Title: Song Selectivity in the Pallial-Basal Ganglia Song Circuit of Zebra Finches Raised without Tutor Song Exposure

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ABSTRACT

Acoustic experience critically influences auditory cortical development, as well as emergence of highly selective auditory neurons in the songbird sensorimotor circuit. In adult zebra finches, these ‘song selective’ neurons respond better to the bird’s own song (BOS) than to songs of other conspecifics. Birds learn their songs by memorizing a tutor’s song, and then matching auditory feedback of their voice to the tutor song memory. Song selective neurons in the pallial-basal ganglia circuit called the anterior forebrain pathway (AFP) reflect the development of BOS. However, during learning they also respond strongly to tutor song, and are compromised in their adult selectivity when birds are prevented from matching BOS to tutor, suggesting that selectivity depends on tutor song learning as well as sensorimotor matching of BOS feedback to the tutor song memory. We examined the contribution of sensory learning of tutor song to song selectivity by recording from AFP neurons in birds reared without exposure to adult conspecifics. We found that AFP neurons in these ‘isolate’ birds had highly tuned responses to isolate BOS. The selectivity was as high, and in the striato-pallidal nucleus Area X, even higher than that in normal birds, due to abnormally weak responsiveness to conspecific song. These results demonstrate that sensory learning of tutor song is not necessary for BOS tuning of AFP neurons. Since isolate birds develop their song via sensorimotor learning, our data further illustrate the importance of individual sensorimotor learning for song selectivity and provide insight into possible functions of song selective neurons.

KEYWORDS

LMAN: Area X; isolation; anterior forebrain pathway; sensorimotor learning
INTRODUCTION

In vocal learners, such as humans and songbirds, the development of their complex vocalizations strongly depends on two types of auditory experience in early life: hearing the adults they will imitate and hearing themselves as they practice (Doupe and Kuhl 1999). In songbirds, a young bird first memorizes the song of an adult conspecific (the tutor) during the sensory phase of learning (Fig. 1A). In the subsequent sensorimotor learning phase, the juvenile begins to sing and refines its vocalizations until they resemble the tutor song (Konishi 1965; Price 1979). Since normal song development in this second phase depends on auditory feedback (Konishi 1965) but not on the continued presence of the tutor, the bird is thought to compare its auditory feedback to the tutor song memory (the ‘template’) and to use the results of this evaluation to guide song motor development.

Auditory neurons within the vocal control system of songbirds appear to reflect the process of song learning. Although these neurons exist throughout the circuit (Coleman and Mooney 2004; Doupe and Konishi 1991; Janata and Margoliash 1999; Margoliash 1983; Margoliash and Fortune 1992; Mooney 2000; Nick and Konishi 2005; Vates et al. 1997; Vicario and Yohay 1993), their development has been studied most extensively in the anterior forebrain pathway (AFP, Fig. 1B), a pallial-basal ganglia circuit that is essential for both sensory and sensorimotor learning (Basham et al. 1996; Bottjer et al. 1984; Scharff and Nottebohm 1991; Sohrabji et al. 1990). By the end of the sensory learning phase and partway through the sensorimotor learning phase (post-hatch day 60, PHD 60), auditory neurons in two nuclei of the AFP, Area X and the lateral portion of the magnocellular nucleus of the anterior nidopallium (LMAN), have developed selective responsiveness for the bird’s own song (BOS) relative to songs of other zebra finches (conspecifics) and temporally altered versions of BOS, even when BOS has been made abnormal by manipulation of the peripheral motor system (Doupe 1997; Solis and Doupe 1997, 1999; Yazaki-Sugiyama and Mooney 2004). This ‘song selectivity’ suggests that these neurons are shaped by the bird hearing its own song during sensorimotor learning.

However, song selectivity appears to be influenced by more than just the sound of the bird’s song. For one, when sensorimotor matching of BOS to tutor song is chronically disrupted by denervation of the vocal organ (syrinx), BOS selectivity is compromised (Solis and Doupe 2000): neurons in Area X of such birds have a lower degree of selectivity
for BOS than do normal adults, and LMAN neurons with auditory responses of any kind are found with a much lower frequency than in normal adults. In contrast, neurons in adult birds that successfully mimic tutor song after presumably reinnervating the syrinx, exhibit normal adult selectivity for BOS. Thus, during sensorimotor learning, the development of selectivity for complex stimuli such as BOS may be influenced by how well the bird can match its motor output to the internal tutor song model.

In addition, by PHD 60, many of the highly BOS selective neurons in Area X and LMAN also respond strongly to the tutor song (Doupe 1997; Solis and Doupe 1997, 1999; Yazaki-Sugiyama and Mooney 2004), even when it differs markedly from BOS (Solis and Doupe, 1999). This raises the possibility that, in addition to depending on successful sensorimotor learning, the development of BOS selectivity requires the sensory learning of tutor song template.

Therefore, to test whether song selectivity obligatorily depends on sensory learning of tutor song, we examined the auditory selectivity of AFP neurons in zebra finches raised in isolation from adult individuals, so that they had not experienced normal tutoring. Although isolate birds do not learn songs of conspecific individuals, their song has many conspecific features, which require hearing in order to emerge (Eales 1987, 1985; Konishi 1965; Marler and Tamura 1964; Morrison and Nottebohm 1993; Price 1979; Scharff and Nottebohm 1991). Thus, isolate birds are thought to develop their song in a sensorimotor learning process, matching their vocalizations to an innate, species-specific template (Adret 2004; Konishi 1965; Marler 1997).

We report here that AFP neurons in birds raised without experience of adult conspecific song develop BOS selectivity comparable to, and in Area X, even greater than that in normally-reared birds. These results indicate that song selectivity can develop in the AFP in the absence of sensory learning of tutor song, and with sensorimotor matching likely limited to innate information about song, rather than to a learned template. Together with the existing evidence on how tightly song selectivity follows the motor development of BOS (Doupe 1997; Nick and Konishi 2005; Roy and Mooney 2003; Solis and Doupe 1997, 1999; Yazaki-Sugiyama and Mooney 2004), our data provide further support for the critical importance of song sensorimotor learning to the properties of song selective neurons.
MATERIALS AND METHODS

Animals and Isolation protocols

Experiments used adult male zebra finches (*Taeniopygia guttata*). The care and treatment of experimental animals was reviewed and approved by the animal care and use committee at the University of California, San Francisco (UCSF). Birds to be isolated were initially raised in sound-attenuating chambers with their parents, and siblings from the same clutch, until PHD 9-11. After this day the male parent was removed. At PHD ~30, the young birds were removed from the female parent and the siblings, and housed alone in individual sound-attenuating chambers. Normal control birds were raised in individual cages in the colony, with their parents and siblings; opaque dividers between cages visually isolated control birds from other conspecifics, which allows them rich song exposure but limits their song learning to the visible tutor (Eales 1989; Price 1979). Control birds were removed from their parents at PHD ~60, and housed in cages with their brothers and/or other conspecifics.

Electrophysiology

Extracellular signals of single LMAN and Area X neurons during song playback were recorded as described by Solis and Doupe (1999), in isolate birds at 216-774 days of age and in normal control birds at 151-427 days of age. Two to seven days prior to the experiment, birds were prepared for neurophysiological recording by affixing a head post to the skull under Equithesin anesthesia (Solis and Doupe 1999) and marking the location of the song nuclei on the skull (see Solis and Doupe, 1997, for details), or creating a craniotomy that was sealed with bone wax. On the day of the experiment, they were anesthetized with a 20 % solution of urethane (60-85 µl, i.m.; Sigma, St. Louis, MO) or sedated with 0.5 % (5 mg/ml) diazepam (10-30 µl, i.m.; Abott Laboratories, North Chicago, IL), secured in the stereotaxic apparatus by the head post, and placed in a double-walled anechoic sound-attenuating chamber. Body temperature was maintained at ~40°C with a temperature controller. Extracellular activity recorded with tungsten electrodes (1-5 MΩ) was amplified and filtered between 300 Hz and 5 kHz (A-M Systems, Everett, WA). Some neurons with high signal-to-noise ratios (approx. >10) without any background units were isolated with an online window discriminator (FHC Inc., Bowdoinham, ME). For
most sites, 1 or at most 2 neurons were sorted offline from a waveform with high
signal-to-noise, containing a small number of units, using spike-sorting software
developed by Michael Lewicki (Caltech) and Brian Wright (UCSF). Briefly, spike
models were constructed from spike waveforms recorded during and before the stimulus
presentation, and spikes were then classified using the spike models with a
template-matching algorithm based on Bayesian probability theory (Lewicki 1994). All
neurons were judged to be single units based on inspection of the waveforms and by
analysis of interspike interval (ISI) violations: any sites where occurrence of ISIs < 0.7
msec constituted more than 1.0% of total ISI occurrences were discarded.

Electrolytic lesions were made at selected locations for reconstructing recording
sites. At the end of each experiment, the bird was deeply anesthetized with Metofane
(Pitman-Moore, Mundelein, IL) or Isoflurane (Abott Laboratories, North Chicago, IL) and
transcardially perfused with 0.9% saline, followed by 3.7% formaldehyde in 0.025 M
phosphate buffer. Brains were postfixed, and 40 µm sections were cut with a freezing
microtome. Sections were stained with cresyl violet, and electrode tracks and lesions
were identified. Neural recording sites where we did not make any lesions were identified
in reference to the lesion sites on the same electrode tracks. Only neurons histologically
confirmed in this manner to be in LMAN or Area X were analyzed.

**Stimuli**

The songs of the isolate birds and of the normal control birds were recorded as
described by Solis and Doupe (1999), at 2-9 days before the neurophysiological recordings.
Prior to the final song recording, isolate songs were recorded 2-3 times at intervals of 1
week to 4 months to make sure that the songs were crystallized. Once songs were
crystallized, a song of the type that was most frequently produced was chosen by visual
inspection of the spectrograms and by listening to the songs. Most bird’s own song (BOS)
stimuli had several introductory notes followed by two song motifs as shown in Fig. 2.
During neural recordings, stimuli were presented from a speaker positioned 20 cm from the
bird. Search stimuli included BOS and the father’s song at different peak volumes (75, 65,
55, 45 dB), and broad-band noise bursts (65 dB at peak volume, 500 msec or 1 sec
duration). At most sites, the stimulus intensity at which BOS or the father’s song evoked
the strongest responses was determined by examining average response amplitude in peristimulus time histograms (PSTH) of spike rasters displayed on line, and song selectivity was then examined by playing all the stimuli at that intensity (usually 65-75 dB). Stimulus types included two to four different songs of normal zebra finches (conspecifics), songs of other isolate birds (isolated conspecifics), at least one song of Bengalese finches and/or white-crowned sparrows (heterospecific songs), BOS, and temporally reversed version of BOS (reverse BOS). Stimuli were presented in a random, interleaved fashion. Intertrial interval was randomly varied within a range of 5-12 sec. An effort was made to present each neuron with 15-30 trials of each stimulus type.

Data analysis

We analyzed only neurons that had an average firing rate during at least one of the auditory stimuli that was significantly different from the background rate (two-tailed paired t test with Bonferroni correction, \( p < 0.01 \)). The response strength (RS) of a neuron to a stimulus was calculated as the difference between the firing rate during the stimulus and the background rate. The period of stimulus presentation was offset by estimates of the latency determined in prior studies, 35 msec for Area X and 50 msec for LMAN. The background firing rate was defined as the mean firing rate of a 2 sec period preceding stimulus presentation. For each neuron, the RS to a stimulus was measured for each trial and then averaged across trials to obtain a mean RS. For presentation of conspecific songs, heterospecific songs, and songs of isolated conspecifics, data from different stimuli but of the same stimulus type were also averaged to obtain the mean RS of a neuron to a particular stimulus type.

The selectivity of an individual neuron for one song stimulus (A) over another song stimulus (B) was quantified using the \( d'_{A-B} \) measure (Green and Swets 1966), as described by Solis and Doupe (1997), where:

\[
d'_{A-B} = \frac{2(RS_A - RS_B)}{\sqrt{\sigma^2_A + \sigma^2_B}}.
\]

In this equation, \( \overline{RS_A} \) and \( \overline{RS_B} \) are the mean RS to stimulus A and B, respectively, and \( \sigma^2 \) is the variance of each RS. If \( d'_{A-B} \) is positive, then stimulus A elicited a greater response than stimulus B. A neuron was considered selective for stimulus A over stimulus
B if it had a $d'_{A-B}$ value $\geq 0.5$.

We also used an additional measure, the selectivity index (SI) (Volman, 1996; Doupe, 1997; Solis and Doupe, 1997), which focuses less on absolute discriminability and more on the relative difference in the strength of the average responses between two stimuli. The SI quantifies responsiveness to one stimulus relative to the other by comparing the mean RS to each stimulus in ratio form (and thus is normalized by response magnitude):

$$SI = \frac{RS_A}{RS_A + RS_B}.$$  

SI will approach 1.0 if a response to stimulus A is much stronger than a response to stimulus B and will be close to 0.5 if the two responses are comparable. When a particular $RS_B$ had a negative value, it was considered to be zero for calculating SI, and thus the SI would be one regardless of $RS_A$.

For the neural data of normal control birds, we had data that were newly collected for the current study (55 Area X neurons from 10 birds and 15 LMAN neurons from 4 birds), as well as data originally collected for a previous paper (Solis and Doupe 2000; 34 Area X neurons from 13 birds and 14 LMAN neurons from 6 birds). Although Solis and Doupe (2000) did not determine optimal amplitude for song playback and only used a fixed volume for each stimulus ranging from 64 to 73 dB, we found that for most neurons their amplitude range was in the saturated range of the response-stimulus amplitude curve that we determined in this study. Moreover, neither spontaneous firing rate, auditory responsiveness, nor BOS selectivity were significantly different between the two groups of data (unpaired $t$ tests, $p > 0.05$): for Area X neurons, spontaneous firing rates (Hz) of the current and previous study were 44.7 ± 4.1 (mean ± SEM) and 39.5 ± 4.8, respectively; RS to BOS (spikes/sec) were 11.3 ± 1.0 and 8.5 ± 1.2; $d'_{BOS-CON}$ was 1.83 ± 0.77 and 1.55 ± 1.73; for LMAN neurons, spontaneous firing rates (Hz) were 2.5 ± 0.8 and 2.1 ± 0.5; RS to BOS (spikes/sec) were 4.7 ± 1.0 and 2.8 ± 0.8; $d'_{BOS-CON}$ was 2.11 ± 0.25 and 1.80 ± 0.17. Given this equivalence, we combined these data for analysis in the present study.

In the great majority of cells recorded from both isolate and normal birds (isolates: 72/79 neurons; normal birds: 112/117 neurons), auditory responses were recorded under urethane anesthesia; the other 7 isolate and 5 normal bird neurons were recorded under diazepam sedation. We included both urethane and diazepam data in the analyses,
because similar properties of auditory responses in the two drug conditions were reported for recordings in the upstream nucleus HVC (Cardin and Schmidt 2004, 2003; Lewicki and Arthur 1996; Margoliash and Fortune 1992; Mooney 2000; Theunissen and Doupe 1998; Volman 1996), and also because we observed no significant difference in auditory responses or song selectivity between the two agents in our preliminary experiments in Area X of normal birds (3 birds, 32 sites with urethane, 33 sites with diazepam, data not shown).

**Song analysis**

To quantify the difference in temporal structure of isolate song from that of normal song, we compared the number of syllables in song motifs and the syllable durations. In most birds (6 of 10 isolate birds; 7 of 10 normal control birds), syllable boundaries were defined by inspection of song oscillograms as points where the song envelope fell to background level. For the songs of the other 7 birds (4 isolate and 3 normal control birds), we developed a more automated and quantitative approach to segmentation: syllable/note boundaries were defined as points where the song’s envelope crossed a threshold, which was set as five to ten times the RMS amplitude of the background noise. Then, silent intervals shorter than 2 msec were discarded and the notes before and after the short intervals were combined into one syllable. For straightforward songs with clearly defined syllables, this method resulted in the same segmentation as the visual inspection. We measured the syllable durations in the first motif of the BOS stimuli that were used in the extracellular recordings in the current study. The number of syllables in a motif was measured for each song and the mean and variance of syllable number across all the different songs in a group (normal or isolate) were calculated.
RESULTS

_Songs of isolate birds_

Consistent with previous reports (Aamodt et al. 1995; Livingston et al. 2000; Morrison and Nottebohm 1993; Price 1979; Volman and Khanna 1995), all the male zebra finches ($n = 10$ birds) isolated from conspecific adults by 9-11 days of age developed stereotyped songs composed of several repetitions of an introductory note followed by one or more renditions of a song motif (Fig. 2A and B). The songs had abnormal acoustic features including broadband noise notes, upward sweeps, an abundance of call-like notes (harmonic stacks), abnormally long and short syllables, and many relatively soft syllables with short neighboring intervals and poorly defined syllable boundaries (asterisks in Fig. 2B). Using our segmentation of the songs (see Materials and Methods), the distributions of syllable durations of all the isolate songs that were used in the extracellular recordings had significantly greater variance than that of normal birds’ songs (Fig. 2C and Table 1; $F$-test for equality of variance, $p < 0.005$). The higher variance of syllable duration in isolate birds is consistent with a previous report by Price (1979). Also, the number of syllables in a motif was larger in isolate songs than in normal birds’ songs. Visual inspection of the spectrograms of the songs showed no sharing of song notes between the songs of isolate birds and of their fathers, except for the generic call-like notes. This contrasted with the songs of normally-raised birds, which usually contained many song notes copied from the father’s song (Fig. 2A, pupil). Thus, our isolate birds appeared to have no evidence for an influence of father’s song on their song development.

_Song selectivity of Area X neurons in isolate birds_

Extracellular recording of single Area X neurons from isolate birds revealed that these cells had a strong preference for BOS over other song stimuli. We recorded 46 Area X neurons from 10 isolate birds that responded significantly to at least one of the stimuli we played to the birds. One Area X neuron that had a significant inhibitory response to BOS and no significant responses to any other stimuli was excluded from the analyses.

Figure 3 shows a typical neuron with relatively high spontaneous firing, which responded substantially more to BOS than to the father’s song or to a song of an isolate conspecific (a sibling of the experimental bird). On average, the mean response strength
(RS; difference between the firing rate during the stimulus and the background rate) of all the auditory neurons in Area X was significantly greater to BOS than to the other stimulus types we played: father’s song, reversed version of BOS (reverse BOS), songs of isolated conspecifics (isolate CON), songs of normally-reared conspecifics (normal CON), and heterospecific song (HET) (Fig. 4A, one-way ANOVA and Tukey-Kramer HSD test, \( p < 0.05 \)). The song selectivity of individual Area X neurons is illustrated with a scatter plot in Fig. 4B, where the mean RS to BOS of each cell is plotted against its mean RS to isolate CON. All the points lie above the diagonal line, indicating stronger responses to BOS than to isolate CON; the difference between BOS and isolate CON responses was significant in the majority of individual neurons (30/42, filled circles, paired \( t \) tests with Bonferroni correction, \( \alpha \)-value for significance = 0.01). We also quantified the preferences of individual neurons with a measure of discriminability, \( d' \) (see Materials and Methods). Neurons were considered to prefer stimulus A over stimulus B if they had \( d'_{A-B} \geq 0.5 \). The \( d'_{BOS-father} \), \( d'_{BOS-reverse BOS} \), and \( d'_{BOS-normal CON} \) of all the Area X neurons recorded are shown in Figure 4C. For all the stimulus types that we compared to BOS, \( d' \) values were \( \geq 0.5 \) in most Area X neurons (39/43 cells for \( d'_{BOS-father} \); 42/45 cells for \( d'_{BOS-reverse BOS} \); 38/42 cells for \( d'_{BOS-isolate CON} \); 44/46 cells for \( d'_{BOS-normal CON} \); 42/45 cells for \( d'_{BOS-HET} \)). These results indicate strong preferences for BOS over the other stimulus types in Area X neurons of isolate birds.

Although isolate birds’ song did not share notes with their father’s song (Fig. 2), this does not rule out the possibility that the isolate birds may have a memory of the father’s song that influences the tuning of AFP neurons, given the fact that these birds were exposed to their father until 10 days after hatching. However, Area X neurons in isolate birds were showed no selective for their father’s song. The group mean RS to father’s song was not significantly different from that to other song stimuli except BOS, and most (39/42) neurons showed no significant difference in RS between father’s song and isolate CON (Fig. 4D). The highly similar distributions of \( d'_{BOS-father} \) and of \( d'_{BOS-reverse BOS} \) and \( d'_{BOS-isolate CON} \) in Figure 4C also indicate little selectivity for father’s song. Thus, Area X neurons in isolate birds had strong preferences for BOS and virtually absent preferences for father’s song or other song stimuli, corresponding to their highly limited auditory experience.
Area X is known to have heterogeneous cell populations, containing low-firing rate striatal neurons (spiny neurons), high-firing rate pallidal-like neurons (aspiny fast-firing neurons), presumptive cholinergic neurons, and other small interneurons (Farries and Perkel 2002). Our Area X neurons had spontaneous firing rates in a wide range (3.8-103.3 Hz) and can be divided into two major classes based on spontaneous activity (Fig. 4E). Nonetheless, BOS evoked stronger responses than father’s song and isolate CON over the entire range of firing rates (Fig. 4F), suggesting that the different firing rate classes show no obvious difference in song selectivity.

**Song selectivity of LMAN neurons in isolate birds**

As in Area X, LMAN neurons of isolate birds \((n = 29\) neurons in 6 birds) had high selectivity for BOS over the other stimulus types (Fig. 5A-C). Auditory neurons in LMAN had relatively low spontaneous firing rates \((2.3 \pm 0.4\) Hz, range 0.1-10.7 Hz; see Fig. 5A), and showed significantly greater mean RS to BOS than to other stimuli (Fig. 5D, one-way ANOVA and Tukey-Kramer HSD test, \(p < 0.05\)). All the individual neurons had larger mean RS to BOS than to isolate CON (Fig. 5E), and the difference between those responses was significant in the majority of neurons \((20/26\) cells, filled circles, paired \(t\) tests with Bonferroni correction). Many LMAN neurons also showed \(d'\) values \(\geq 0.5\) for BOS over the other stimulus types \((25/28\) cells for \(d'_{\text{BOS-father}}\); 27/29 cells for \(d'_{\text{BOS-reverse BOS}}\); 24/26 cells for \(d'_{\text{BOS-isolate CON}}\); 28/29 cells for \(d'_{\text{BOS-normal CON}}\); 25/29 cells for \(d'_{\text{BOS-HET}}\), Fig. 5F).

As with Area X neurons, LMAN neurons in isolate birds were not selective for their father’s song. The group mean RS to father’s song was not significantly different from that of isolate CON (Fig. 5D), and this was also true for the RS values of individual neurons in all but one neuron (Fig. 5G). The very similar distributions of \(d'_{\text{BOS-father}}\) and of \(d'_{\text{BOS-reverse BOS}}\) and \(d'_{\text{BOS-CON}}\) in Figure 5F likewise indicate little selectivity for father’s song. Thus, both LMAN neurons and Area X neurons of isolate birds are strongly and exclusively selective for BOS.

**Comparison of isolate song selectivity to that of normal birds**

In normal adult birds, neurons in both Area X and LMAN have high selectivity for
BOS relative to conspecific and heterospecific songs. Area X neurons differ slightly from those in LMAN, however: they tend to have significant responses to a variety of conspecific songs in addition to BOS (albeit lower than to BOS), and are less likely than LMAN neurons to be significantly inhibited by non-BOS songs (Doupe 1997). In contrast to normal birds, Area X neurons in adult birds raised with a deficit in sensorimotor learning created by denervating the syrinx have abnormally low BOS selectivity, due to unusually low BOS responses along with higher than usual responses to conspecific and reversed songs (Solis and Doupe 2000); LMAN in these birds is compromised as well, with markedly low auditory responsiveness to any stimuli. Consistent with the idea that disruption of sensory learning due to a lack of tutor song exposure might also affect song selectivity, a previous preliminary report had suggested that AFP neurons in isolate birds were not highly song-selective (Maekawa 1998). However, we found that AFP neurons in isolate birds had BOS selectivity as high as, and in the case of Area X, even higher than that in normal birds.

Figure 6 illustrates this result with a comparison of responsiveness and selectivity of Area X neurons between isolate and normal birds (89 neurons from 23 birds; combined data from Solis and Doupe 2000 and the present study; see Methods for comparison of the two groups). Auditory responses to BOS in isolate birds were as strong as in normal birds (Fig. 6A; two-way ANOVA, \( p < 0.0001 \); unpaired \( t \) test with Bonferroni correction, \( \alpha \)-value for significance = 0.013, \( p = 0.16 \)). Area X neurons in isolate birds, however, had virtually no responses to reverse BOS, normal CON, and HET, and therefore these responses were much lower than those in normal birds (Fig. 6A, \( p < 0.001 \) for reverse BOS; \( p < 0.0001 \) for normal CON; \( p < 0.01 \) for HET). This pattern of lower responses to non-BOS stimuli but not to BOS in isolate birds was observed over the entire range of spontaneous firing rates in the Area X neurons (Fig. 6B, C), indicating that the different responsiveness to non-BOS stimuli between isolate and normal birds is not due to different fractions of cell types collected between the two groups.

Along with the lower responses to non-BOS songs, we found higher selectivity for BOS relative to other songs in isolate Area X neurons (Fig. 6D): the mean \( d'_{\text{BOS-reverse BOS}} \) was significantly greater in isolate birds than in normal birds (two-way ANOVA, \( p < 0.0001 \); unpaired \( t \) test with Bonferroni correction, \( \alpha \)-value for significance = 0.017, \( p < 0.0001 \).
0.012) and the mean $d'_{\text{BOS-normal CON}}$ and the mean $d'_{\text{BOS-HET}}$ had the same trend, although the differences were not significant ($p = 0.021$ and 0.13, respectively). When we quantified the selectivity using a measure that quantifies average responsiveness to one stimulus relative to the other (see Methods), the selectivity index (SI), the difference between isolate birds and normal birds was even more evident (Fig. 6E): the mean SI$_{\text{BOS-reverse BOS}}$ and SI$_{\text{BOS-CON}}$ were significantly greater in isolate birds than in normal birds (two-way ANOVA, $p < 0.0001$; unpaired $t$ test with Bonferroni correction, $\alpha$-value for significance $= 0.017$, $p < 0.01$ for SI$_{\text{BOS-reverse BOS}}$, $p < 0.0001$ for SI$_{\text{BOS-CON}}$); the mean SI$_{\text{BOS-HET}}$ revealed the same trend but was not significantly different between the two groups ($p = 0.018$). Together, these results demonstrate that song selectivity in Area X neurons does not require sensory learning of tutor song, and may also reflect birds’ experience of songs other than BOS and tutor song, with normally reared birds showing a broader range of responsiveness to non-BOS stimuli.

LMAN neurons in isolate birds, like those in isolate Area X, had responses to BOS similar in strength to those of normal birds (29 neurons from 10 birds; Fig. 7A, B). However, in contrast to Area X, responses to the other songs in isolate LMAN neurons were not lower than that in normal birds: normal LMAN neurons were already sharply tuned to BOS with virtually no excitatory responses to non-BOS stimuli, and thus were similar to isolate LMAN neurons in this respect (Fig. 7A, C). Because of this response property in LMAN of normal birds, BOS selectivity relative to the other song types was not higher in isolate birds than in normal birds: the mean $d'$ or SI values for BOS relative to the other song stimuli (reverse BOS, normal CON, or HET) in LMAN were not significantly different between the two groups of birds (Fig. 7D, E). Thus, deprivation of conspecific song experience had little effect on song selectivity in LMAN neurons, which contrasts with higher selectivity in Area X neurons.

In LMAN of adult birds that were raised with a denervated syrinx and thus did not successfully mimic their tutor song, auditory neurons were encountered at a much lower frequency than in normal birds (Solis and Doupe 2000). In isolate birds, however, auditory responsiveness of LMAN neurons was not compromised, and was as high as that of normal birds: of the 9 isolate birds in which LMAN was sampled, auditory responses were obtained from 6 birds (67.0%), comparable to 4/8 normal birds (50.0%; here we used
only data collected for the current study). Of the 52 LMAN neurons recorded in the isolate birds, 29 neurons yielded auditory responses (55.8%). This frequency at which we encountered auditory neurons in isolate birds was not significantly different from that in normal birds, where 15/25 neurons showed auditory responses (60.0%, $\chi^2$ test, $p = 0.81$). Together with the selectivity data, these results support the idea that normal auditory selectivity of LMAN neurons can develop without sensory learning of tutor song.
DISCUSSION
This study demonstrates that sensory learning of tutor song is not obligatory for the emergence of song selectivity in AFP neurons. In birds raised in isolation from adult conspecific song, AFP neurons developed selectivity for BOS relative to other song stimuli as high as that in normal birds, and in Area X, higher than in normal birds, due to normal BOS responses and abnormally weak responses to non-BOS stimuli. Together with the existing evidence for the dependence of song neural selectivity on song sensorimotor learning, this result stresses the critical role of sensorimotor learning of BOS in shaping song selective neurons. Moreover, our data suggest that a lack of conspecific experience can even sharpen BOS selectivity.

High BOS selectivity of AFP neurons in isolate birds, and possible functions of AFP song selectivity

A variety of evidence suggests that AFP song selective neurons can independently develop sensitivity to both BOS and tutor song, at least transiently. At PHD 60, AFP neurons show stronger and more temporally sensitive responses to both these stimuli than to other conspecific songs (Solis and Doupe 1997; Yazaki-Sugiyama and Mooney 2004), even when BOS has been manipulated to be very different from the tutor song (Solis and Doupe 1999). An obligatory requirement for tutor exposure and for subsequent matching of BOS to the sensory memory of tutor song was further suggested by the impaired song selectivity evident both in preliminary experiments in birds raised in isolation (Maekawa 1998) and in birds chronically prevented from matching their song to the tutor song (Solis and Doupe 2000). Our results clearly demonstrate, however, that, despite the presumed lack of an acquired song template in isolate birds, maximal development of BOS selectivity does not require sensory learning of tutor song. The findings here provide further evidence that BOS selectivity is strongly shaped by the bird’s experience of its own song during learning, and support the idea that song-selective neurons represent an auditory version of the bird’s current song (Margoliash 1983; Nick and Konishi 2005; Solis and Doupe 1997, 1999; Volman 1993; Yazaki-Sugiyama and Mooney 2004).

Our results also speak to several further functions that have been suggested for song selective neurons. For one, the dual tuning to BOS and tutor song at PHD 60 and the
compromised responses of AFP neurons in adult birds raised with a denervated syrinx (Solis and Doupe 2000) raised the possibility that AFP neurons are important in the comparison of BOS to the tutor template: that is, they might develop strong BOS-selective responses only when auditory feedback of the bird’s own vocalizations is close enough to the tutor song target to evoke activation of both BOS- and tutor-tuned inputs. In this respect, AFP song selective neurons may be comparable to their counterparts in mammalian basal ganglia-cortical circuits, which have been shown to acquire responses predictive of or dependent on reward (for recent reviews see (Hikosaka et al. 2006; Salzman et al. 2005; Schultz et al. 2003). Isolate birds are also thought to match their vocalizations to a template, one that is innate and species-specific (Adret 2004; Konishi 1965; Marler 1997). Thus, although isolate birds do not learn any songs during the sensory phase, they may nonetheless experience successful matching to a song template during sensorimotor learning, which could result in high BOS selectivity. To be certain of this, however, it would be necessary to know more about the nature of the innate template.

A second possible function of song selective neurons is that they represent a neural mapping between auditory feedback and vocal motor output, which may be necessary for song learning. This has been suggested by accumulating evidence that song selectivity is not a purely sensory property, but is found of necessity in neurons with both sensory and motor-related responses. A specific relationship between sensory and motor responses that may reflect such a sensorimotor mapping has been described in the premotor song nucleus RA (the robust nucleus of the arcopallium), where playback of a set of syllables evokes a song-selective firing pattern that resembles the premotor activity for the next syllable in the song (Dave and Margoliash 2000). The AFP, like mammalian cortical-basal ganglia circuitry (Graybiel et al. 1994; Hikosaka et al. 2000), has both auditory responses and premotor activity (Hessler and Doupe 1999a, 1999b; Leonardo 2004), and thus could also be involved in developing a sensorimotor map during learning. Syringeally-denervated birds may have impaired AFP song selectivity because they cannot create a reliable relationship between motor commands and auditory feedback. In contrast, isolate birds are able both to control and to hear their vocalizations as normal birds do, so that they should have normal sensorimotor mapping, which may explain their strong BOS selectivity.
Higher BOS selectivity of AFP neurons in isolate birds and effects of sensory experience

Not only was BOS selectivity in isolate AFP neurons not compromised by tutor song deprivation, it was in fact higher than in normal birds, in Area X, which includes both striatal and pallidal neurons. This increased selectivity was the result of weaker responses to non-BOS song stimuli in isolate birds compared to controls, while BOS responses did not differ between the two groups. This could reflect the unusual and variable structure of isolate songs, which may result in lower overall acoustic similarity both between isolate and normal songs and among isolate birds than among normal songs. Such lower acoustic similarity may have weakened conspecific responses of Area X neurons tuned to isolate BOS, and thus increased neural BOS selectivity relative to conspecific songs.

Alternatively, the increased selectivity of isolate Area X neurons may reflect their different, highly impoverished experience of conspecific song. Isolate birds were raised in sound-attenuating chambers without hearing any other adult zebra finch songs, whereas normal birds were raised in a colony where they could hear their siblings and other conspecifics. Thus, the isolate birds’ sensory experience of song was narrowly focused on BOS, and this may have decreased their responses to non-BOS song stimuli. If so, it suggests that the responsiveness of song neurons in normal birds includes a component shaped by the sounds of other birds in addition to BOS and tutor song. Examination of song selectivity in birds exposed to a tutor (and thus with acoustically normal BOS) but otherwise in isolation from conspecific song might be informative in distinguishing effects of passive sensory experience of others from effects of sensory experience of BOS on Area X neuron responsiveness.

Unlike Area X, responses to non-BOS song stimuli in the pallial nucleus LMAN of isolate birds were not different from those of normal birds, and thus BOS selectivity was not different between the two experimental groups. In general, LMAN neurons of normal birds are more narrowly responsive to BOS and much less likely than Area X neurons to show excitatory responses to non-BOS stimuli (Doupe 1997). Our result that conspecific song deprivation has differential effects on neurons in Area X and its pallial target LMAN appears to reflect this different responsiveness to conspecific songs between the two nuclei in normal birds: the already narrowly BOS-tuned LMAN neurons would be less
susceptible to isolation from conspecific songs than the more broadly tuned Area X neurons.

The neural dynamics underlying the differential conspecific responsiveness between nuclei in this pallial-basal ganglia circuit remain unclear. The differences may reflect changes in AFP firing properties from Area X to LMAN due to strong inhibitory synapses in the thalamic nucleus DLM (the medial part of the dorsolateral anterior thalamic nucleus), where a post-inhibitory rebound spike is thought to participate in the synaptic transmission of auditory responses (Luo and Perkel 1999; Person and Perkel 2005). The large difference in spontaneous firing rate between most LMAN neurons and the high firing Area X neurons could also contribute to the different conspecific responsiveness between the two nuclei. Because LMAN neurons have very low spontaneous activity, only strong synaptic inputs such as BOS responses may be able to evoke supra-threshold postsynaptic potentials and subsequent spike firing. The functional role of the differential conspecific responsiveness and the resulting differences in BOS selectivity between Area X and LMAN is a matter for speculation. The stronger conspecific responsiveness of Area X neurons could be useful for a comparison between conspecific song and BOS, which has been hypothesized to involve the AFP (Scharff et al. 1998). Alternatively the very high BOS selectivity seen in LMAN may simply be emerging gradually along the AFP circuit. To understand the function of the different degrees of song selectivity in AFP nuclei, as well as the function of song selectivity in song learning more generally, further studies of song auditory responses in relation to premotor song activity in awake birds will be required.

Effects of adult song deprivation on the song auditory and vocal control systems may differ

Song selectivity does not originate in the AFP – it is already robustly present in HVC (Margoliash, 1983; Margoliash and Fortune, 1992; Mooney, 2000; Nick and Konishi, 2005), and even in the interfacial nucleus of the nidopallium (NIf), although less strongly (Janata and Margoliash, 1999; Coleman and Mooney, 2004). Selectivity likely emerges gradually through a hierarchy of telencephalic auditory and early song system areas. The primary auditory area Field L is the main structure transmitting auditory information from the thalamus to this hierarchy (Fortune and Margoliash 1995; Karten 1968; Katz and
Our results from the very high-level song neurons of the AFP raise the question of whether a similar resilience to tutor and conspecific song deprivation exists at all levels of this network including Field L. Strikingly, studies of Field L in European starlings deprived of adult song in early life (Cousillas et al. 2004) show marked effects: Field L had highly abnormal properties, including altered tonotopy and a large non-selective auditory area. This result contrasts strongly with the normal BOS selectivity of AFP neurons observed in isolate zebra finches, and raises the possibility that isolate zebra finches have the same abnormalities in Field L neurons as isolate starlings do, but nonetheless develop normal song selectivity. Given the importance of sensorimotor learning to shaping song-selective neurons, song selective neurons in the AFP and elsewhere may be able to emerge using less organized auditory information, as long as this information relates systematically to song output.

Consistent with possible abnormalities in Field L, isolate zebra finches have deficits in processing of sounds (Sturdy et al. 2001). In contrast, disruption of AFP function has been shown to impair discrimination between BOS and conspecific songs but not between conspecific and heterospecific songs (Scharff et al. 1998). This suggests that the AFP song neurons may be specialized to monitor the bird’s own vocalizations. If song selective neurons function in such perception, our results suggest that isolate birds would have intact perception of self vs. other. Investigating the joint changes in Field L and the song system in response to altered sensory experience should provide insights into the hierarchical emergence of song selectivity and into the function of both these sets of areas in auditory perception and song learning.
GRANTS

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FIGURE LEGENDS

Figure 1.  A, Time course of zebra finch song learning. The sensory phase ends at ~PHD 60; the sensorimotor phase begins at ~PHD 30 and continues until PHD 90-110.  B, Simplified schematic view of the two major pathways in the song system. The motor pathway nuclei are gray and the anterior forebrain pathway (AFP) nuclei are black; primary auditory stations are indicated in white.  DLM, medial nucleus of the dorsolateral thalamus; LMAN, lateral magnocellular nucleus of the anterior nidopallium; NIf, interfacial nucleus of the nidopallium; RA, robust nucleus of the arcopallium.

Figure 2.  A, Songs of a father from nest 67, its normally-reared offspring (pupil), and four additional siblings reared in isolation from the father (is01-03 and is05). Sonograms plot frequency versus time, and the energy in each frequency band is indicated by its darkness. For each bird, introductory notes and the subsequent two motifs are shown except for is01, which sang only one motif in most bouts. Song motif is indicated by a bar underneath the sonogram.  B, Songs of a father from nest 27 and its isolation-reared offspring, is04. Asterisks indicate examples of relatively soft syllables with short neighboring intervals and poorly defined syllable boundaries. Conventions are as in A.  C, Distributions of syllable durations in isolate and normal birds’ songs. Bin size is 10 msec.

Figure 3.  BOS selective responses of an Area X neuron recorded from an isolate bird.  A, Raster plots and peristimulus time histograms (PSTHs) show the greater responses of a single Area X neuron to BOS than to father’s song and isolate conspecific song, which here is the bird’s brother’s song; 20 trials of each song were presented. Bin size of the PSTHs is 30 msec.  A raw extracellular recording trace of the first trial of each raster plot is shown at the top. Songs are shown underneath each PSTH as sonograms (frequency vs. time) and oscillograms (amplitude waveform vs. time).  B, Overlaid waveforms of 100 spike events that were randomly chosen from the BOS recording shown in A.  C, Histogram of interspike intervals calculated from the spike events in the BOS recording shown in A. Note that the horizontal axis has a logarithmic scale.

Figure 4.  Group data of Area X auditory responses in isolate birds.  A, Mean response
strength (RS) to BOS, father’s song, reverse BOS, isolate conspecific song (“isolate CON”), normal conspecific song (“normal CON”) and heterospecific song (“HET”) in all the auditory neurons recorded. One site with significant inhibitory responses to BOS is excluded from the analysis. Error bars denote SEM for all the subsequent figures. Asterisks indicate significant differences after paired comparisons of the mean RS to BOS and the other song stimuli ($p < 0.05$, one-way ANOVA and Tukey-Kramer HSD test).

B, The mean RS to BOS of each Area X neuron is plotted against the mean RS to isolate CON. The diagonal line marks where cells would lie if the RS to the two stimuli were equal; filled circles indicate those neurons with significantly greater responses to one of the stimuli versus the other (paired $t$ tests with Bonferroni correction, $\alpha$-value for significance $= 0.01$).

C, The cumulative distribution of preferences of individual Area X neurons for BOS over the songs of the father (circles), reverse BOS (triangles), and isolate CON (squares), as quantified with $d'$ values. The cells in the area between the two dashed lines (-0.5 < $d$ < 0.5) are considered to respond equally to the songs being compared.

D, The mean RS to father’s song of each neuron is plotted against the mean RS to isolate CON. Conventions are as in B.

E, Histogram showing the distribution of spontaneous firing rates of all the isolate auditory Area X neurons recorded.

F, Mean RS to BOS (red circles), father’s song (blue triangles), and isolate CON (black squares) in individual neurons, plotted against spontaneous firing rate.

Figure 5. Song selectivity of LMAN neurons recorded from isolate birds. All conventions are as in Fig. 3 and 4. A, Raster plots and PSTHs show the greater response of a single LMAN neuron to BOS than to father’s song and isolate conspecific song, which is the bird’s brother’s song; 20 trials of each song were presented. B, Overlaid waveforms of 100 spike events that were randomly chosen from the BOS recording shown in A. C, Histogram of interspike intervals calculated from the spike events in the BOS recording shown in A. D, Mean RS to BOS, father’s song, reverse BOS, isolate CON, normal CON and HET in all the auditory LMAN neurons recorded. Asterisks indicate significant differences after paired comparisons of the mean RS to BOS and the other song stimuli ($p < 0.05$, one-way ANOVA and Tukey-Kramer HSD test). E, The mean RS to BOS of each LMAN neuron is plotted against the mean RS to isolate CON. The filled circles indicate
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G, The mean RS to father’s song of each neuron is plotted against the mean RS to isolate CON.

Figure 6. BOS selectivity of Area X neurons in isolate birds relative to that found in normal birds.  

A, The histograms compare the mean RS to different stimulus types (BOS, reverse BOS, normal CON, and HET) measured from Area X neurons in isolate birds (closed bars) and normal birds (open bars).  Asterisks indicate significant differences between isolate and normal birds (two-way ANOVA and paired t tests with Bonferroni correction, α-value for significance = 0.013, p < 0.01).  Isolate birds had significantly smaller mean RS to non-BOS stimuli than normal birds.  

B, The mean RS to BOS in individual neurons from isolate (filled triangles) and normal (open squares) birds plotted against spontaneous firing rate.  

C, The mean RS to normal CON in individual neurons from isolate and normal birds plotted against spontaneous firing rate.  Note that the majority of neurons in normal birds have positive mean RS while most neurons in isolate birds have virtually no responses to CON.  

D, The degree of BOS selectivity in Area X neurons of isolate (closed bars) and normal (open bars) birds, quantified using the d’ values for BOS over normal CON, reverse BOS, and HET.  Asterisks indicate significant differences between isolate and normal birds (two-way ANOVA and paired t tests with Bonferroni correction, α-value for significance = 0.017, p < 0.012).  The p values for d’BOS-normal CON and d’BOS-HET are 0.021 and 0.13, respectively.  

E, BOS selectivity quantified using the selectivity index (SI).  Asterisks indicate significant differences between isolate and normal birds (two-way ANOVA and paired t test with Bonferroni correction, α-value for significance = 0.017, p < 0.01).  The p value for SI_{BOS-HET} is 0.018.

Figure 7. BOS selectivity of LMAN neurons in isolate birds relative to that found in normal birds.  All conventions are as in Fig. 6.  

A, Histograms of the mean RS to different stimulus types in LMAN neurons of isolate and normal birds.  No significant difference
was found in mean RS between isolate and normal birds.  

B & C, The mean RS to BOS (B) and normal CON (C) in individual neurons from isolate (filled triangles) and normal (open squares) birds plotted against spontaneous firing rate. The distributions of neurons from isolate and normal birds overlap for both BOS and normal CON at all firing rates.  

D & E, The degree of BOS selectivity of LMAN neurons in isolate and normal birds, quantified using the $d'$ values (D) and selectivity index (E). No significant difference in BOS selectivity relative to any other stimulus types was found between isolate and normal birds.
TABLE 1
Temporal properties of songs from isolate and normal birds

<table>
<thead>
<tr>
<th></th>
<th>Isolate bird (n = 10)</th>
<th>Normal bird (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syllable duration (msec)</td>
<td>Mean (range)</td>
<td>78.06 (3.7-520.0)</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>54.25</td>
</tr>
<tr>
<td></td>
<td>S.D.</td>
<td>76.62**</td>
</tr>
<tr>
<td>Number of syllables</td>
<td>Mean (range)</td>
<td>10.30 (5-19)*</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td>S.D.</td>
<td>4.27</td>
</tr>
</tbody>
</table>

Mean and standard deviation (S.D.) of syllable durations were calculated from the songs of isolate and normal birds. Asterisks indicate significant differences between isolate’s and normal bird’s songs: *p < 0.05; **p < 0.005. Comparisons of mean values were made using the unpaired $t$ test (parametric) or the Mann-Whitney $U$-test (non-parametric). Comparisons of S.D. were made using the $F$-test for equality of variance.
Figure 1. A, Time course of zebra finch song learning. The sensory phase ends at ~PHD 60; the sensorimotor phase begins at ~PHD 30 and continues until PHD 90-110. B, Simplified schematic view of the two major pathways in the song system. The motor pathway nuclei are gray and the anterior forebrain pathway (AFP) nuclei are black; primary auditory stations are indicated in white. DLM, medial nucleus of the dorsolateral thalamus; LMAN, lateral magnocellular nucleus of the anterior nidopallium; NIf, interfacial nucleus of the nidopallium; RA, robust nucleus of the arcopallium.
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