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Patterns of FOS protein induction in singing female starlings

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Abstract

Females of many songbird species produce song, but information about the neural correlates of singing behavior is limited in this sex. Although well studied in males, activity in premotor song control regions and social behavior regions has not been examined in females during song production. Here, we examined the immediate early gene protein product FOS in both song control and social behavior brain regions after female starlings defending nest boxes responded to an unfamiliar female in a naturalistic setting. We found that females that sang in response to the intruder had much higher numbers of fos-immunoreactive neurons (fos-ir) in the vocal control regions HVC, the robust nucleus of the arcopallium (RA), and the dorsomedial part of the nucleus intercollicularis (DM of the ICo). In HVC, fos-ir correlated positively with song length. In RA, DM and Area X, fos-ir correlated positively with number of songs produced. In social behavior regions, singers showed higher fos-ir in the nucleus taeniae of the amygdala, the dorsal part of the bed nucleus of the stria terminalis, and the ventromedial hypothalamus than non-singers. Overall, patterns of fos-ir in song control regions in females were similar to those reported for males, but differences in fos-ir were identified in social behavior regions. These differences may reflect a distinct role for brain regions involved in social behavior in female song, or they may reflect differences in the social function of female and male song.

Keywords

female song; communication; ventral tegmental area; medial preoptic area; lateral septum; immunocytochemistry

1. Introduction

Recent work has established that females of both tropical and temperate songbirds sing [1–5]. However, most of our understanding of the physiological and neural correlates of song has come from studies on males. This is in part because females of two important laboratory models (e.g., zebra finches *Taeniopygia guttata* and canaries *Serinus canaria*) sing little or not at all [6], and because song production generally is relatively male biased [7]. Because of this bias, a number of interrelated factors may inadvertently influence how we understand song production. For instance, in males, song is often under intense sexual selection; it is often tied strongly to breeding and mate attraction behaviors; it is often highly seasonal in

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production; and it is (in temperate species) influenced by the male sex hormone, testosterone [7]. In females, these factors are not present, reduced, or their influence unknown. Understanding female song, therefore, represents an opportunity to identify distinct mechanisms underlying vocal communication, because female song is presumably under different selection pressures and hormonal and neural constraints than that of males [8].

Singing in oscine passerines is controlled by a distinct and specialized neural circuit known as the song control system [reviewed in 9]. The system consists of two pathways, the anterior forebrain pathway and the caudal pathway. The former governs vocal learning in birds and includes Area X. The latter includes HVC (used as a proper name) and the robust nucleus of the arcopallium (RA), which underlie vocal production. Unlearned (innate) vocalizations appear to be generated or controlled by the dorsomedial part of the nucleus intercollicularis (DM of the ICo). This region receives input from other song control regions, and projects to many of same regions as RA [10–13]. The DM may be, in concert with avian midbrain central gray, the analog of the mammalian vocal control region, the periaqueductal gray (PAG) [14]. Production of vocalizations also involves a suite of regions underlying social behavior [e.g. 15, 16– 18]. This suite seems to be responsible for production of song in the appropriate social context. A number of these regions, in particular the medial preoptic area (mPOA), the ventral tegmental area (VTA) and the PAG, project directly to vocal control regions, [19–21].

Involvement of these brain regions in song production has been studied almost exclusively in male songbirds, but it is clear that the same networks exist in females that sing [22]. In some species, the neuroendocrine correlates of song appear to be similar to those of males [8, 23]. However, a major gap in our understanding of song production is the short-term neural correlates of song production in females. Only one study to our knowledge has demonstrated activity in the song control system that correlates to vocal production in a female songbird. Specifically, in white-crowned sparrows (*Zonotrichia leucophrys oriantha*) calls predicted ZENK protein induction in several vocal control regions [24], The authors of that study note that in three individuals that sang, induction appeared high in those regions, though singing was not statistically linked to ZENK [24].

The present study is a first step toward understanding the neural regulation of female song. Female starlings sing to defend nesting sites against other females, and unlike many male songbirds do not sing to attract mates [25–29]. After presenting a female with an unfamiliar female competitor, we analyzed the number of cells that showed immunoreactivity for FOS (fos-ir) in several vocal control regions and a suite of social behavior regions to determine whether FOS protein induction in those regions correlated with song production.

2. Methods

2.1 Subjects

Wild starlings (35 females) were captured in Madison, WI. Photosensitive starlings were implanted using standard techniques with silastic implants containing 17β-estradiol (two, 17 mm in length of i.d., 1.47 mm; o.d. 1.96 mm; Dow Corning, Midland, MI USA, packed with 13-mm 17β-estradiol,Sigma Aldrich, St. Louis, MO, USA) to facilitate breeding season typical endocrine conditions necessary to promote singing behavior [18, 30]. After hormone implantation, individuals were placed in same-sex groups of six on 11h light:13h dark in indoor aviaries with four nest boxes. They were provided nest material (dry grass) to stimulate interest in nest boxes. All procedures were approved by the University of Wisconsin Institutional Animal Care and Use Committee and all procedures adhered to methods approved by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

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2.2 Observations

Behavioral observations were performed from behind one-way glass at least two days after two individuals in a room began singing, entering and occupying nest boxes, and carrying nest material to boxes. Each group of six individuals was exposed to an unfamiliar female starling (the stimulus individual) for 30 minutes. During this period the number of song bouts was recorded to the nearest second, as was the length of each song to the nearest second. These values were used to calculate rate of song production (number of songs in 30 minutes) and the mean duration of each song (song bout length). While all groups were audio-recorded with a Marantz 661 PMD recorder and a Sennheiser ME67 microphone, because only two individuals ever sang simultaneously, song starts and ends were easily notated by the observer. We also noted where the song was produced (i.e. from floor, free perch, nest box perch, or nest box roof). The number of eating, drinking, bill-wiping and preening events were recorded for each individual Additionally, breeding behaviors, specifically the number of wing-waves (a visual display produced while singing) and the number of nest box entries (to any nest box) and the number of times a subject picked up nest material. We also tracked the number of times females displaced each other from nest box perches. After 30 minutes, the stimulus individual was removed. Different stimulus females were used for each group. Thirty minutes after stimulus removal two subjects from the group were sacrificed. Individuals were selected based on nest box occupancy; a female was defined as occupying a specific nest box if she carried nest material to that box repeatedly without being displaced, if she entered a specific nest box repeatedly without being displaced, or if she sang most of her songs from a nest box perch (but not roof). Subjects were sacrificed by rapid decapitation and their brains submersion fixed overnight in 5% acrolein, cryoprotected for 4 days in 30% sucrose, flash frozen, and stored at −80°C until processing. In all, 22 females were sacrificed. In some cases, the group remaining in the room was supplemented with additional individuals to form a new group of six. In these cases, after one week the new group was again subjected to the same observation regime. Most females went through the paradigm once, and none more than twice.

2.3 Immunohistochemistry and Data Analysis

Brain tissue was sectioned at 40 microns and stored in antifreeze at −20°C. Every 3rd section was immunolabeled via standard protocols [31] with 1:18,000 FOS primary antibody (K-25, sc253; Santa Cruz) and 1:250 biotinylated goat anti-rabbit secondary (Vector Labs), and visualized with diaminobenzadine. This antibody has been validated using preadsorption in starlings in our lab previously [16]. All individuals were run in a single batch to eliminate batch variability. Cells showing FOS-immunoreactive label (fos-ir) were quantified on a Nikon microscope with a Spot camera (Diagnostics Instruments, Inc.) and MetaVue software (Universal Imaging Corp) in standardized boxes and ovals placed on digital photomicrographs within the boundaries of the following regions (Figure 1): HVC, RA, DM, Area X, the bed nucleus of the stria terminalis (dorsal and ventral; BNSTd and BNSTv), nucleus taeniae (TnA), lateral septum (LS), ventromedial hypothalamus (VMH), VTA, PAG and mPOA. The MetaVue autoscale function was used to calculate the correct exposure of each image as a percentage of the total range of light, helping to reduce the variation in background among individuals, although background labeling was fairly uniform across individuals. Each region was thresholded separately because of the difference in labeling patterns from region to region. The threshold was set such that it automatically recorded the number of cells that showed labeling. Two independent observers checked the thresholds for each region to agree that each setting was marking obviously labeled tissue but not extraneous background regions. A single threshold was used for each region across all subjects, but each measurement was visually checked to confirm that extraneous objects were not included in the measure. For most regions, six measures were made from three sections, measuring the same region on each side of the midline. In

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cases where tissue was damaged, we either used adjacent sections or dropped a measure. We were able to make an average of 5.7 ± 0.6 (mean ± standard deviation) for each subject. Because HVC, DM, and TnA are relatively long when cut coronally, we made measurements on four sections, for a maximum of eight measures per subject (7.2±1.2).

2.4 Statistics

All statistical analyses were performed in R or JMPv5.1. For all variables that did not meet the requirements of parametric statistics, we transformed data with the natural log to meet those requirements. To examine how singing behavior related to fos induction in the brain, we analyzed whether singing status (song produced or no song produced) related to fos-ir across all brain regions in an omnibus one-way ANOVA. However, because data loss in individual areas drastically reduced sample size and power, we ran Student's t-tests to compare singers and non-singers for each brain region, followed by sequential Bonferroni tests for each set of variables to account for multiple comparisons [32] (one correction for analyses of song control regions and a separate correction for analyses social brain regions). To further assess the exact relationships of fos-ir to singing behavior, we performed separate simple linear regressions for each brain region with the number of songs produced, and, of the birds that sang, mean song length, examining how these variables statistically predicted fos. These variables could not be combined in a single model due to their high levels of correlation. We additionally assessed relationships between non-sexual maintenance behaviors (i.e. feeding, drinking, preening and bill-wiping) and the fos data for each region using a backwards step-wise approach to linear mixed models. All behavioral variables were added to the model and removed until all remaining variables were significant at the $\alpha = 0.05$ level. Then we assessed whether non-song behaviors associated with singing and nest box occupancy (i.e. wing-waving, nest material carrying, and nest box entry) statistically predicted fos labeling in any region, again using linear mixed models with a backwards stepwise regression approach. In cases where multiple variables remained in the model, we report β values to indicate the strength of those relationships with each individual variable.

3. Results

3.1 Singing Behavior

All the individuals sacrificed for the study (n = 22) were observed perching on and entering nest boxes. Sample sizes (shown in figures and reported below) for the measures of the numbers of cells labeled for fos (fos-ir) in some cases are smaller than the nominal sample size of 22 individuals because tissue damage occasionally made accurate assessment of fos-ir in some sections impossible for particular individuals. The minimum sample size was 17 subjects and the maximum was 20 for any one brain region (specific sample sizes included in statistical reports below). R^2 and *p*-values were determined using these reduced datasets. Of the 22 subjects, 13 sang and 9 did not. Singers produced on average 5.6 ± 5.0 songs, of an average song bout length of 11± 8.5 s.

Student's t-tests comparing fos-ir in three vocal control regions, HVC, RA and DM, showed that singers had substantially higher fos-ir than non-singers (Figure 2, 3; HVC: Figure 3a; n = 19, $t_{17} = -3.2$, $p = 0.005$; RA: Figure 3b; 2d; n = 20, $t_{18} = 7.2$, $p < 0.0001$; DM: Figure 3c; 2g; n = 20, $t_{18} = 3.5$, $p = 0.003$). In Area X, however, there was not a significant correlation between fos-ir and singer status (Figure 3d). In each region, there were linear relationships between other measures of song production and fos-ir. In HVC, song length (Figure 4b) but not number of songs (Figure 4a) showed a linear relationship with fos-ir ($R^2 = 0.63$, n = 19, $p < 0.0001$). In contrast, fos-ir in DM and RA showed linear relationships with number of songs (DM: Figure 4e, $R^2 = 0.26$, n = 20, t = 2.6, p = 0.019; RA: Figure 4c; $R^2 = 0.42$, n = 20, t = 3.63, p = 0.002) but not mean song length (Figure 4f, 4d). In Area X, fos-ir related

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linearly to number of songs produced (Figure 4g; $R^2 = 0.21$, n = 19, t = 2.45, p = 0.025). All relationships were significant after sequential Bonferroni corrections.

In three areas outside of the song control circuit, BNSTd, VMH and TnA, singers showed much higher fos-ir than non-singers (Figure 5; TnA: Figure 6a; n = 17, $t_{17} = 3.2$, p = 0.005; VMH: Figure 6b; n = 21, $t_{19} = 5.4$, $p < 0.0001$; BNSTd: Figure 6c; n = 21, $t_{19} = -2.6$, $p < 0.016$ [not significant after sequential Bonferroni correction]). In VMH, there was also a strong positive linear relationship between fos-ir and number of songs produced (Figure 7c; $R^2 = 0.24$, n = 20, $F_{1,19} = 2.5$, p = 0.022). Fos-ir in PAG, LS and mPOA showed no relationships to song measures.

3.2 Non-song Behaviors

In VMH, breeding behaviors, specifically nest material gathering and nest box entry, contributed significantly to variance in fos-ir with fos-ir (Table 1). In the DM (Table 1), fos-ir was predicted by a model including all three sexual behaviors; nest material gathering related negatively to fos-ir while nest box and wing waves were positively correlated with fos-ir. A negative correlation was found between fos-ir in the VMH and feeding (Table 1). Fos-ir in the DM correlated negatively with drinking (Table 1).

4. Discussion

To our knowledge, this is the first study to examine relationships between song production and FOS protein labeling in a female songbird. We examined relationships between song production and fos-ir in parts of two interacting neural networks, the song control network and the social behavior network, in females singing in a territorial context. All examined regions of the song control network showed positive linear relationships between song production and fos-ir, with singers showing more fos-ir than non-singers in three of the vocal control regions. Fos-ir was also higher in some regions of the social behavior network, but linear relationships were fewer and less well-defined than in the song control regions.

While numerous studies have examined fos induction in female songbirds in response to song playback [33–35], FOS induction in singing females has not been examined previously. Indeed, in the preferred model species for investigating song, the zebra finch, females do not sing and lack or possess extremely reduced song control regions [6]. However, in the many songbird species in which females sing, females show the same clearly defined song control regions including HVC, RA, and Area X, though these areas are often reduced in size compared with those of males [22, 36]. Female starlings show clearly defined regions that are known to be involved primarily in song production, reflecting the fact that they sing throughout most of the year and produce relatively complex songs [25, 27].

Given the roles of HVC and RA in male song production [37, 38], it is unsurprising that these regions would show fos induction in singing females. Similar relationships between song production and fos have been found in male starlings and zebra finches [16, 37, 38]. It is of interest that this study shows statistically stronger relationships between activity in HVC and song length than between HVC activity and the number of songs produced. While comparative data are limited, in species with variable song lengths or song bout lengths, there is some evidence that various characteristics of HVC correlate with mean song length more so than other singing variables such as number of songs [e.g. 39]. The behavioral relevance of these song parameters is unclear in female starlings. In male starlings, older birds sing longer songs, which are more attractive to females [40], and longer songs tend to be more complex [40, 41]. With respect to female song rates, in female superb fairy-wrens, high song rates are associated with increased territoriality [42].

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Our findings for fos-ir in Area X are novel, but somewhat difficult to interpret. At least four studies have examined fos-ir in this region in males [16, 37, 38, 43]. None found correlations between song production and fos-ir. In zebra finches, no fos-ir cells were detected in Area X [37]. In starlings and house sparrows only a lack of correlation to song or nest box ownership was reported [38, 43]. Contrasting to results with fos, singing female white-crowned sparrows showed induction of ZENK protein product in Area X [24]. In comparison with results for fos in other studies, female starlings in our study showed a positive correlation between fos-ir cell counts in this region and the number of songs produced, though this effect was not strong. Area X is involved in song learning and can be active when songbirds sing in the absence of a communication target (usually a conspecific female), probably when singers are practicing [44, 45]. Given this probable function it is unclear why Area X would show fos-ir in females singing territorial song. Such a result could be a function of different song production processes in females, though this seems unlikely. Instead, it may reflect prior practice and our method of assessing fos-ir. It is possible that prior to the introduction of the unfamiliar stimulus bird that females were singing “undirected song”, and the detected correlation reflects fos induction from that period.

Of interest is the correlation between fos-ir in DM and song production. DM is demonstrably active during the production of unlearned vocalizations in oscine passerines [10, 12, 13, 46–49]. Although it projects to the same suite of regions as RA [11, 50], it is unclear what role DM plays in song production. In zebra finches, fos-ir in DM showed no relationship to song production, but call production was not measured [37]. It remains to be determined what role DM plays in song compared with production of unlearned vocalizations. Associations of fos-ir in DM with several motor behaviors may have several explanations. Wing-waving is performed while singing, so correlations of wing-waving with fos-ir in DM may be related the vocal production during this period, rather than wing waving per se. Nest building, entering the nest box, and drinking negatively related to fos-ir in DM, but this may be because individuals spent time singing rather than performing other motor behaviors.

Of the social behavior network regions we analyzed, three showed differences in fos-ir in singers and non-singers. Effects in the VMH and the BNSTd are consistent with those found in male starlings [30], suggesting that fos induction in these regions may occur in association with production of song regardless of social context. Specifically, in our study females sang to defend nest boxes, whereas in prior studies males sang to attract potential mates. VMH fos-ir also correlated with eating, and with female sexual behaviors such as nest material gathering and nest box entry, both behaviors associated with VMH activity in prior studies [51–53]. TnA, which has been implicated previously in communication in a sexual context [54, 55], showed fos-ir correlations in our study. This however contrasts with results from males [54, 55]. Conversely, we found no correlations between song and fos-ir in the mPOA or the VTA of females in this study, which have been shown in male starlings singing in a mate attraction context [38]. Male song sparrows (*Melospiza melodia*) also show fos-ir in the VTA when singing song to defend a territory, but this finding may be due to one strong singer [56]. mPOA is known to regulate several aspects of male sexual behavior [57], and VTA is involved in motivation [58]. Studies of male starlings exposed subjects to potential mates, which could generate high levels of sexual motivation, accounting for the fos-ir in mPOA and VTA. Females were under no such pressure. However, because these two groups differ both in the context and function of song and the sex of singer, we cannot rule out sex differences in fos induction in these regions regardless of context. We found no correlations between song and FOS induction in PAG or SL, results which are consistent with those from males singing directed song. Finally, the fact that fos-ir

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did not differ in PAG, LS, and mPOA (or area X) in singers and non-singers indicates that differences were not attributable to overall, brain-wide staining differences.

Understanding the neural correlates of song in females offers an expansion of an already extremely important model system for investigating the production of learned behaviors and determining how sex specific behaviors are controlled. Here we have shown that the premotor vocal control regions in females show patterns of FOS induction (an indirect marker of neural activity) that relate to song production. Social control regions in females showed patterns different than those previously reported in males, possibly reflecting the social context or the sex of the singer. Further work should examine whether females show similar or different patterns to males in other social contexts, and should investigate whether the social behavior networks in the sexes influence vocal production similarly.

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Research Highlights

- Little is known about the neural correlates of female song production
- Female starlings show fos-ir in song control regions after singing
- Brain regions implicated in social behavior also show fos-ir related to song
- Patterns of fos-ir in social brain regions differ from those in song control regions

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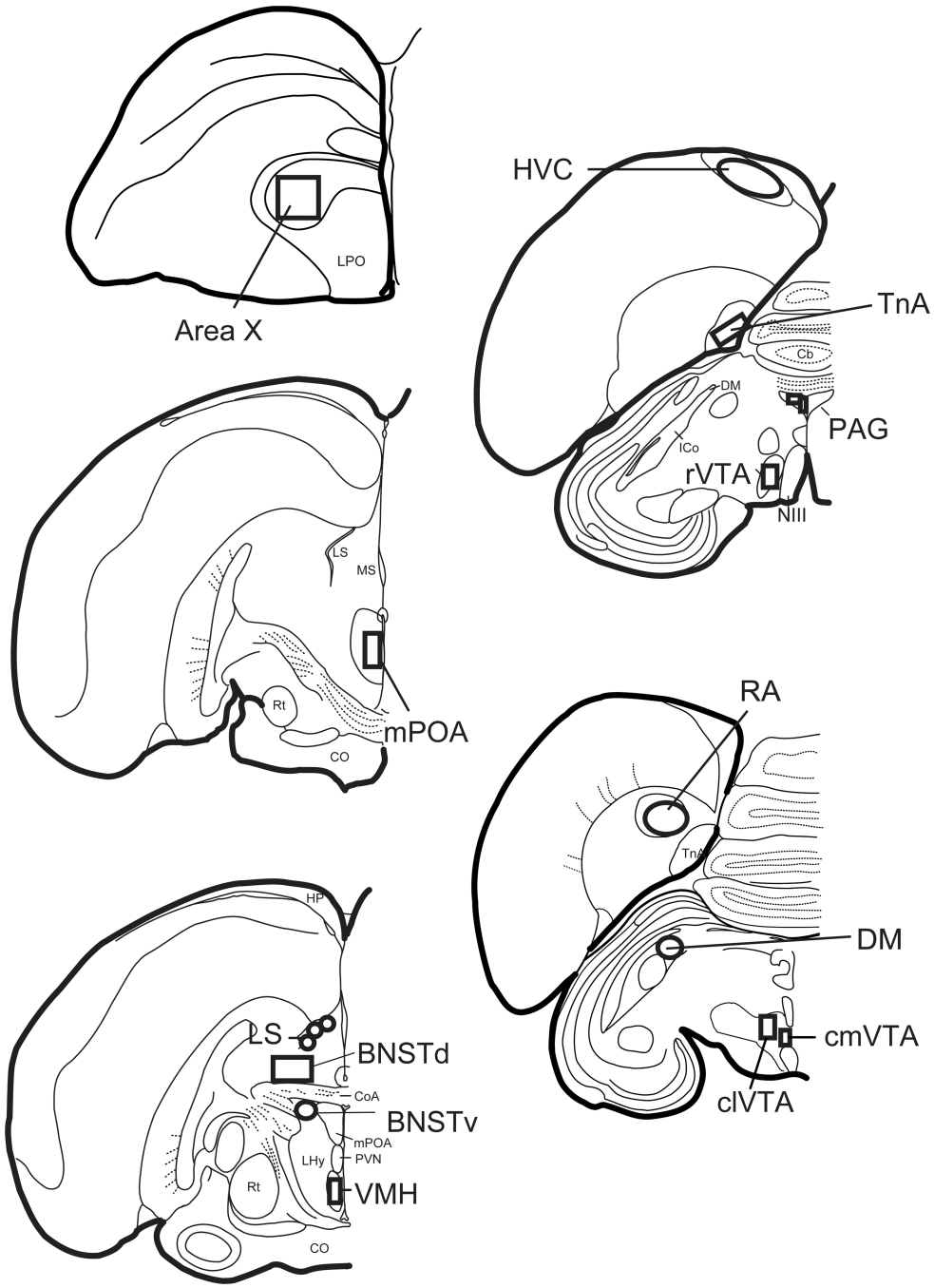

Figure 1.

Brain regions examined via immunohistochemistry for fos protein. Heavy boxes and circles indicate measurement regions. Large text indicates brain regions examined. Area X (0.233mm^2); BNST: bed nucleus of the stria terminalis, d: dorsal (0.19 mm^2); v: ventral (0.152 mm^2); CoA: anterior commissure; Cb: cerebellum; CO: optic chiasm; DM: dorsomedial part of the nucleus intercollicularis (0.092 mm^2); HP: hippocampus; HVC: used as a proper name (0.095 mm^2); ICo: nucleus intercollicularis; LHy: lateral hypothalamus; LS: lateral septum (0.10 mm^2); mPOA: medial preoptic nucleus (0.092 mm^2); MS: medial septum (0.072 mm^2); NIII: oculomotor nerve; PAG: periaqueductal gray (0.124 mm^2); PVN: paraventricular nucleus; RA: robust nucleus of the arcopallium (0.070 mm^2); Rt:

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nucleus rotundus; TnA: nucleus taeniae of the amygdala (0.146 mm^2); VMH: ventromedial nucleus of the hypothalamus (0.117 mm^2); VTA: ventral tegmental area; caudolateral: clVTA (0.138 mm^2); rostral: rVTA(0.138 mm^2); caudomedial: cmVTA (0.088mm^2)

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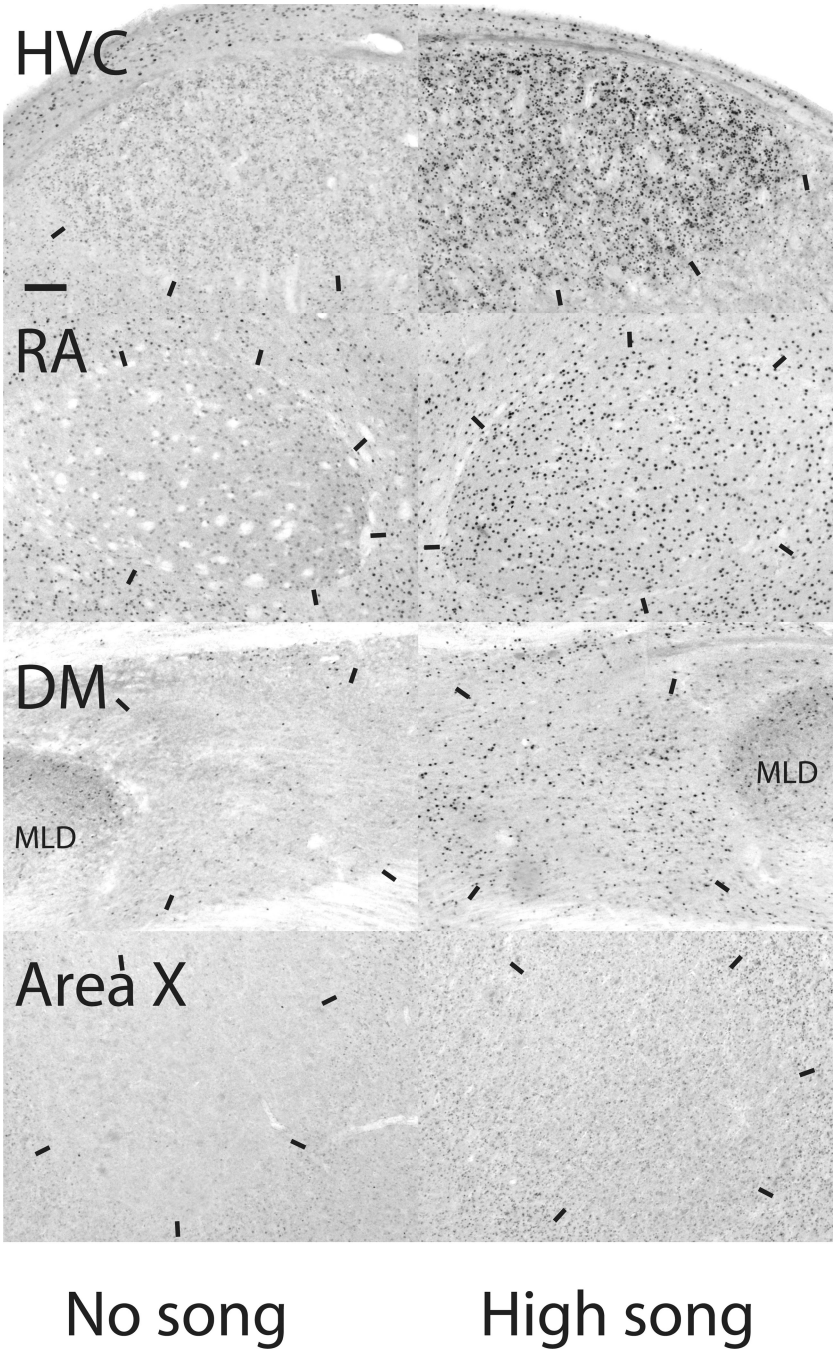

Figure 2.
Representative photomicrographs of vocal control regions. Right images are from individuals that produced higher numbers of songs (in HVC, longer songs), left images from individuals that produced no song (in HVC, short song). Horizontal bar in HVC is 100εm, ticks indicate boundaries of regions. MLD = nucleus mesencephalicus.

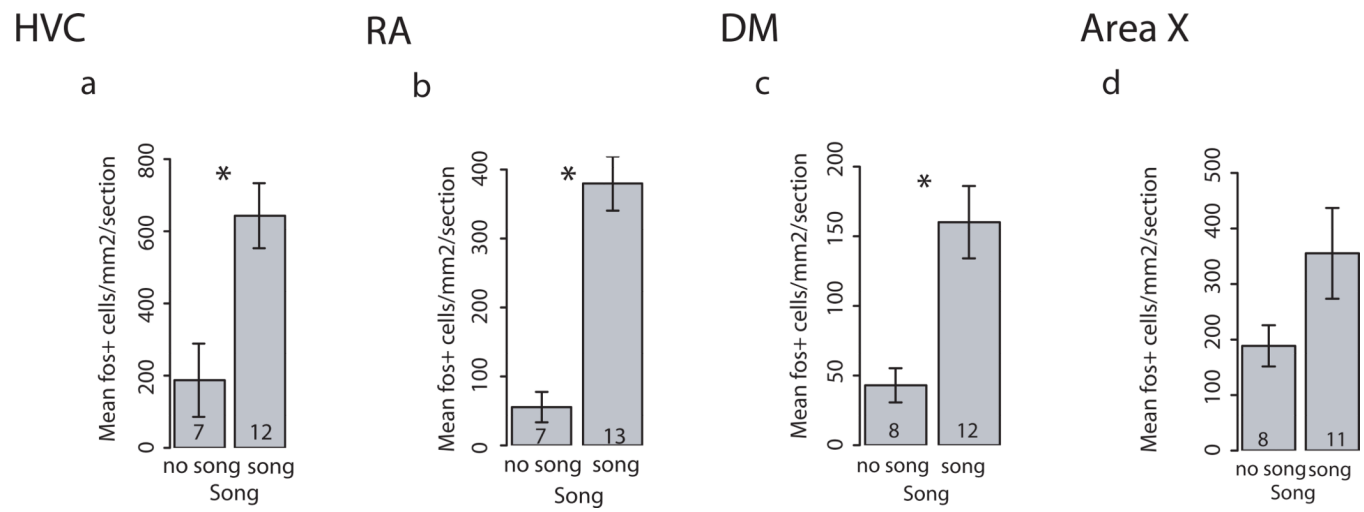

Figure 3.

Fos-ir in vocal control regions as a function of singer status, comparing individuals that sang at least one song with those that did not sing. Y-axis shows density of fos-positive cells in the measurement area, averaged over three consecutive sections and both left and right sides. Asterisks indicate significant differences at $\alpha = 0.05$.

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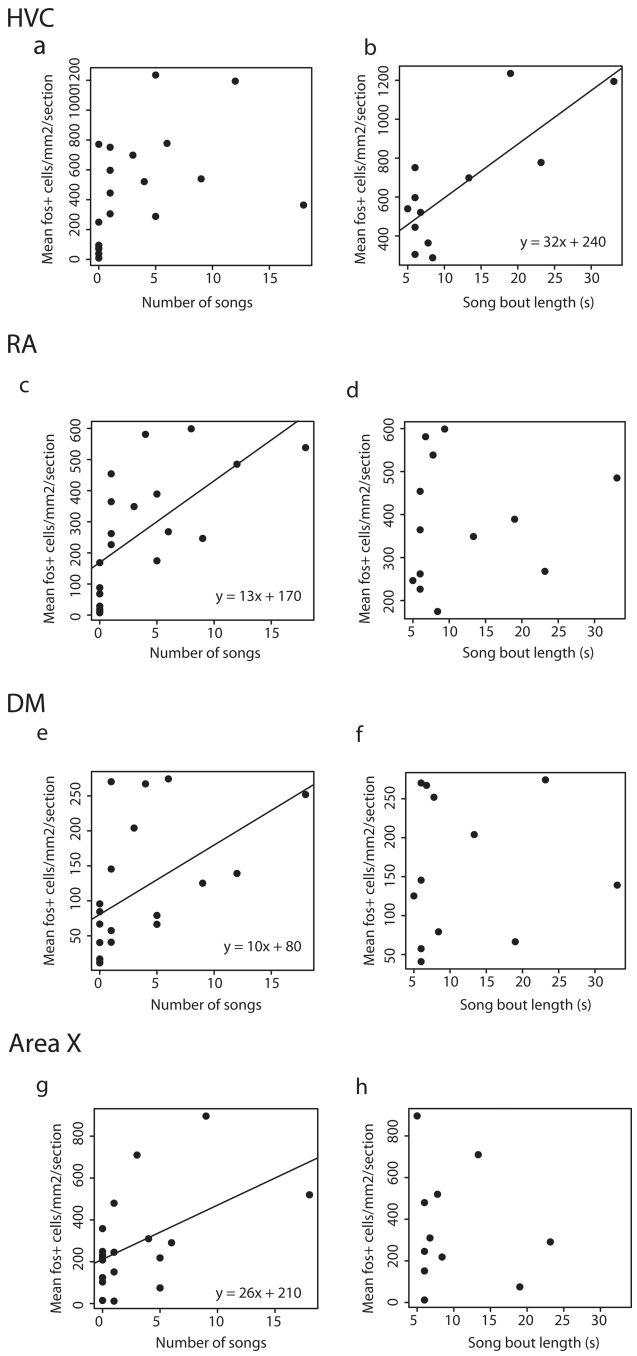

Figure 4.

Fos-ir in vocal control regions showing significant linear relationships with number of songs produced and mean song length (dropping non-singers), respectively. Solid lines indicate $p < 0.05$. Each point represents a single individual.

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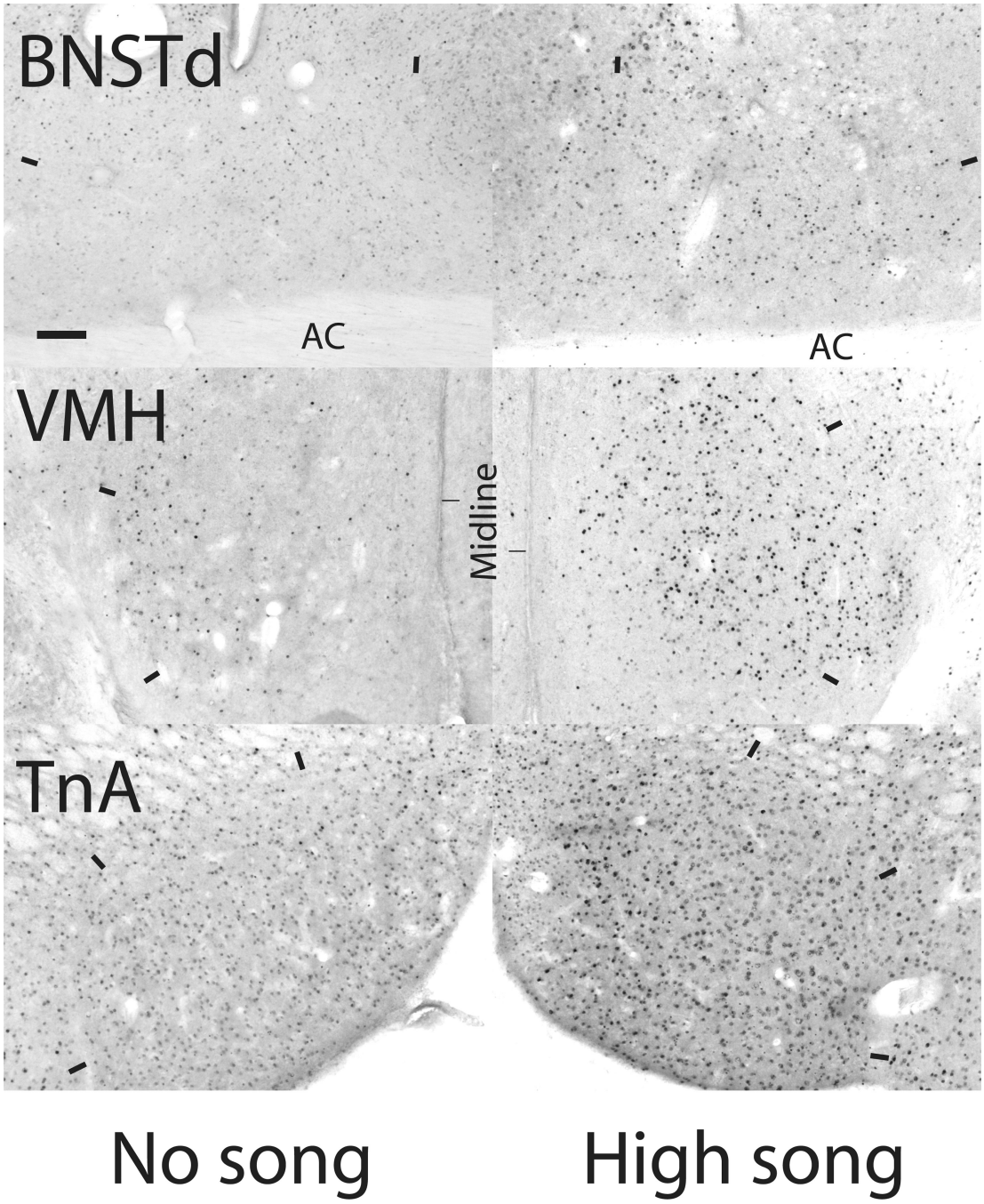

Figure 5.
Representative photomicrographs of social behavior regions. Right images are from individuals that produced higher numbers of songs, left images from individuals that produced no song. Horizontal bar in BNSTd is 100μm, ticks indicate boundaries of regions. AC = anterior commissure.

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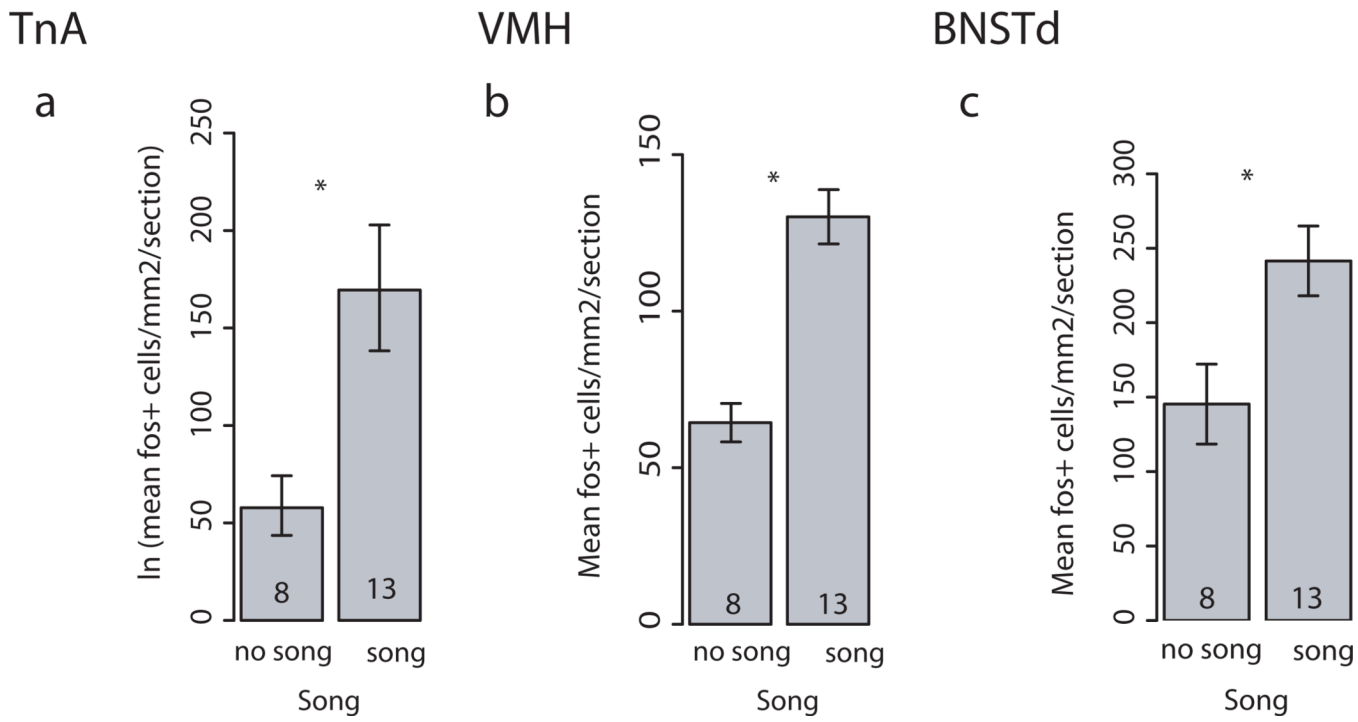

Figure 6.

Fos-ir in social behavior brain regions as a function of singer status, comparing individuals that sang at least one song with those that did not sing. Asterisks indicate significant differences at $\alpha = 0.05$.

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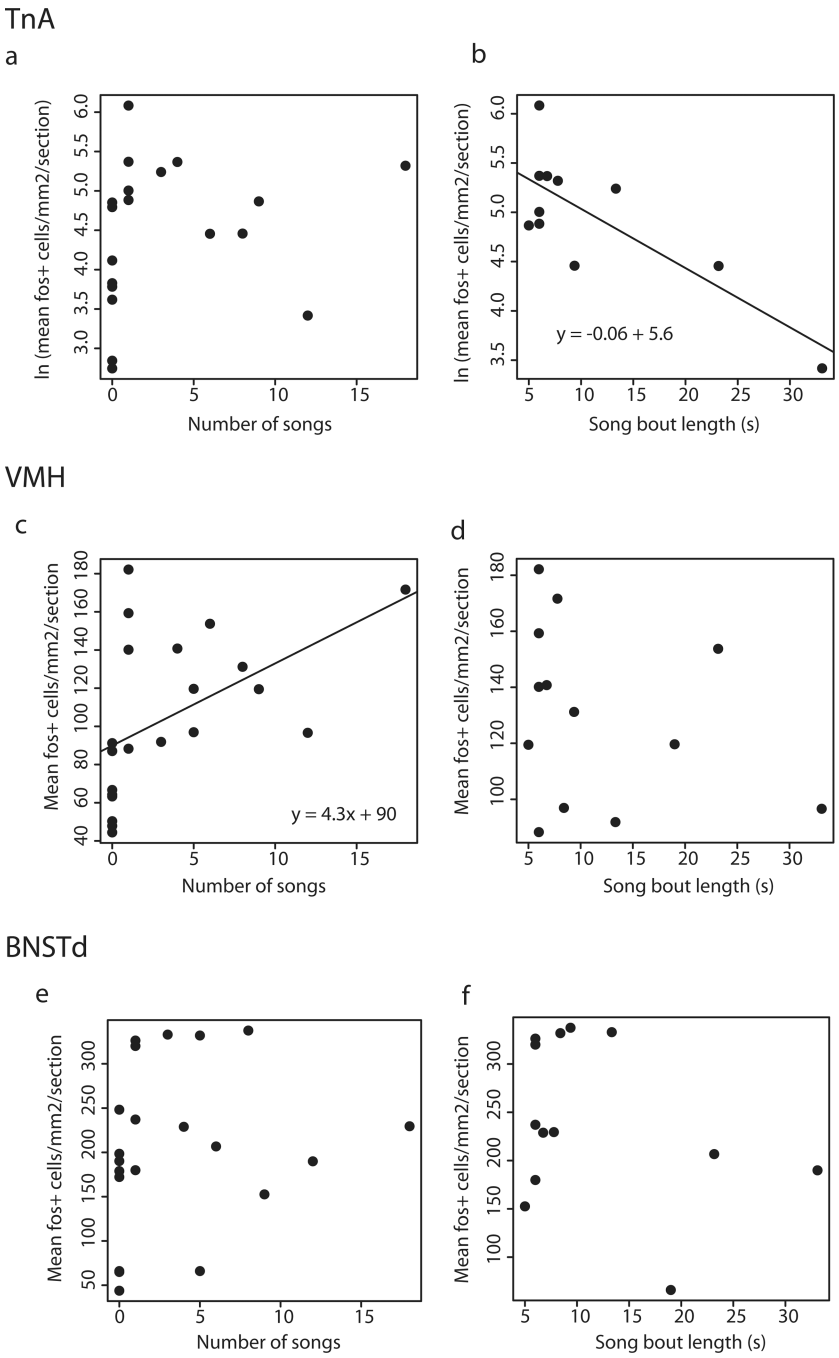

Figure 7.

Fos-ir in social behavior brain regions showing significant linear relationships with number of songs produced and mean song length, respectively. Solid lines indicate $p < 0.05$. Each point represents a single individual.

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Table 1

Statistics for relationships between fos-ir and breeding and non-breeding motor behaviors (β values reported for multiple linear regressions, R^2 when only one variable contributed).

Region	Behavior type	Predictor behavior	Variance statistic	n	F	*p*
	breeding		β			
VMH		nest box entry	0.34	20	6.9	0.017
		nest material				
		gathering	−0.63	20	5.6	0.03
DM		nest box entry	0.31	20	10	0.006
		nest material				
		gathering	−0.67	20	11	0.004
		wing waves	0.5	20	7.5	0.014
	non-breeding		R^2			
VMH		feeding	0.28	20	7.5	0.013
DM		drinking	0.28	20	7.1	0.015