*). Pairwise post-hoc comparisons demonstrated that, BOS FPRs were significantly bigger than those of all other stimulus categories (all *p*s < .002) and CS+ FPRs were significantly greater than CS- FPRs ($p = .014$) in the right hemisphere. In contrast, in the left hemisphere, while BOS elicited the

largest mean RM, the only significant difference was that FPRs were higher for BOS than for CS- (p = .005).

In addition to investigating the differences in FPR magnitudes among stimulus categories, the rate at which RMs decreased for different stimulus categories was also examined by analyzing the Slopes of the linear regression fits. The differences between the Slopes among stimulus categories were analyzed by a two-way mixed ANOVA with between-subjects variable Hemisphere (Left, Right) and within-subjects variable Stimulus Category (BOS, CS+, CS-, PasFam, Novel). No significant main effect of Hemisphere (F(1,82) = 1.061, p = .306) or interaction between Hemisphere and Stimulus Category (F(4,328) = .689, p = .600) was observed, however there was a main effect of Stimulus Category (F(4,328) = 29.058, p < .001), which was marked by significantly higher (less negative) Slopes for BOS than for all the other stimulus categories (all ps < .001) in post-hoc comparisons. In a further analysis, five separate one sample t-tests were conducted to test whether the Slope of each of five stimulus categories was significantly different than 0. While BOS Slopes were not different than 0 (t(83) = .581, p = .080), all other stimulus categories had Slopes significantly lower than 0 (all ps < .001, Figure 6*C*).

It can be argued that between-stimulus category comparisons would be more accurate if Slopes are divided by the mean RM on the same trials to normalize the response decrease seen with repetition across the different RM levels for different stimulus types. Linear decreasing trends are indeed analyzed this way in other zebra finch auditory brain regions (Phan et al., 2006). However, it is important to note that there was no significant correlation between mean RMs and Slopes among recording sites in the present study (r = -.044, p = .370). When normalization was carried out (Slopes divided by mean RMs), the

variances among different stimulus categories were unbalanced (Mauchley's sphericity test, p <.001). Thus, normalized Slopes were analyzed by multiple non-parametric Wilcoxon singed-rank tests. Results were identical to the raw Slopes: BOS normalized Slopes were significantly higher than those of all the other Stimulus Categories (all *p*s < .001) and no other pairwise difference was statistically significant. When tested with one-sample t-tests, none of the normalized Slopes among stimulus categories were significantly different from 0.

BOS-biases increase with stimulus repetition and are higher in right than in left HVC

Since there were differences in the linear trends between BOS and other stimulus categories across the whole experiment, BOS-biases over Novel stimuli, as measured by *d'* values (see Materials and Methods), for the first and last 10 trials were calculated separately and analyzed by a two-way mixed ANOVA with between-subjects variable Hemisphere (Left, Right) and within-subjects variable Trial (First 10, Last 10). A significant main effect of Trial ($F(1,82) = 27.106$, $p < .001$, Figure 7) indicated higher *d'* values for BOS versus other stimuli on the Last 10 as compared to First 10 trials. The interaction between Hemisphere and Trial was not significant ($F(1,82) = 1.499$, $p = .224$), however there was a strong, but non-significant, main effect of Hemisphere ($F(1,82) = 3.839$, $p = .054$), revealing higher *d'* values for Right than for Left hemisphere. A between-subjects t-test on *d'* values including all trials across the whole experiment, i.e., not separately for first and last 10 trials but all 20 trials, confirmed that BOS-biases in the right hemisphere were significantly higher than those in the left hemisphere ($t(82) = 2.242$, $p = .028$).

BOS linear trends are related to behavioral state

To assess whether the number of both-eyes-closed trials (as an index of behavioral alertness) changed across the experiment (200 trials), eyes-closed trials were counted in three different time windows: 100 trials (half), 50 trials (quarter), and 25 trials (one-eighth). None of these three analyses revealed a significant difference across the experiment: Half 1 vs 2, $t(6) = .083$, $p = .937$; Quarters 1 to 4, $F(3,18) = .526$, $p = .670$; One-eighths 1 to 8, $F(7,42) = .586$, $p = .763$. This suggests that eyes-closed trials did not increase or decrease during the experiment. Nevertheless, the relationships between number of eyes-closed trials and linear trends were analyzed by five separate correlation analyses, one for each stimulus category. There was a moderate, but not significant, positive correlation between number of eyes-closed trials and BOS Slopes ($r = .703$, n = 7, $p = .078$, Figure 8). None of the other stimulus categories had a correlation coefficient significantly different than 0 (all ps > .402).

HVC Shelf responses decrease with repetition for all stimuli

HVC and HVC Shelf RMs were analyzed by a two-way mixed ANOVA with between-subjects variable Region (HVC, HVC Shelf) and within-subjects variable Stimulus Category (BOS, CS+, CS-, PasFam, Novel). HVC Shelf RMs were significantly higher than HVC RMs ($F(1,113) = 36.662$, $p < .001$, Figure 9*A*). The main effect of Stimulus Category was also significant ($F(4,452) = 39.642$, $p < .001$). In post-hoc comparisons, BOS RMs were significantly higher than RMs of all other stimulus categories (all ps < .001) and CS- RMs were significantly smaller than CS+ ($p < .001$), Novel ($p < .001$), and PasFam RMs ($p < .025$). Furthermore, the interaction between Region and Stimulus Category was significant ($F(4,452) = 5.284$, $p < .001$). Bonferroni post-hoc tests revealed that, in HVC,

BOS RMs were significantly bigger than the RMs of all other stimuli (all *p*s < .001) and Novel RMs were significantly higher than CS- RMs ($p = .015$). However, in HVC Shelf, BOS, CS+ and Novel RMs were significantly greater than CS- RMs (all *p*s < .015), but not different from each other; and no other pairwise comparison was statistically significant.

FPRs were calculated from regressions of HVC Shelf RMs across trials, as described above. A two-way mixed ANOVA with between-subjects variable Region (HVC, HVC Shelf) and within-subjects variable Stimulus Category (BOS, CS+, CS-, PasFam, Novel) was also conducted to analyze FPRs. Similar to RMs, FPRs in HVC Shelf were significantly bigger than in HVC ($F(1,113) = 62.639$, $p < .001$, Figure 9*B*). A significant main effect of Stimulus Category ($F(4,452) = 8.235$, $p < .001$) was marked by significantly higher FPRs for Novel and CS+ than for CS- FPRs (both *p*s < .001) and significantly higher FPRs for BOS than for CS- and PasFam (both *p*s < .001) . There was also a significant interaction between Region and Stimulus Category ($F(4,452) = 3.360$, $p = .010$). Post-hoc comparisons indicated that BOS FPRs were significantly greater than CS-, PasFam, and Novel FPRs (all *p*s < .020) and CS+ FPRs were significantly higher than CS- FPRs ($p = .021$) in HVC, whereas, in HVC Shelf, the only significant difference was higher FPRs for Novel than for CS- ($p = .013$).

HVC Shelf Slopes were analyzed by five separate one-sample t-tests, one for each stimulus category, to test whether they were different than 0. Slopes for all five stimulus categories were found to be significantly lower than 0 (all *p*s < .001, Figure 9*C*), which suggests that the RMs of all stimulus categories decreased as the experiment proceeded. To analyze HVC Shelf Slopes in relation to those of HVC, a two-way mixed ANOVA with between-subjects variable Region (HVC, HVC Shelf) and within-subjects variable

Stimulus Category (BOS, CS+, CS-, PasFam, Novel) was conducted. HVC Shelf Slopes were significantly lower than HVC Slopes ($F(1,113) = 54.695$, $p < .001$), which suggests that HVC Shelf RMs decreased at faster rates than HVC RMs during the experiment. There was also a significant main effect of Stimulus Category ($F(4,452) = 26.261$, $p < .001$), which showed significantly higher (less negative) Slopes for BOS than for all other stimulus categories in post-hoc comparisons (all ps $< .001$). There was no interaction between Region and Stimulus Category ($F(4,452) = .882$, $p = .474$). Although HVC Shelf Slopes were smaller than those of HVC when analyzed as raw Slopes, when analyzed as Slopes normalized by their mean RMs, there was no significant difference between the two regions ($F(1,113) = .153$, $p = .696$).

Single-unit HVC responses resemble multi-unit RMs except decreasing linear trends

For each single-unit, a within-subjects t-test comparing the firing rates of baseline periods to the firing rates of stimulus periods for all 200 trials was conducted to test whether there was a change in the firing rate in response to repeated auditory stimuli. The same type of analysis was also conducted for only the 20 BOS trials since there can be BOS-selective neurons that only respond to BOS and no other stimulus in HVC. Out of 52 isolated neurons, 8 showed no significant change in firing rate between the baseline and the stimulus periods in either analysis. Of the 44 driven neurons, 6 neurons significantly decreased their firing rates during stimulus presentations, thus were found to be “inhibited” neurons. Thirty-seven neurons increased their firing rate in response to auditory stimuli as revealed in the 200-trials analysis. One neuron showed a significant increase in firing rate only in response to BOS but not to auditory stimuli in general. These 37 neurons and one

BOS-selective neuron were combined as "excited" neurons and examined in further statistical analyses.

RMs of excited neurons were analyzed by a two-way mixed ANOVA with between-subjects variable Hemisphere (Left, Right) and within-subjects variable Stimulus Category (BOS, CS+, CS-, PasFam, Novel). The main effect of Hemisphere and the interaction between Hemisphere and Stimulus Category were not statistically significant ($F(1,36) = 2.996$, $p = .092$; $F(4,144) = .054$, $p = .995$, respectively). However, there was a significant main effect of Stimulus Category ($F(4,144) = 12.056$, $p < .001$, Figure 10*A*). Post-hoc Bonferroni tests showed that BOS RMs were significantly higher than all other stimulus categories (all ps $< .001$) and no other pairwise comparisons were significant. Nevertheless, CS+ and Novel RMs were found to be significantly greater than CS- RMs when compared in two separate dependent-samples t-tests ($t(37) = 2.943$, $p = .006$ for CS+; $t(37) = 2.897$, $p = .006$ for Novel), similar to the results seen in multi-unit responses. A two-way mixed ANOVA with between-subjects variable Hemisphere (Left, Right) and within-subjects variable Stimulus Category (BOS, CS+, CS-, PasFam, Novel) on FPRs revealed no significant main effect of Hemisphere ($F(1,36) = 1.410$, $p = .243$), Stimulus Category ($F(4,144) = .860$, $p = .490$), or interaction between Hemisphere and Stimulus Category ($F(4,144) = .153$, $p = .962$).

To test whether single-unit Slopes for excited units were significantly different from 0, five separate one-sample t-tests, one for each stimulus category, were conducted. There was a strong tendency for BOS Slopes to be higher than 0, however this difference did not reach significance with the Bonferroni-corrected alpha level of .01 ($t(37) = 2.392$, $p = .022$, Figure 10*B*). None of the other stimulus categories had Slopes significantly

different from 0 (all *p*s < .368). To compare the Slopes among stimulus categories, a two-way mixed ANOVA with between-subjects variable Hemisphere (Left, Right) and within-subjects variable Stimulus Category (BOS, CS+, CS-, PasFam, Novel) was conducted. The main effect of Stimulus Category was significant ($F(4,144) = 8.847$, $p < .001$), indicating higher Slopes for BOS than for all other stimulus categories (all *p*s < .002). No significant main effect of Hemisphere ($F(1,36) = .333$, $p = .567$) or interaction between Hemisphere and Stimulus Category ($F(4,144) = .407$, $p = .804$) was observed.

Discussion

The overall results of the current study demonstrate that the auditory responses of the songbird premotor nucleus HVC to various categories of stimuli in awake zebra finches are robust, complex, and dynamic. In line with previous findings (Margoliash, 1986; Raksin et al., 2012), responses to BOS were higher than to any other stimulus right from the beginning and remained higher throughout the experiment. Moreover, novel and reward-predicting CS+ stimuli elicited greater responses than neutral CS- stimuli, both on the first presentation and during the whole experiment. Responses declined with stimulus repetition for all stimuli except BOS. The linear trend of BOS responses showed a tendency to be positively correlated with the number of eyes-closed trials; as a bird spent more trials with its eyes closed, BOS responses trended higher across the experiment. Due to the differences in linear trends between BOS and other stimuli, BOS-biases in the latter half of the experiment were bigger than those in the first half. Furthermore, BOS-biases were generally higher in right than in left HVC. Single neurons in HVC showed BOS-bias and preferential activity to CS+ and Novel as compared to CS- stimuli similar to the multi-unit activity, however they did not show decreasing responses as the experiment proceeded. Last, HVC Shelf, the area surrounding the ventral border of HVC, showed greater auditory responses which decreased at a faster rate as compared to HVC, did not demonstrate BOS-bias, and responded more to CS+ and novel than to CS- stimuli.

Complex and dynamic auditory responses support a new view of HVC processing

Auditory responses in the premotor songbird nucleus HVC have traditionally been believed to be highly selective to BOS with little or no activity induced by any other acoustic signal, based on studies on anesthetized birds. Conceptually, this made sense because it suggested

that auditory responses in HVC were primarily related to its role as a vocal motor command structure for producing BOS. My current results add to these findings: BOS is a special stimulus for HVC as revealed by different activity profiles in response to BOS than any other stimulus in virtually all measures: BOS responses are higher, even on the very first presentation; they do not decrease with repeated presentation as the responses to other stimuli do; and they may have a distinct relationship with the behavioral state of the bird, which is not seen for other stimuli. However, the present results also challenge the earlier view that HVC is BOS-selective (instead of BOS-biased) as I found robust and dynamic auditory activity in HVC for all trained, passively familiar, and novel conspecific songs; furthermore, responses differentiated trained stimuli according to their predictive value. My results are consistent with and extend the limited existing research on awake birds which confirms the general finding that BOS responses are higher, but found HVC responses to other auditory stimuli, sometimes as great as those to BOS (Prather et al., 2010; Raksin et al., 2012). Thus, the current view on the perceptual role of HVC needs to be modified to fully capture the complexity of neural responses to various acoustic signals.

A major finding of the present study is lower HVC responses to CS- as compared to CS+ and novel stimuli. In the behavioral training before electrophysiological recordings, CS+ stimuli predicted a water reward whereas CS- stimuli did not signal any outcome. Assuming that, at the beginning of the training when they were novel to the birds, the arbitrarily chosen CS+ and CS- stimuli induced responses of similar magnitude to those elicited by novel stimuli during the electrophysiological recording, CS- responses decreased as a result of behavioral training, while CS+ responses stayed the same. Although this is the most parsimonious explanation of the present results, more complex

interpretations are possible. In any case, an intriguing question is where the source of the differential activity to CS+ and CS- stimuli in HVC might be. There is no data available regarding the responses of two major auditory inputs to HVC, i.e., NIf and Avalanche, to learned predictive values of auditory signals. However, the general region in which Avalanche resides, Caudal Mesopaillium (CM), was found to show higher responses to rewarded than to unrewarded conspecific songs in anesthetized European starlings (Jeanne et al., 2011), which is in line with the current findings. Nevertheless, the same study reported that responses to novel stimuli were lower than rewarded and comparable to unrewarded stimuli, which is in direct contrast with the present results in HVC. The activity in Avalanche may not be exactly the same as in the general CM region; however, if it is, then the current findings in HVC may not exclusively reflect Avalanche activity but may be partly due to NIf projections and/or local circuitry mechanisms. It should be noted that not all inputs to HVC are well characterized anatomically.

Does HVC play an active role in learning and/or recognition of the predictive value of acoustic stimuli in behavioral tasks? Although the current study cannot give a direct answer to this question, the differential activity to reward-predicting and neutral signals shows that this information reaches HVC. Previous work indicated that HVC lesions do not disrupt the behavioral discrimination of previously acquired categories of sounds but increase the latency to learn new associations (Gentner et al., 2000). Thus, it is suggested that HVC is involved in acquisition, but not in recognition, of the predictive value of auditory signals. If this view is correct, then the activity patterns I found in HVC in well trained birds might represent non-functional residual activity in the afferent structures to HVC. However, the HVC lesions in Gentner and colleagues' (2000) study were partial,

which raises the question whether recognition might be affected with complete lesions. It is possible that HVC actively contributes to and/or supports other brain structures in learning and/or recognition of predictive value of sounds. The current project represents the first step of a comprehensive research line that will investigate the perceptual role of HVC in behavioral tasks.

In the present study, no significant difference in HVC activity was observed between novel stimuli and stimuli that were passively heard <=20 hours earlier. A forebrain auditory region of the zebra finch brain, NCM, shows neuronal memory for passively heard conspecific songs within 20 hours of testing, as demonstrated by lower neural response magnitudes and lower rates of response decrease with repeated presentation (the phenomenon of stimulus-specific adaptation, see below for detailed explanation) for these stimuli than for novel stimuli (Chew et al., 1996a). Neither occurred in HVC. Thus, the current findings suggest that HVC may not be involved in this kind of auditory memory. Alternatively, encoding of passively heard auditory signals in HVC might require more exposure, or be shorter lasting than in other structures.

The present results revealed that responses to all auditory stimuli, with the exception of BOS, decreased as the experiment proceeded. Since, the experimental stimuli were presented in shuffled rather than in serial order, one can argue that this decline was not stimulus-specific and occurred across the time experiment instead of occurring separately for each stimulus. However, if this was the case, one would expect this non-specific decline to affect BOS responses too, and this was <u>not</u> true; thus, I consider the decreases to be stimulus-specific. A similar phenomenon called stimulus-specific adaptation (SSA) is a well-defined characteristic of higher-order auditory regions of the

waking songbird brain, NCM and CM (Chew et al., 1996a, 1996b; Smulders & Jarvis, 2013). SSA is a process of neural memory formation and reflects stimulus familiarity: more familiar stimuli adapt at a slower rate. Based on these findings in other auditory structures, one would expect CS+ and CS- stimuli, with which the birds were familiarized over many presentations, to decrease at a slower rate than novel stimuli. Yet, the present study did not find any difference in the rates of decrease among non-BOS stimuli. Thus, the mechanisms underlying this phenomenon in HVC are unclear. Decreasing responses in the awake bird may represent a process of encoding the predictive value of the experimental stimuli in a novel context (the electrophysiological recording booth). Since no stimulus predicted any outcome or was associated with anything during electrophysiological recordings, responses for all stimuli may have dropped as a process of encoding this non-predictive value. This interpretation is in line with the results showing decreased overall responses to CS-, which did not predict any outcome in behavioral training, than CS+ stimuli. Future studies using methods in which novel stimuli would predict an outcome during the electrophysiological recordings will clarify the encoding of associative/predictive value of auditory signals in HVC.

BOS responses seem to be resistant to decline with repeated stimulus presentation. In fact, the current results support previous findings suggesting that there is a special relationship between behavioral state and BOS responses (Rauske et al., 2003; Cardin & Schmidt, 2003). The present findings also indicate that this relationship may only hold true for BOS responses and is not seen for other stimuli (see below for further discussion). An important finding of the current study is the elevated BOS-biased activity in the second half of the experiment as compared to the first half. This was a result of the fact that HVC

activity in response to BOS stayed constant throughout the experiment while responses to all other stimuli decreased. The implications of this finding are far reaching regarding the study of auditory activity in HVC. Previous studies found either no or very little responses to auditory signals other than BOS; hence, BOS-biases (or BOS-selectivities) were much higher than what is reported here (Margoliash, 1986; Theunissen & Doupe, 1998; Mooney et al., 2001; Cardin & Schmidt, 2003). These studies, however, used the same stimulus or set of stimuli over and over again to elicit HVC responses as a single electrode or a few electrodes were positioned sequentially at multiple sites. As a result of the decreasing responses to non-BOS stimuli (that probably occurred, but were undocumented), BOS-biases reported in these studies are likely to have been much higher than if BOS responses had been compared to new novel stimuli at each recording site. A recent fMRI analysis in anesthetized zebra finches demonstrated that HVC activity did not decrease with repetition in response to BOS or other conspecific songs (Poirier et al., 2009), thus the above argument might not apply to anesthetized recordings. Nevertheless, the original interpretation that HVC responds poorly to stimuli other than BOS should be viewed with caution, especially for awake recordings.

Despite all the above considerations, BOS-bias in HVC probably cannot be solely explained by this phenomenon (response decrease to all other stimuli), since BOS-biased activity was observed even for the very first stimulus presentation. However, the current experimental design has a problem that leaves some uncertainty with respect to this conclusion. During the initial penetrations that lowered the electrodes towards HVC, search stimuli were played to elicit HVC-typical activity: these consisted of BOS and another novel conspecific song, played not more than 20 times each. That original novel song was

not included in the experimental stimulus list. The result is that BOS, but no other experimental stimulus, being presented up to 20 times right before electrophysiological data were recorded. It is thus possible that BOS responses decreased during these early presentations to an asymptotic level that did not show any further decrease during the recordings. Nonetheless, the FPR for BOS was higher than for other stimuli, suggesting that, even at an asymptote, responses to BOS were higher.

The response magnitudes of single neurons in HVC revealed patterns of activity similar to those of multi-units. My data confirmed previous findings showing BOS-bias even at the single neuron level in HVC (Raksin et al., 2012). In addition, single neurons increased their firing rates more in response to CS+ and novel than to CS- stimuli. However, the biggest difference between single-unit and multi-unit data was that responses of single-units as a group did not decrease in response magnitude with stimulus repetition. In fact, BOS responses showed a strong trend towards increasing throughout the experiment. Interestingly, the multi-unit trend to higher slopes for BOS than for other stimuli was replicated in the single-unit responses. The lack of decreasing responses might have stemmed from greater variability and smaller sample size in the single-unit data. Some neurons in our sample indeed show decreasing responding with repeated stimulus presentation, however there was no tendency to increase or decrease responding as a group. Alternatively, our electrodes and/or our spike sorting technique might have selectively isolated a class of neurons that do not significantly decrease their responding during the experiment.

Hemispheric difference in BOS-biases

Several functional hemispheric differences underlie the auditory and the motor areas of the songbird brain (Moorman & Nicol, 2014). The current results indicated that BOS-biases over novel songs were higher in right than in left HVC. Moreover, BOS induced higher responses than all the other stimuli in the very first presentation only in right HVC, while, in left HVC, BOS responses were only greater than CS- responses. These results suggest that right HVC processes BOS versus other stimuli more differentially than does left HVC. In line with this finding, in an fMRI study with anesthetized zebra finches, Poirier et al. (2009) found that blood oxygen level dependent responses elicited by BOS were significantly greater than conspecific cage mate songs only in right, but not in left, HVC. Furthermore, in juvenile zebra finches, an hour of novel conspecific song exposure led to more immediate early gene Zenk expression in left than in right HVC, which suggests that left HVC is more responsive to songs other than BOS (Moorman et al., 2012). However, since this study did not test the effects of BOS exposure, it cannot fully be compared to the present findings. In addition to right-lateralized BOS processing in HVC, the motor production of song is described as right-dominant in zebra finches based on finding more severe song deterioration in birds that received right HVC lesion (Williams et al., 1992) or right tracheosyringeal nerve transection (Floody & Arnold, 1997). Taken together, it seems that there may be a coupling in the hemispheric bias of both perceptual and motor processing of BOS in the zebra finch HVC. A similar phenomenon is observed in premotor and motor speech areas in the human brain, although the side of the dominance is reversed: left motor cortex and Broca's area (or inferior frontal cortex) are more active than their right counterparts during both speech production and comprehension (for a

review, Hickok & Poeppel, 2004). One can speculate that, in the zebra finch, right HVC is more involved in auditory and motor processing of BOS, while left HVC is more dedicated to processing of other sounds (including conspecific vocalizations), perhaps as an adaptation to increase processing efficiency by limiting bilateral duplication (Vallortigara & Rogers, 2005). However, such a strong claim is not supported by other work (Long & Fee, 2008; Wang et al., 2008). An overarching framework that brings all lateralization findings together in songbirds (or in any one species) in a functionally comprehensible way is yet to be established and requires further empirical evidence.

Behavioral State and BOS responses

It is well-established that auditory responses in HVC are highly sensitive to behavioral state (Rauske et al., 2003; Cardin & Schmidt, 2003). Under anesthesia, during sleep and sedation, HVC responds selectively and stereotypically to BOS with little or no activity to any other auditory stimulus. During regular wakefulness, responses are seen to multiple sounds, are more variable and less BOS-biased. In contrast, a rapid transition from sedation to aroused state (as induced by an alerting signal) completely suppresses the auditory responses in HVC to any stimulus. Furthermore, these sleep-to-waking and sedation-to-arousal activity differences are seen within milliseconds (Nick & Konishi, 2001; Cardin & Schmidt, 2003). The present study was conducted on un-anesthetized birds, however it might still be argued that the linear trends and activity differences among experimental stimuli are results of differences in behavioral state throughout the experiment and in response to different stimuli, respectively. To get a measure of behavioral state, trials in which both of the bird's eyes were closed were coded. Although there was considerable variability in the proportion of eye-closed trials among birds, there were not enough trials

to compare the responses in eyes-closed and eyes-open trials for each stimulus for each bird. Nonetheless, when analyzed on a bird-by-bird basis, behavioral state showed a weak relationship to the linear trend of BOS responses: birds with more eyes-closed trials had BOS responses that increased during the experiment (although this strong trend was not statistically significant). At first sight, this finding may seem perfectly in line with the previous work showing BOS responses increase from wakefulness to sleep. However, it is important to note that the number of eyes-closed trials did not increase as the experiment proceeded. It was the total number of eyes-closed trials in the entire experiment that positively correlated with the increasing BOS linear trends. This perplexity may stem from the fact that closing eyes is not a perfect indication of sleep; perhaps, in some trials the bird just closes its eyes, while in others it falls asleep. Future work should adopt more valid measures of behavioral state, such as EEG (Nick & Konishi, 2001), to further illuminate the correlation between changing behavioral states and HVC responses. Regardless, the decreasing linear trends of stimuli other than BOS did not show any relation to the number of eyes-closed trials, which suggests that the decrease in these responses as a function of stimulus repetition results from a process independent of behavioral state. Further support for this claim comes from one bird that never closed its eyes during the whole recording session, but still showed clear decreasing linear trends for stimuli other than BOS.

As for the differences in response magnitudes among experimental stimuli, it is unlikely that the results were artifacts of different behavioral states induced by different stimuli, although the possibility was not experimentally ruled out, Taking into account the previous work, one can argue that some stimuli can be more arousing, leading to lower HVC responses for those stimuli as compared to the others. However, in the present study,

CS- stimuli elicited the lowest responses and there is no a priori reason why they would be more arousing than CS+ or novel stimuli. Furthermore, the number of eye-closed trials among these stimuli were comparable. Hence, I conclude that possible behavioral state differences in response to different categories of sounds cannot explain the present findings.

Auditory responses in HVC Shelf

HVC Shelf is a region defined by the terminals of the projections of auditory brain areas Field L1 and L3 and CLM to the region surrounding the ventral border of HVC (Vates et al., 1996; Mello et al., 1998). Although right next to each other, HVC and the Shelf share limited anatomical connectivity. The present study revealed both similarities and differences between HVC and the Shelf auditory responses. First, while the well-established phenomenon of BOS-biased activity was seen in HVC, recordings from the Shelf did not show that property. Previous work on BOS-selectivity in HVC Shelf suffered from methodological problems and did not yield conclusive results (Poirier et al., 2009; Poirier et al., 2011). The lack of BOS-biased activity in HVC Shelf is consistent with the non-BOS-biased response profiles in the afferents to the Shelf, i.e., Field L1 and L3 and CLM (Amin et al., 2007). A similar BOS-related response difference between HVC and the Shelf was in linear trends: BOS-responses in HVC did not decrease with repetition, but they did in the Shelf. Moreover, auditory responses started higher and decreased at a faster rate in the Shelf than in HVC. This suggests that HVC Shelf is more similar in its auditory activity profile to the proper auditory regions of the songbird brain, such as NCM and CM (Chew et al., 1996a; Smulders & Jarvis, 2013) than is HVC. However, the source of decreasing auditory responses in the Shelf is not yet clear. Some neurons in Fields L1 and

L3 do show an adaptation phenomenon although the rate of response decrease is much smaller than observed in the other forebrain auditory structures (Smulders & Jarvis, 2013). Thus, it is more likely that this response profile reflects CLM inputs and/or local circuit properties.

The main similarity between HVC and the Shelf auditory activities was the decreased response to CS- as compared to CS+ and novel stimuli. Although it is possible that this correlation is due to the reciprocal connections between the two regions, it is unlikely since these projections are extremely limited (Mello et al., 1998). An alternative account is that the general CM region conveys this differential activity information separately to HVC, via Avalanche, and to the Shelf, via CLM. CM in general (both Medial and Lateral Caudal Mesopallium) indeed is shown to encode the learned predictive values of acoustic stimuli in European starlings (Jeanne et al., 2011). Recent work from our laboratory also demonstrated that the auditory forebrain nucleus NCM shows higher responses to reward-predicting than to punishment-predicting stimuli after operant auditory discrimination training (Bell et al., 2013). Although anatomical studies did not reveal any direct connection between NCM and HVC or the Shelf, this information in NCM may reach these structures via NCM's projections to CM. The observation of differential activity between reward-predicting and neutral auditory signals in HVC Shelf raises the intriguing question whether this region actively participates in learning and/or performance of auditory discrimination. Future studies testing the effects of selective lesioning of either afferent projections to the Shelf or the whole HVC Shelf on behavioral discrimination tasks may enhance our understanding of the exact role of this region in perception and learning.

Conclusion

The present study provides the first step towards a comprehensive understanding of the role of the premotor vocal nucleus HVC in perception of learned sound categories in zebra finches. Complex and dynamic auditory responses in HVC raise intriguing research questions and demand further detailed examination of the specific role played by this premotor brain area in perception. In addition to enhancing our knowledge of the songbird brain, the present project also provides a potential avian model for investigating the role of premotor systems in speech perception in the human brain. At this stage, it is difficult to reconcile all the findings in the zebra finch HVC with data from human premotor language processing areas; however, parallel patterns of activity, such as processing of learned sound categories and functional lateralization, emerge. Understanding neural mechanisms underlying auditory perception in the zebra finch model will potentially reveal principles that can be applied to deepen our knowledge of normal and abnormal speech perception and production in humans.

References

Amin, N., Doupe, A., & Theunissen, F. E. (2007). Development of selectivity for natural sounds in the songbird auditory forebrain. *Journal of neurophysiology*,*97*(5), 3517-3531.

Bell, B. A., Phan, M. L., & Vicario, D. S. (2013). *Auditory responses in the songbird forebrain reflect acquired salience and individual learning rates in a behavioral discrimination paradigm.* Poster presented at the annual meeting of the Society for Neuroscience, San Diego, CA.

Brenowitz, E. A. (1991). Altered perception of species-specific song by female birds after lesions of a forebrain nucleus. *Science*, *251*(4991), 303-305.

Cardillo, E. R., Watson, C. E., Schmidt, G. L., Kranjec, A., & Chatterjee, A. (2012). From novel to familiar: tuning the brain for metaphors. *Neuroimage*,*59*(4), 3212-3221.

Cardin, J. A., Raksin, J. N., & Schmidt, M. F. (2005). Sensorimotor nucleus NIf is necessary for auditory processing but not vocal motor output in the avian song system. *Journal of neurophysiology*, *93*(4), 2157-2166.

Cardin, J. A., & Schmidt, M. F. (2003). Song system auditory responses are stable and highly tuned during sedation, rapidly modulated and unselective during wakefulness, and suppressed by arousal. *Journal of neurophysiology*,*90*(5), 2884-2899.

Chew, S. J., Vicario, D. S., & Nottebohm, F. (1996a). Quantal duration of auditory memories. *Science*, *274*(5294), 1909-1914.

Chew, S. J., Vicario, D. S., & Nottebohm, F. (1996b). A large-capacity memory system that recognizes the calls and songs of individual birds. *Proceedings of the National Academy of Sciences*, *93*(5), 1950-1955.

Coleman, M. J., Roy, A., Wild, J. M., & Mooney, R. (2007). Thalamic gating of auditory responses in telencephalic song control nuclei. *The Journal of Neuroscience*, *27*(37), 10024-10036.

Del Negro, C., Gahr, M., Leboucher, G., & Kreutzer, M. (1998). The selectivity of sexual responses to song displays: effects of partial chemical lesion of the HVC in female canaries. *Behavioural brain research*, *96*(1), 151-159.

Doupe, A. J., & Kuhl, P. K. (1999). Birdsong and human speech: common themes and mechanisms. *Annual review of neuroscience*, *22*(1), 567-631.

Embick, D., Marantz, A., Miyashita, Y., O'Neil, W., & Sakai, K. L. (2000). A syntactic specialization for Broca's area. *Proceedings of the National Academy of Sciences*, *97*(11), 6150-6154.

Fiebach, C. J., Schlesewsky, M., Lohmann, G., Von Cramon, D. Y., & Friederici, A. D. (2005). Revisiting the role of Broca's area in sentence processing: syntactic integration versus syntactic working memory. *Human brain mapping*, *24*(2), 79-91.

Floody, O. R., & Arnold, A. P. (1997). Song lateralization in the zebra finch.*Hormones and behavior*, *31*(1), 25-34.

Gentner, T. Q., Hulse, S. H., Bentley, G. E., & Ball, G. F. (2000). Individual vocal recognition and the effect of partial lesions to HVc on discrimination, learning, and categorization of conspecific song in adult songbirds. *Journal of neurobiology*, *42*(1), 117-133.

Hickok, G., & Poeppel, D. (2004). Dorsal and ventral streams: a framework for understanding aspects of the functional anatomy of language. *Cognition*, *92*(1), 67-99.

Jeanne, J. M., Thompson, J. V., Sharpee, T. O., & Gentner, T. Q. (2011). Emergence of learned categorical representations within an auditory forebrain circuit. *The Journal of Neuroscience*, *31*(7), 2595-2606.

Kirn, J. R., Alvarez-Buylla, A., & Nottebohm, F. (1991). Production and survival of projection neurons in a forebrain vocal center of adult male canaries. *The Journal of neuroscience*, *11*(6), 1756-1762.

Liberman, A. M., & Mattingly, I. G. (1985). The motor theory of speech perception revised. *Cognition*, *21*(1), 1-36.

Long, M. A., & Fee, M. S. (2008). Using temperature to analyse temporal dynamics in the songbird motor pathway. *Nature*, *456*(7219), 189-194.

Lotto, A. J., Hickok, G. S., & Holt, L. L. (2009). Reflections on mirror neurons and speech perception. *Trends in cognitive sciences*, *13*(3), 110-114.

Margoliash, D. (1986). Preference for autogenous song by auditory neurons in a song system nucleus of the white-crowned sparrow. *The Journal of neuroscience*, *6*(6), 1643-1661.

Mello, C. V., Vates, E., Okuhata, S., & Nottebohm, F. (1998). Descending auditory pathways in the adult male zebra finch (Taeniopygia guttata). *Journal of Comparative Neurology*, *395*(2), 137-160.

Meltzer, J. A., McArdle, J. J., Schafer, R. J., & Braun, A. R. (2010). Neural aspects of sentence comprehension: syntactic complexity, reversibility, and reanalysis. *Cerebral cortex*, *20*(8), 1853-1864.

Menenti, L., Petersson, K. M., Scheeringa, R., & Hagoort, P. (2009). When elephants fly: differential sensitivity of right and left inferior frontal gyri to discourse and world knowledge. *Journal of Cognitive Neuroscience*, *21*(12), 2358-2368.

Mooney, R. (2000). Different subthreshold mechanisms underlie song selectivity in identified HVc neurons of the zebra finch. *The Journal of Neuroscience*,*20*(14), 5420-5436.

Mooney, R. (2009). Neural mechanisms for learned birdsong. *Learning & Memory*, *16*(11), 655-669.

Mooney, R., Hoese, W., & Nowicki, S. (2001). Auditory representation of the vocal repertoire in a songbird with multiple song types. *Proceedings of the National Academy of Sciences*, *98*(22), 12778-12783.

Moorman, S., Gobes, S. M., Kuijpers, M., Kerkhofs, A., Zandbergen, M. A., & Bolhuis, J. J. (2012). Human-like brain hemispheric dominance in birdsong learning. *Proceedings of the National Academy of Sciences*, *109*(31), 12782-12787.

Moorman, S., & Nicol, A. U. (2014). Memory-related brain lateralisation in birds and humans. *Neuroscience & Biobehavioral Reviews*.

Nick, T. A., & Konishi, M. (2001). Dynamic control of auditory activity during sleep: correlation between song response and EEG. *Proceedings of the National Academy of Sciences*, *98*(24), 14012-14016.

Okanoya, K., Ikebuchi, M., Uno, H., & Watanabe, S. (2001). Left-side dominance for song discrimination in Bengalese finches (Lonchura striata var. domestica). *Animal cognition*, *4*(3-4), 241-245.

Phan, M. L., Pytte, C. L., & Vicario, D. S. (2006). Early auditory experience generates long-lasting memories that may subserve vocal learning in songbirds.*Proceedings of the National Academy of Sciences of the United States of America*, *103*(4), 1088-1093.

Poirier, C., Boumans, T., Vellema, M., De Groof, G., Charlier, T. D., Verhoye, M., ... & Balthazart, J. (2011). Own song selectivity in the songbird auditory pathway: suppression by norepinephrine. *PloS one*, *6*(5), e20131.

Poirier, C., Boumans, T., Verhoye, M., Balthazart, J., & Van der Linden, A. (2009). Own-song recognition in the songbird auditory pathway: selectivity and lateralization. *The Journal of Neuroscience*, *29*(7), 2252-2258.

Prather, J. F., Peters, S., Nowicki, S., & Mooney, R. (2010). Persistent representation of juvenile experience in the adult songbird brain. *The Journal of Neuroscience*, *30*(31), 10586-10598.

Raksin, J. N., Glaze, C. M., Smith, S., & Schmidt, M. F. (2012). Linear and nonlinear auditory response properties of interneurons in a high-order avian vocal motor nucleus during wakefulness. *Journal of neurophysiology*, *107*(8), 2185-2201.

Rauske, P. L., Shea, S. D., & Margoliash, D. (2003). State and neuronal class-dependent reconfiguration in the avian song system. *Journal of Neurophysiology*, *89*(3), 1688-1701.

Roskies, A., Fiez, J., Balota, D., Raichle, M., & Petersen, S. (2001). Task-dependent modulation of regions in the left inferior frontal cortex during semantic processing. *Cognitive Neuroscience, Journal of*, *13*(6), 829-843.

Scharff, C., Nottebohm, F., & Cynx, J. (1998). Conspecific and heterospecific song discrimination in male zebra finches with lesions in the anterior forebrain pathway. *Journal of neurobiology*, *36*(1), 81-90.

Stromswold, K., Caplan, D., Alpert, N., & Rauch, S. (1996). Localization of syntactic comprehension by positron emission tomography. *Brain and language*, *52*(3), 452-473.

Smulders, T. V., & Jarvis, E. D. (2013). Different mechanisms are responsible for dishabituation of electrophysiological auditory responses to a change in acoustic identity than to a change in stimulus location. *Neurobiology of learning and memory*, *106*, 163-176.

Tchernichovski, O., Nottebohm, F., Ho, C. E., Pesaran, B., & Mitra, P. P. (2000). A procedure for an automated measurement of song similarity. *Animal Behaviour*, *59*(6), 1167-1176.

Theunissen, F. E., & Doupe, A. J. (1998). Temporal and spectral sensitivity of complex auditory neurons in the nucleus HVc of male zebra finches. *The Journal of neuroscience*, *18*(10), 3786-3802.

Thompson, J. V., & Gentner, T. Q. (2010). Song recognition learning and stimulus-specific weakening of neural responses in the avian auditory forebrain.*Journal of neurophysiology*, *103*(4), 1785-1797.

Vallortigara, G., & Rogers, L. J. (2005). Survival with an asymmetrical brain: advantages and disadvantages of cerebral lateralization. *Behavioral and brain sciences*, *28*(4), 575-588.

Vates, G. E., Broome, B. M., Mello, C. V., & Nottebohm, F. (1996). Auditory pathways of caudal telencephalon and their relation to the song system of adult male zebra finches (Taenopygia guttata). *Journal of Comparative Neurology*,*366*(4), 613-642.

Vicario, D. S., Naqvi, N. H., & Raksin, J. N. (2001). Behavioral discrimination of sexually dimorphic calls by male zebra finches requires an intact vocal motor pathway. *Journal of neurobiology*, *47*(2), 109-120.

Vignal, C., Andru, J., & Mathevon, N. (2005). Social context modulates behavioural and brain immediate early gene responses to sound in male songbird. *European Journal of Neuroscience*, *22*(4), 949-955.

Wang, C. Z., Herbst, J. A., Keller, G. B., & Hahnloser, R. H. (2008). Rapid interhemispheric switching during vocal production in a songbird. *PLoS biology*,*6*(10), e250.

Whitney, C., Kirk, M., O'Sullivan, J., Ralph, M. A. L., & Jefferies, E. (2010). The neural organization of semantic control: TMS evidence for a distributed network in left inferior frontal and posterior middle temporal gyrus. *Cerebral Cortex*, bhq180.

Williams, H., Crane, L. A., Hale, T. K., Esposito, M. A., & Nottebohm, F. (1992). Right-side dominance for song control in the zebra finch. *Journal of neurobiology*, *23*(8), 1006-1020.

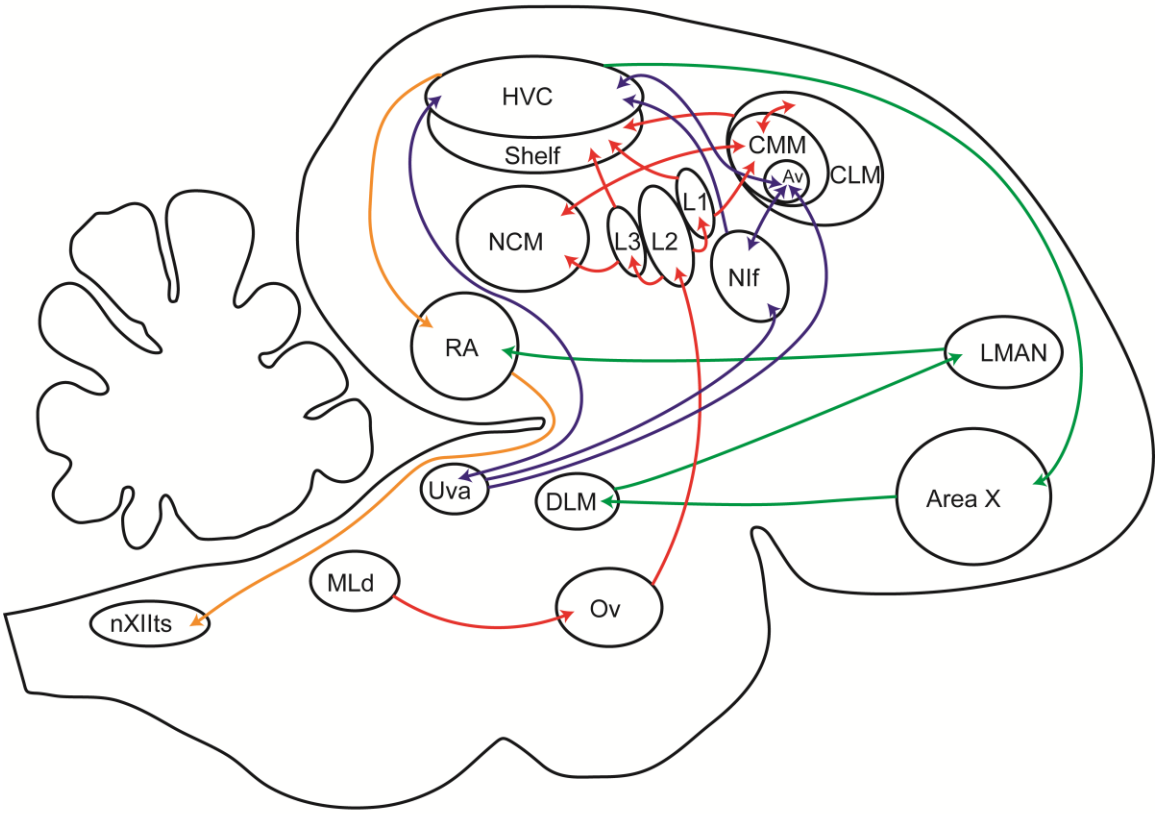

Figure 1. Schematic representation of the anatomy of the auditory and vocal motor structures of the zebra finch brain. Red connections denote the conventional auditory thalamus-driven ascending auditory pathway, blue projections show the ascending auditory and motor feedback pathway, orange connections indicate the song production pathway, and green projections show the corticostriatal anterior forebrain pathway,. Abbreviations: HVC (used as a proper name); Shelf: HVC Shelf; NCM: Caudal Medial Nidopallium; CMM: Caudal Medial Mesopallium; CLM: Caudal Lateral Mesopallium; Av: Avalanche; L1, L2, and L3: Subregions 1, 2, and 3 of the general Field L region; Nif: Nucleus Interfacialis of the Nidopallium; RA: Robust Nucleus of the Archopallium; LMAN: Lateral Magnocellular Nucleus of the Anterior Neostriatum; Area X (used as a proper name); Uva: Uveaformis; DLM: Medial Nucleus of the Dorsomedial Thalamus; Ov: Nucleus Ovoidalis; MLd: Mesencephalicus Lateralis Dorsalis; nXIIts: Tracheosyringeal Motor Nucleus.

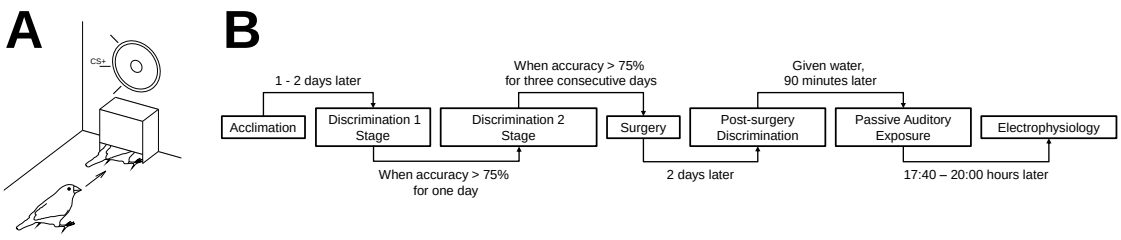

Figure 2. Schematic representation of the **A,** differential classical conditioning apparatus and **B,** the experimental procedure.

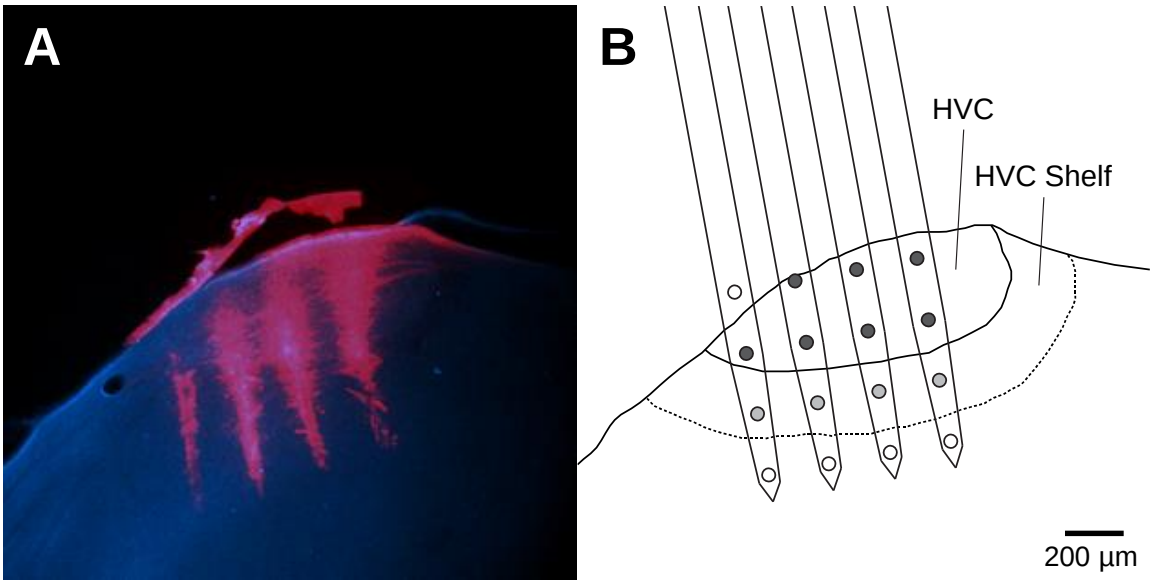

Figure 3. Histological verification of electrode placement. **A,** An example of a superimposed image of pseudo-colored unstained anatomical markers (blue, 345/455 nm excitation/emission filters) and DiI-stained silicon probe track (red, 570/576 nm excitation/emission filters) at 20 X magnification. **B,** The diagram showing the anatomical boundaries and the scaled silicon probe placement for the image in **A.** Recording sites denoted by black and gray are verified to be in HVC and HVC Shelf, respectively. White recording sites are excluded from further analyses.

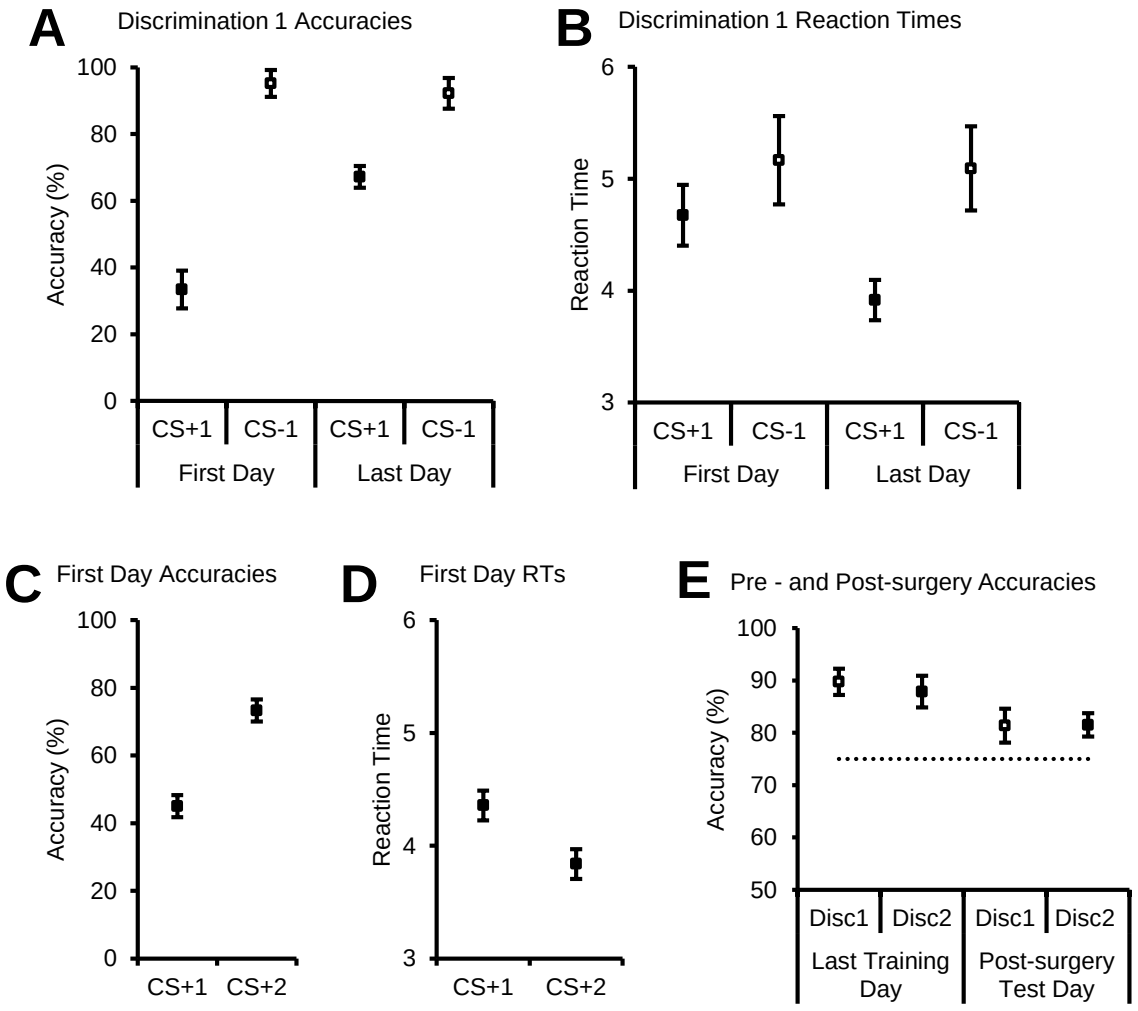

Figure 4. Behavioral performance in the differential classical conditioning task. **A,** Accuracies for CS+1 and CS-1 in the first and the last day of discrimination 1 stage. While CS- accuracies did not significantly change from the First to the Last training day, CS+ accuracies significantly increased ($p < .001$). **B,** Reaction times (RT) for CS+1 and CS-1 in the first and the last day of discrimination 1 stage. CS+ RTs show a strong trend towards decreasing from the First to the Last day of training ($p = .044$, not significant at the Bonferroni-corrected alpha level of .025), whereas no such difference was observed for CS-. **C,** Accuracies for the CS+1 and CS+2 stimuli in their corresponding first days of training. CS+2 stimulus is significantly higher than CS+1 accuracy on its first training day ($p = .005$). **D,** RTs for the CS+1 and CS+2 stimuli in their corresponding first days of training. CS+2 RTs were lower than CS+1 RTs, although this difference was not significant ($p = .097$). **E,** Birds were able to perform over success criterion accuracy after the surgery which was not significantly different from the performance right before the surgery. All boxes and error bars are means and within-subjects SEMs, respectively.

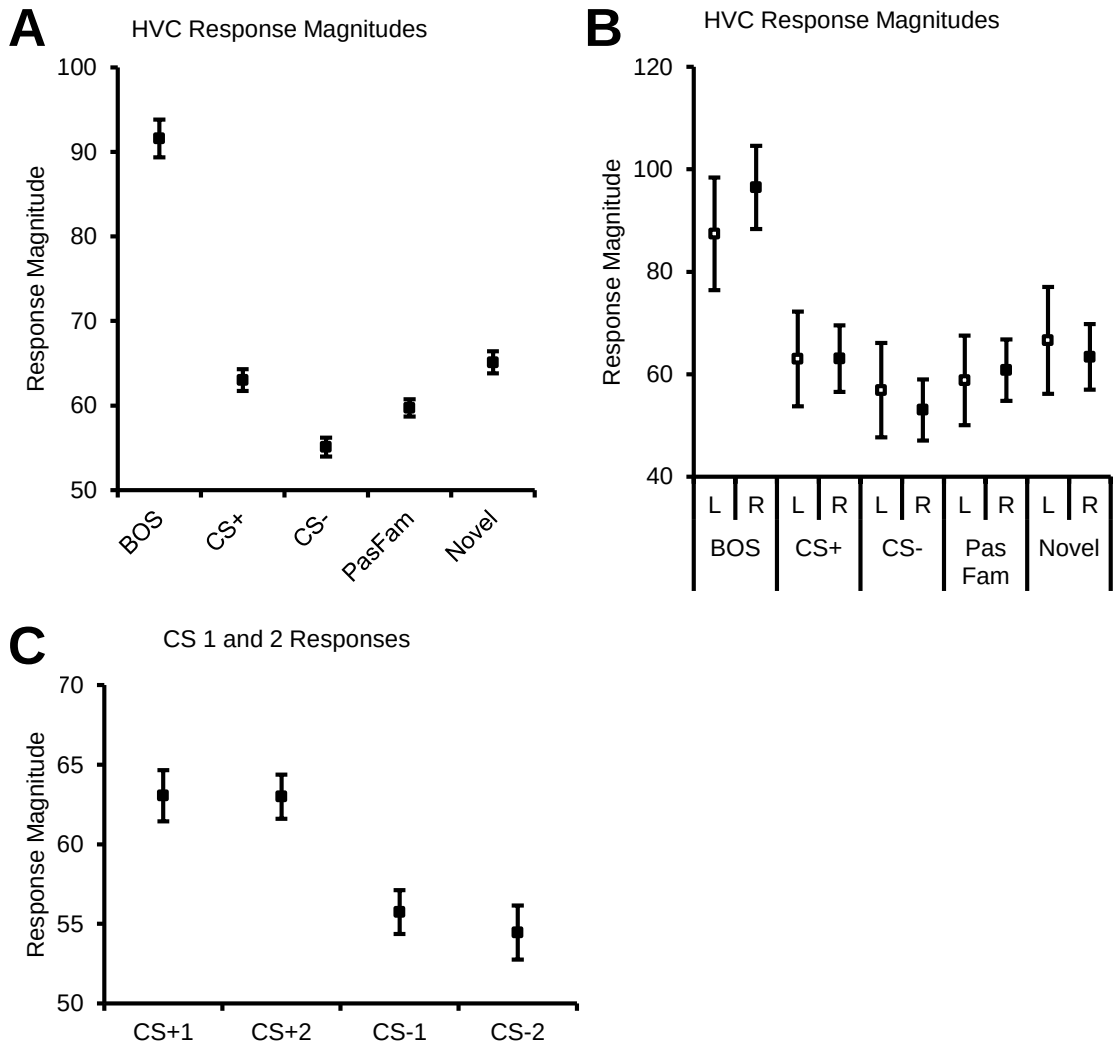

Figure 5. HVC response magnitudes (RM) to all stimulus categories and pairs of CS separately. **A,** BOS RMs are significantly higher than those of all the other stimulus categories (all *p*s < .001). Also, CS- RMs are significantly lower than CS+ ($p = .006$) and Novel ($p < .001$) RMs. **B,** HVC RMs to all stimulus categories for two hemispheres separately. ANOVA revealed a significant interaction between Hemisphere and Stimulus Category ($F(4,328) = 2.577$, $p = .038$), however post-hoc comparisons showed the identical pattern for both hemispheres: BOS RMs were significantly higher than RMs of all the other stimulus categories (all *p*s < .001). **C,** Both CS+1 and CS+2 RMs are significantly greater than both CS-1 and CS-2 RMs (all *p*s < .005), while there is no significant difference between the two CS+ or two CS- stimuli. All boxes and error bars in **A** and **C** are means and within-subjects SEMs, respectively. Error bars in **B** are between-subjects SEMs.

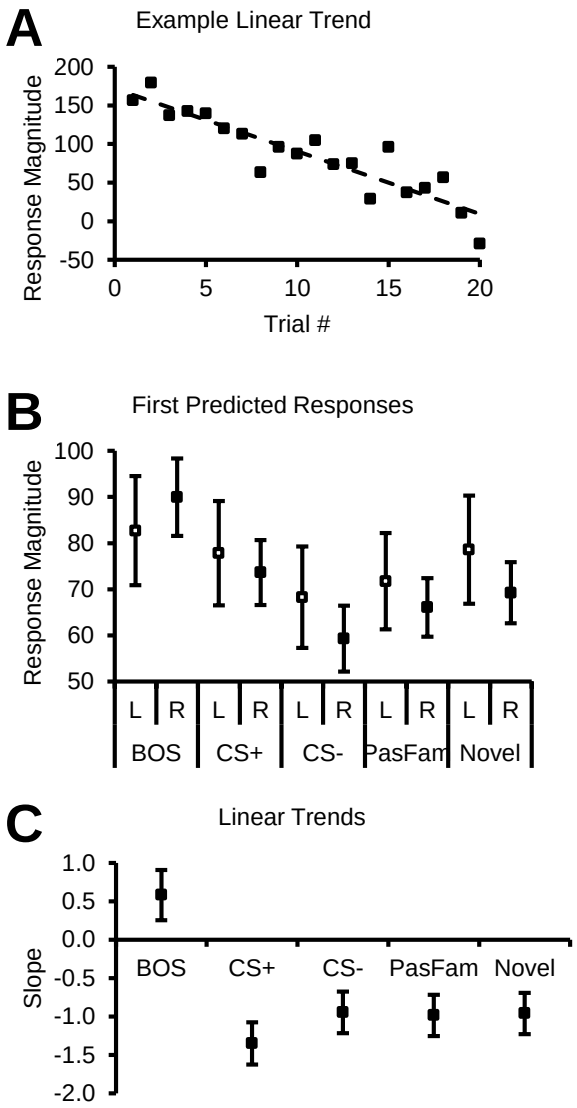

Figure 6. HVC first predicted responses (FPR) and linear trends (Slopes) to all stimulus categories. **A,** An example linear trend of a recording site to one novel stimulus. Black boxes are the response magnitudes to the auditory stimulus at each 20 presentations and the dashed line is the linear fit of the data. The first predicted response from the fitted line and the slope of the line are analyzed further for each stimulus category. **B,** While, in the right hemisphere, BOS FPRs are significantly bigger than those of all other stimulus categories (all *p*s < .002) and CS+ FPRs are significantly greater than CS- FPRs ($p = .014$), in the left hemisphere, the only difference is significantly higher FPRs for BOS than for CS- ($p = .005$). **C,** Separate one sample t-tests are conducted to test whether the slope of each of five stimulus categories was significantly different than 0. While BOS Slopes are not different than 0, all other stimulus categories have slopes significantly lower than 0 (all *p*s < .001). Moreover, BOS slopes are significantly higher than the slopes of all the other stimulus categories (all *p*s < .001). Boxes and error bars in **B** and **C** are means and between-subjects SEMs, respectively.

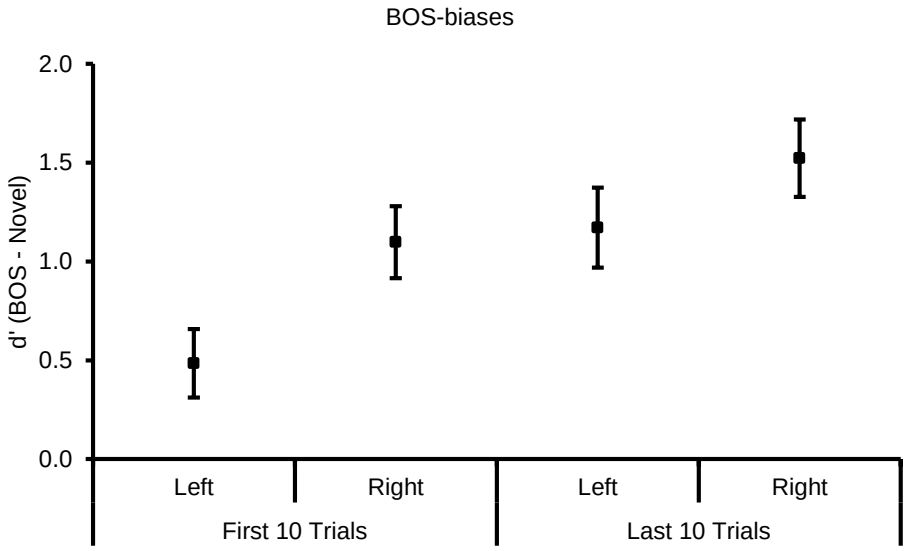

Figure 7. BOS-biases (as measured by *d'* values taking into calculation BOS and Novel responses) in two hemispheres in the first and the second half of the experiment. BOS-biases in Last 10 trials are higher than in First 10 trials (p <.001). Also, an analysis of *d'* values including all trials across the whole experiment, i.e., not separately for first and last 10 trials but all 20 trials, showed that BOS-biases in the right hemisphere are significantly higher than those in the left hemisphere (p = .028). Boxes and error bars are means and between-subjects SEMs, respectively.

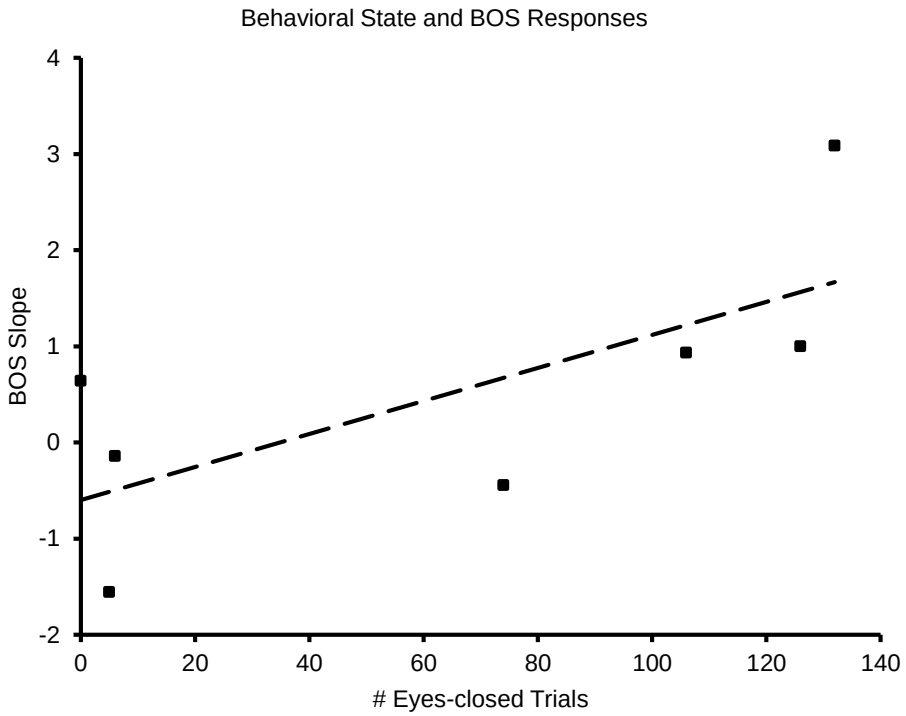

Figure 8. BOS linear trends and behavioral state among birds. Each black box indicates the number of eyes closed trials and the slope of the BOS responses for each experimental bird. Dashed line is the linear fit of the data. The correlation between number of eyes-closed trials and BOS Slopes is moderate, but not significant (r = .703, p = .078).

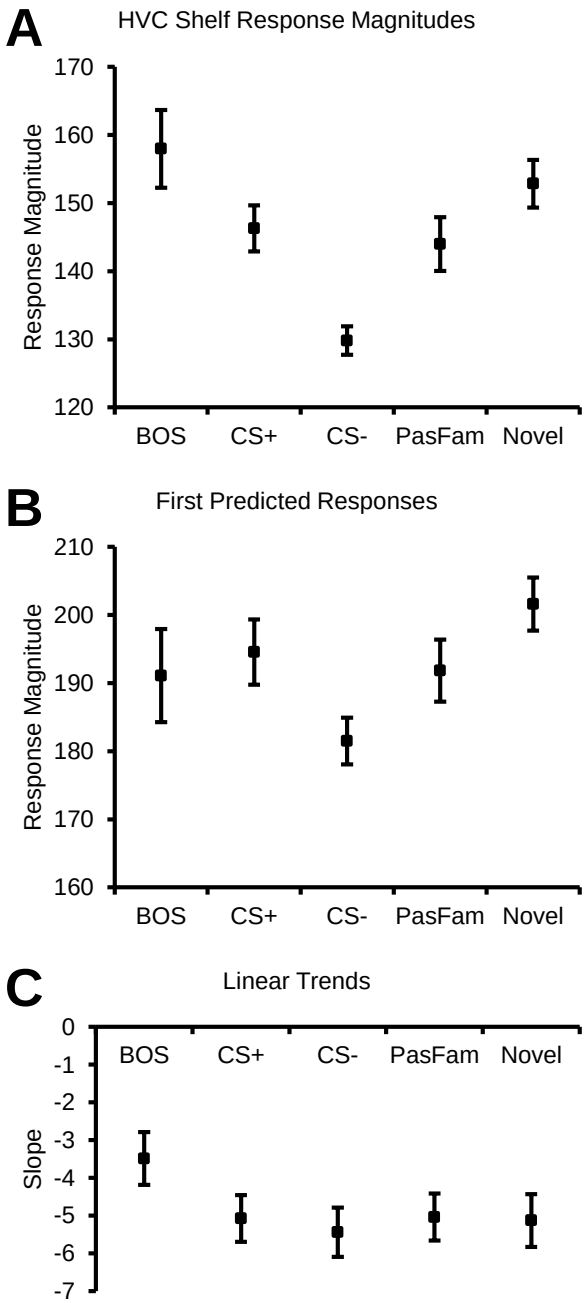

Figure 9. HVC Shelf response magnitudes (RM), first predicted responses (FPR), and linear trends (Slopes). **A,** BOS, CS+ and Novel RM are all significantly greater than CS- RMs (all *p*s < .015). **B,** FPRs for Novel stimuli are significantly higher than for CS- (p = .013) with no other difference among stimulus categories. **C,** Slopes for all five stimulus categories were found to be significantly lower than 0 (all *p*s < .001). Moreover, slopes of BOS stimuli are significantly higher than those of all other stimulus categories separately (all *p*s < .001). All boxes and error bars are means and within-subjects SEMs, respectively.

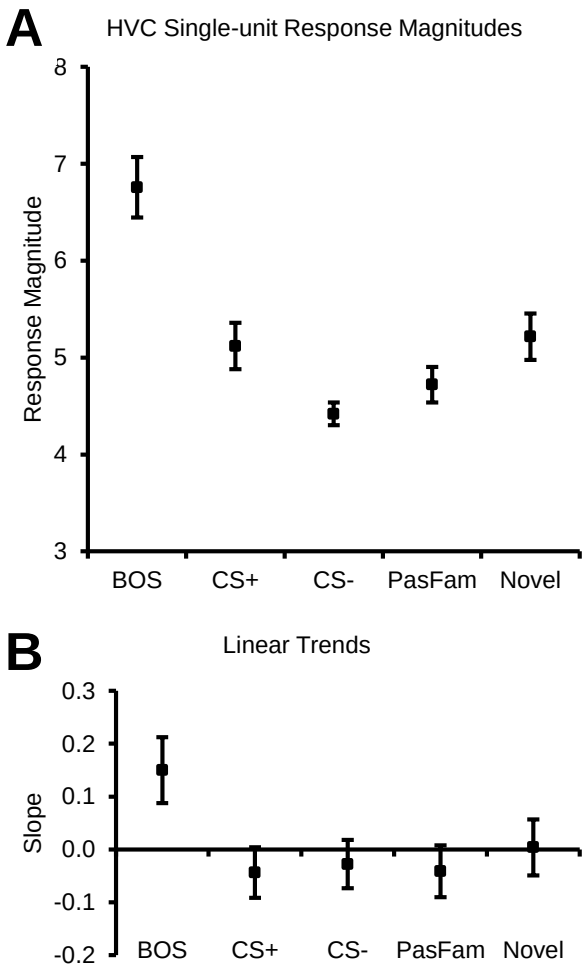

Figure 10. HVC single-unit response magnitudes (RM) and linear trends (slope) for spike-sorted 'excited' single neurons. **A,** Single-unit RMs reveal similar results to those seen in multi-unit responses. RMs for BOS stimuli are significantly higher than for all other stimulus categories (all *p*s < .001). CS+ and Novel RMs are found to be significantly greater than CS- RMs when compared in two separate dependent-samples t-tests ($p = .006$ for CS+; $p = .006$ for Novel). **B,** Contrary to what is found in multi-unit slopes, single-unit slopes do not significantly differ from 0 for any stimulus category. Indeed, there is a strong tendency for BOS slopes to be higher than 0, however this difference did not reach significance with the Bonferroni-corrected alpha level of .01 ($p = .022$). Furthermore, BOS slopes are significantly higher than slopes of all the other stimulus categories separately (all *p*s < .002). All boxes and error bars are means and within-subjects SEMs, respectively.