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Research report

Immediate early gene expression following exposure to acoustic and visual components of courtship in zebra finches

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Abstract

Sensory driven immediate early gene expression (IEG) has been a key tool to explore auditory perceptual areas in the avian brain. Most work on IEG expression in songbirds such as zebra finches has focused on playback of acoustic stimuli and its effect on auditory processing areas such as caudal medial mesopallium (CMM) caudal medial nidopallium (NCM). However, in a natural setting, the courtship displays of songbirds (including zebra finches) include visual as well as acoustic components. To determine whether the visual stimulus of a courting male modifies song-induced expression of the IEG ZENK in the auditory forebrain we exposed male and female zebra finches to acoustic (song) and visual (dancing) components of courtship. Birds were played digital movies with either combined audio and video, audio only, video only, or neither audio nor video (control). We found significantly increased levels of Zenk response in the auditory region CMM in the two treatment groups exposed to acoustic stimuli compared to the control group. The video only group had an intermediate response, suggesting potential effect of visual input on activity in these auditory brain regions. Finally, we unexpectedly found a lateralization of Zenk response that was independent of sex, brain region, or treatment condition, such that Zenk immunoreactivity was consistently higher in the left hemisphere than in the right and the majority of individual birds were left-hemisphere dominant.

Keywords: Zenk; Egr-1; Birdsong; *Taeniopygia guttata*; Lateralization; cmHV

1. Introduction

In songbirds the auditory brain regions specialized for processing conspecific vocalizations were first identified by their increased expression of immediate early genes (IEGs) following exposure to conspecific song. Regions including caudal medial nidopallium (NCM; formerly called caudomedial neostriatum) and caudal medial mesopallium (CMM; formerly called caudomedial hyperstriatum ventrale, cmHV) exhibit increased expression of the IEG *ZENK* (also known as *zif*-268, *egr*-1, *ngf-Ia* and *krox*-24) following exposure to conspecific song compared with exposure to other sounds [20]. The importance of these regions for processing conspecific song has since been verified through tract tracing, electrophysiology, and lesion studies (e.g. [3,8,18,29]). Studies of other avian taxa have also revealed sensory driven IEG expression specific to conspecific vocalizations [6,28]. Moreover, levels of ZENK expression in CMM and NCM of male and female birds is related to previous experience and/or the behavioral saliency of the particular vocalizations heard (e.g. [2,6,19,26]). Thus CMM and NCM are implicated as important regions for the perceptual processing of conspecific vocalizations.

Birdsong is a vocal signal critical for successful courtship in many bird species. However, song is usually not the only component of courtship behavior. Courtship signals may contain visual, postural, kinetic, and vocal components and typically involves coordinated interaction between males and females. In zebra finches (*Taeniopygia guttata*) courtship involves all of these components. Male visual signals include ornamental plumage and bright red beak color. Males actively

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court females by singing while performing a hop-pivot dance [21,30]. Thus courtship involves color signals, body and beak movements, and complex sound production. Few studies, however, consider more than one of these variables at a time when assessing mate choice behavior or the neural mechanisms underlying perception of courtship song (but see [4]). Although the effects of compound acoustic and visual stimuli have been investigated in regards to song learning (e.g. [1,13,14]) the effect, if any, of visual stimuli on perceptual processing of song by adult songbirds remains unexplored.

The purpose of this study was to determine whether song-induced IEG expression in the songbird auditory forebrain is modulated by the visual stimulus of a courting male. For example, perhaps the visual stimulus of a singing bird would focus attention on the associated song, and result in greater activation of auditory areas in the brain [16]. To test this, we presented male and female zebra finches with playback of male zebra finch courting behavior in one of four conditions: (a) audio and video, (b) audio only, (c) video only, and (d) control (no audio or video). Following playback we measured the number of cells containing the Zenk protein via immunocytochemistry to determine whether the visual stimulus affected the auditory areas CMM and NCM. Although only males produce song in this species, we assessed the responses of both males and females to test for potential sex differences in IEG expression.

2. Material and methods

Herein we use brain nomenclature set forth by Reiner et al. [23]. We refer to the immediate early gene as *ZENK* and its protein product as Zenk.

This experiment used 11 female and 17 male adult zebra finches (see Table 1). Prior to the experiment, birds were housed in groups and food and water were available ad libitum. Care and all experimental procedures were conducted in accordance with the University of Western Ontario's animal use regulations.

2.1. Stimuli

We made stimuli by video-recording male zebra finch courtship behavior (singing and dancing) in a customized recording cage measuring 70 cm × 45 cm × 45 cm. Two 70 cm perches ran the length of the cage to an opening in which a digital video camera (DCR-TRV 120, Sony) was placed. A smaller (36 cm × 25 cm × 31 cm) female holding cage was directly beside the video camera.

Table 1
Treatment groups and sample sizes

Treatment group	*n*	Audio stimulus	Video stimulus
Video and audio (A+V+)	4 males, 3 females	Song playback	Courting male
Video only (A−V+)	4 males, 3 females	None	Courting male
Audio only (A+V−)	5 males, 3 femlaes	Song playback	Blank screen
Control (A−V−)	4 males, 2 females	None	Blank screen

To record courtship behavior and singing, a male was placed alone in the recording cage overnight. The next day the video camera was turned on and a female zebra finch was placed in the smaller adjacent cage for approximately 30 min after which the female and male were returned to their respective housing cages. This resulted in video recordings of males approaching the video camera along the perches while dancing and singing. A sample of these video recordings are available on the internet http://publish.uwo.ca/∼smacdou2/finchmovie.html.

Video recordings of three males were used to create three 30 min playback sequences for use as experimental stimuli (each sequence using only a single male). Playback sequences were prepared using iMovie software (vers. 3.0, Apple Computer). Each playback sequence was six repetitions of a 5 min recording. The 5 min recordings were comprised of alternations between 30 s movie sequences of an actively courting and singing male and 30 s movie sequences of an empty cage.

2.2. Playback procedure

Prior to the playback, each bird was isolated for approximately 24 h in a 36 cm × 25 cm × 31 cm cage inside a sound-attenuating chamber (50 cm × 70 cm × 50 cm). Each chamber was equipped with a playback speaker and video screen (a 13 cm × 9.5 cm thin film transistor (TFT) liquid crystal display screen) for stimulus presentation. Images on TFT screens have been shown to be sufficiently realistic to elicit courtship behavior by male zebra finches [15], and use of these screens in particular have elicited approach behavior by female songbirds [12]. Playback of the video sequences on these screens resulted in an image of the courting male that was approximately life-sized. We could remotely monitor each chamber using microphones and video cameras mounted inside the chambers. The TFT screens produced no detectable noise. When turned on but with no image, the screens provided extremely dim illumination within the chamber. When turned on with an image, the screens provided much more illumination within the chamber.

Stimuli presented in each condition are presented in Table 1. Thirty minutes prior to each playback, the lights inside the chamber were turned off. Following this the TFT screen was turned on and the 30 min playback began. For each subject one of the three playback sequences was selected in a block-randomized fashion for use as a stimulus. In the control condition and audio only condition the TFT screen was turned on but no stimuli were presented on it. For the three treatment conditions we presented the playback sequence through the TFT screen only (video only), the speaker only (audio only), or both (video and audio).

We recorded the number of hops, the number of calls and the number of song bouts performed by each bird during the 30 min of playback. When counting number of calls, we did not discriminate among short (a.k.a. "tet"), medium (a.k.a. "stack") and long (a.k.a. "distance") calls [22,33]; rather we counted the sum total of all these calls (although the large majority of them were long calls). Once the playback finished the TFT screen was turned off and the bird remained in the dark for one further hour.

Next the bird was given an overdose of ketamine and xylazine (1:1) and then transcardially perfused with heparanized 0.1 M phosphate buffered saline (PBS) and then 4% paraformaldehyde. Following perfusion, the brain was immediately removed and placed in 4%

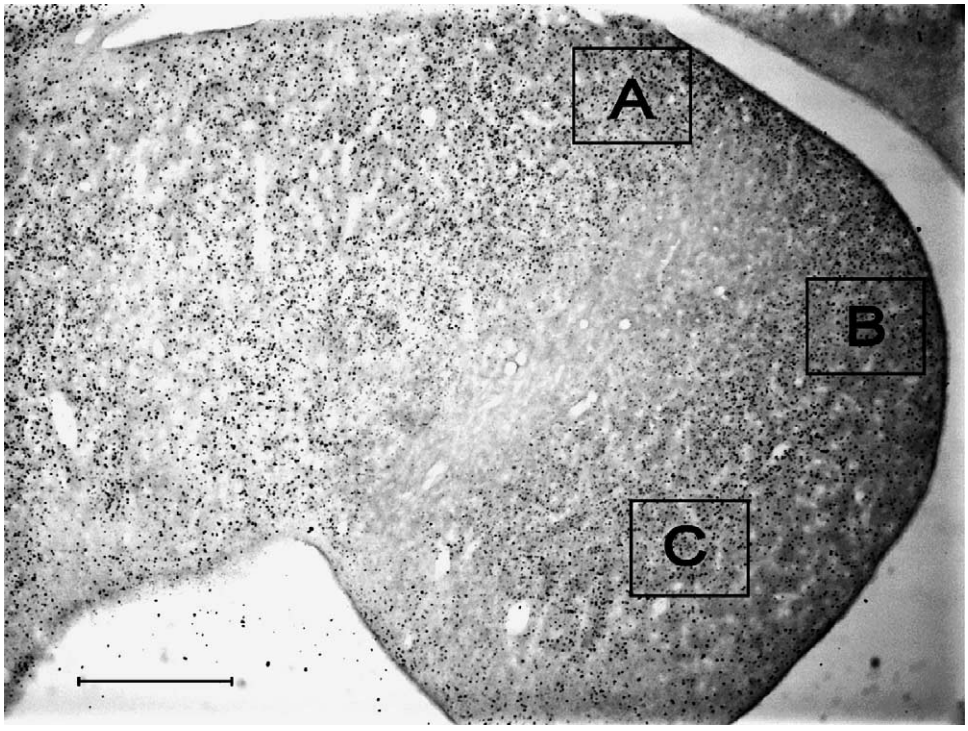

Fig. 1. Photomicrographs of a sagittal section of a female zebra finch auditory forebrain illustrating Zenk immunoreactivity in the audio and video playback condition. The boxes indicate regions sampled to quantify Zenk-ir in CMM (A), NCMd (B) and NCMv (C). Left is rostral and up is dorsal. Scale bar is 0.5 mm.

paraformaldehyde for 24 h then placed in 30% sucrose in PBS for approximately 24 h until saturated. The brains were then frozen in dry ice and stored at −70 °C until immunocytochemistry (ICC) for Zenk protein was performed.

2.3. Immunocytochemistry

For each bird, twenty-four 40 μm sagittal sections starting from the midline were sectioned on a cryostat for each hemisphere and collected into PBS. We processed brains in batches balanced across each of the four treatment groups. Thus, although male and female brains were processed in separate runs playback treatment was balanced across runs.

Sections were washed in 0.1 M PBS, incubated in 0.5% H_2O_2 for 15 min, and washed again in 0.1 M PBS. Next, sections were blocked in 10% normal goat serum for 1 h, followed by incubation in the primary antibody (anti Egr-1, Santa Cruz Biotechnology, catalog # sc-189) at a concentration of 1:20 000 in PBS containing Triton X-100 (PBS/T) for approximately 24 h. Then sections were washed in PBS/T and incubated in biotinylated goat-anti rabbit antibody for 1 h (1:200 dilution in PBS/T). Next, sections were washed in PBS/T, incubated in avidin-biotin horseradish peroxidase (ABC Vectastain Elite Kit) for 1 h and washed in 0.1 M PBS. Finally, the sections were visualized using 3′,3-diaminobenzidine tetrachloride (Sigma FastDAB), mounted on gelatin-coated microscope slides, dehydrated in ethanol and protected with coverslips affixed with Permount (Sigma).

Zenk immunoreactivity (Zenk-ir) was quantified for three auditory brain regions: the dorsal and ventral parts of the caudal medial nidopallium (NCMd, NCMv) and the caudal medial mesopallium (CMM; Fig. 1). The lateral ventricle defined the dorsal, ventral, and caudal borders of NCM, and Field L defined the rostral border. Zenk-ir in NCM was assessed at a dorsal and ventral location (Fig. 1). Zenk-ir in CMM was quantified in the same sections used for NCM and was assessed in the most caudal area bounded by the lateral ventricle and the caudal-ventral boundary of the mesopallial lamina (LaM). The dorsal and caudal borders of HVC were defined by the lateral ventricle and the border with NCM defined its rostral and ventral borders.

For each zebra finch, eight sections per hemisphere (to 480 μm from the midline) were measured for Zenk-ir. Quantification began with the first section in which NCM was attached to the rest of the brain to guarantee that it was mounted in the correct orientation, and continued for the next seven sections moving laterally. For each bird 16 images (0.39 mm × 0.29 mm) of each brain region were captured using a Zeiss Axiophot microscope and SPOT Insight digital camera. Images were captured from locations used in previous studies (e.g. [9,12,26]) and sampled dorsal and ventral portions of NCM as well as CMM. The locations of these areas are illustrated in Fig. 1. For CMM, the image was captured from the most caudal part of the region. For NCM, a dorsal image was captured from the most dorso-caudal part of NCM and a ventral image was captured from the centre of the ventro-rostral area of relatively high immunoreactivity. This sampling method thus captured images from the areas with highest density of immunopositive cells within these auditory regions.

For each image, we counted the number of immunoreactive cells following a semi-automated protocol using SigmaScan Pro software (SPSS Science). In a pilot study we confirmed that this protocol produced almost identical results to cell counts performed manually. In brief, we converted each image to grey scale and set a threshold mask that highlighted all immunopositive cell nuclei. In a subset of images ($n = 5$ birds) we recorded the minimum and maximum sizes

of cells (determined visually) counted by the system, and then averaged across birds. We used this range of cell sizes to determine what objects counted by the system were actually immunopositive cells. Objects that were smaller or larger than this range (7.7–34.5 μm^2) were eliminated from the automated counts to give a more accurate estimate of the number of immunopositive cells in each image. An observer blind to treatment condition and sex of the subjects performed all cell counts.

3. Results

3.1. Auditory brain regions

To determine potential effects of sex, treatment condition, brain region, hemisphere and medial-lateral position we initially conducted an omnibus multi-way ANOVA including sex and treatment group as between-subjects factors and brain region (CMM, NCMv or NCMd), hemisphere (left, right) and medial-lateral position (section numbers 1–8) as within-subject factors. This analysis revealed main effects of treatment group ($F(3,19) = 3.2$, $P = 0.046$), brain region ($F(2,38) = 18.6$, $P < 0.001$), and hemisphere ($F(1,19) = 19.9$, $P < 0.001$). Neither sex ($F(1,19) = 1.6$, $P = 0.23$), nor medial-lateral position ($F(7,133) = 1.2$, $P = 0.32$) were significant main effects. In addition, there were several significant interaction terms. In particular there was a significant interaction of brain region × sex ($F(2,38) = 23.3$, $P < 0.001$), of brain region × treatment group ($F(6,38) = 2.7$, $P = 0.03$), and of brain region × medial-lateral position ($F(14,266) = 2.3$, $P = 0.006$). The interaction of treatment group × medial-lateral position was also significant ($F(21,133) = 1.8$, $P = 0.03$).

Differences in Zenk-ir between the three auditory brain regions may result from a number of factors independent of the experimental manipulations of interest here. Because of this, and because there were significant interactions between brain region and several other factors of interest (see above) we ran separate multi-way ANOVAs for each of these three brain regions. As well, because there was no significant main effect of medial-lateral position in our omnibus ANOVA we used the average number of Zenk-ir cells across the eight sampled sections for each hemisphere as the dependent measure in these separate sex × treatment group × hemisphere ANOVAs.

In CMM there was significant variation in Zenk-ir between sexes, between treatment groups and between hemispheres (Fig. 2). A multi-way ANOVA revealed a significant main effect of sex ($F(1,20) = 7.1$, $P = 0.015$), treatment group ($F(3,20) = 6.9$, $P = 0.002$), and hemisphere ($F(1,20) = 9.9$, $P = 0.005$) with no significant interactions among these factors. Thus in CMM, Zenk-ir was greater in females than in males and was greater in the left hemisphere. Post hoc analyses (Tukey's HSD) revealed that in CMM Zenk-ir in the audio/video and audio only conditions were both significantly different from the no audio/video condition ($P = 0.002$ and $P = 0.03$ respectively). The video only condition, however, did not differ significantly from any of the other three conditions (Fig. 2). Thus, in CMM male and female zebra finches exposed to the two playback conditions with an audio component had the greatest Zenk-ir response and those exposed to the video only condition had an intermediate response. To further analyze the effect of laterality, we compared the amount of Zenk-ir the left and right hemisphere individually for each bird and found that in CMM, 21 of 28 birds had greater Zenk-ir in the left hemisphere.

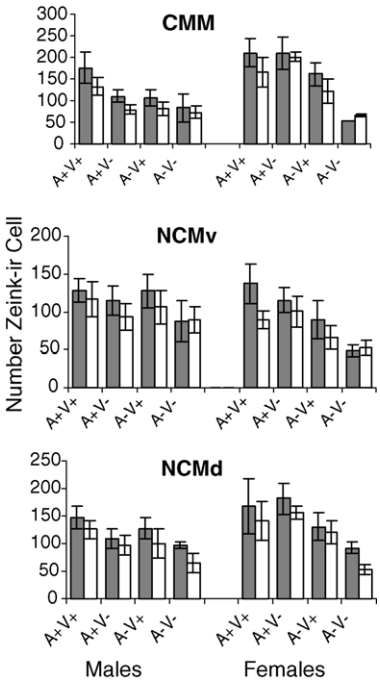

Fig. 2. Mean (±S.E.) number of Zenk-ir cells in CMM, NCMv and NCMd for male and female zebra finches exposed to both audio and video (A+V+), audio only (A+V−), video only (A−V+), or neither audio nor video (A−V−) of a digital movie of a courting male zebra finch. Cell counts are the number cells per field of view samples (see Section 2). Filled bars are means for the left hemisphere, open bars are means for the right hemisphere.

In NCM (both ventral and dorsal regions) there was a significant difference in Zenk-ir between hemispheres (NCMv: $F(1,20) = 23.3$, $P < 0.001$; NCMd: $F(1,20) = 10.3$, $P = 0.004$), but there was no significant main effect of sex (NCMv: $F(1,20) = 1.4$, $P = 0.25$; NCMd: $F(1,20) = 1.9$, $P = 0.19$), nor treatment group (NCMv: $F(3,20) = 2.4$, $P = 0.10$; NCMd: $F(3,20) = 1.7$, $P = 0.19$), nor any significant interactions. Thus, in NCM Zenk-ir was greater in the left hemisphere but did not vary between the sexes or treatment groups. To further analyze the effect of laterality, we compared the amount of Zenk-ir the left and right hemisphere individually for each bird and found that in NCMv, 23 of 27 birds had greater Zenk-ir in the left hemisphere (one bird showed no dominance), and in NCMd, 20 of 28 birds had greater Zenk-ir in the left hemisphere.

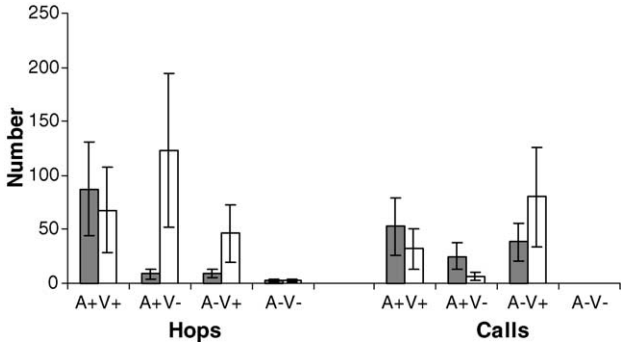

Fig. 3. Mean (±S.E.) number of hops and calls performed by male (filled bars) and female (open bars) zebra finches in response to a digital movie of a courting male zebra finch. Treatment groups were exposed to both audio and video (A+V+), audio only (A+V−), video only (A−V+), or neither audio nor video (A−V−).

3.2. Behavioral responses

To determine if birds in different playback groups differed in their behavioral responses to the playback we compared groups in separate ANOVAs for the number of calls, the number of songs (for males only) and number of hops performed during the 30 min playback session. For calls and hops we conducted two-way ANOVAs of sex × playback condition. For songs we conducted a one-way ANOVA of playback condition because female zebra finches do not sing. There were no significant main effects of sex on the number of calls and hops (Fig. 3; calls $F(1, 20) = 0.006$, $P = 0.94$; hops $F(1, 20) = 2.29$), $P = 0.15$). Although the control group (no audio or video) appeared to have the fewest behavioral responses compared to the other groups, there was no significant main effect of playback group for any behavior (Fig. 3; calls $F(3,20) = 1.55$, $P = 0.23$; hops $F(3,20) = 1.14$, $P = 0.36$; song bouts $F(3,13) = 1.13$, $P = 0.37$).

To determine whether the birds' own behavioral responses related to their Zenk responses, we tested the correlations between behavioral responses (calls, hops, song bouts), and Zenk-ir cell counts in CMM and NCM separately for each group. We combined data from both sexes for correlations with calls and hops, but examined correlations with song bouts only for males. There were no significant correlations among any behavior and Zenk-ir in the three areas, indicating that Zenk-ir counts in CMM and NCM were not related to vocal behavior or general activity in individual birds of each group.

4. Discussion

4.1. Response to playback

Overall, we found that the Zenk response in CMM was significantly elevated in birds exposed to the acoustic stimulus of song playback. There was limited evidence that visual stimulus of a male zebra finch or the behavioral response of the subject during playback altered this Zenk response. Birds in the audio and video, and audio only groups had significantly greater Zenk-ir in CMM than birds in the control group (no audio or video). Birds exposed to video only had levels of Zenk-ir that differed neither from the birds hearing audio stimuli nor birds in the control group suggesting some intermediate level of activation. In particular, Zenk-ir in the CMM of females exposed to only visual stimuli was closer to levels found in females exposed to song than to control females (Fig. 2). The source of this intermediate level of activation is unclear, but could have resulted from sounds generated by the birds moving about the cage or calling.

Although females had significantly greater levels of Zenk-ir than males, there was no significant interaction between sex and playback condition, thus the effects of stimulus type on Zenk-ir in CMM occurred in both sexes. We also found no differences in amounts of behavioral activity (calls, hops, song bouts) among the playback groups, nor was there any relationship between these activities and Zenk-ir in any region (CMM and NCM) for individuals within any group. Therefore, it seems that individual birds' behavior during playback did not affect the results.

It has been well demonstrated that both CMM and NCM are part of the auditory processing system in songbirds (see Section 1). However, in this study the only brain region that differed in Zenk-ir among our playback treatments was CMM. Zenk-ir in NCM followed a similar trend to that observed in CMM (see Fig. 2), with highest numbers of Zenk-ir cells found in the two treatment groups receiving audio playback. Thus the lack of a statistically significant main effect may have resulted from insufficient sample size. In general however, increased IEG response to conspecific vocalizations has more consistently been found in CMM than in NCM in a variety of avian taxa (reviewed in [28]).

Few studies have presented multimodal stimuli in the study of song learning and song perception. Recently it was found that habituation of the Zenk response to song in male zebra finches was dishabituated if a visual stimulus (colored lights) were presented in synchrony with song [16]. This study suggests that visual stimuli modulate activity in the auditory forebrain through associative or attentional mechanisms. In our own study, the visual stimulus of a courting male was not associatively paired with song during the experiment, however the subjects would have experienced a lifetime of the pairings of these two types of stimuli. In our experiment visual stimuli paired with song did not increase Zenk-ir over that observed to song alone. However, birds presented with only visual stimuli and no song had Zenk-ir intermediate to those hearing song and control birds (Fig. 3). This result could result from either prior associations of the visual stimulus with song, or enhanced attention to acoustic stimuli other than song (e.g., cage noise, calling, or ventilation fans). We are thus unable to differentiate between the attentional and associative models proposed by Kruse et al. [16]. Our study, in conjunction with Kruse et al., does suggest that the Zenk

response in the auditory forebrain may be modulated by other sensory modalities. Further work is required to explore this topic.

4.2. Lateralization

We found a lateralization of Zenk-ir response in specialized auditory brain regions (CMM, NCM). The lateralized response was an unexpected finding, and the experiment was not designed to test for lateralization of Zenk response per se. There are no simple histological explanations for the consistently greater immunoreactivity in the left hemispheres so we are left to conclude that most birds did indeed have lateralized brain activity during the playback session. Lateralization of song production has been previously reported (e.g. [7,25,31]). There is also prior evidence that avian perceptual systems are lateralized and left-dominated. Lateralization of both anatomy and function of the visual system in a variety bird species is generally evidenced by greater use of the right eye and left hemisphere [11,24]. Indeed, lateralization is reported for IEG response to sexually imprinted stimuli [17]. In zebra finches, Workman and Andrew [32] found a right-eye and left-hemisphere dominance in the perception of courtship behavior (but see [27]). In the present study, the auditory areas CMM and NCM also responded with greater Zenk-ir activation in the left hemisphere.

Several studies have now also reported lateralization in the auditory processing of song. In a lesion study, Cynx et al. [5] found hemispheric differences in auditory processing of song by zebra finches, with the right hemisphere being more important in discriminating precise auditory information within songs, and the left hemisphere being more important in discriminating their own from a cage-mate's song. Electrophysiological studies have also revealed hemispheric differences in Field L in starlings (*Sturnus vulgaris*) with the right hemisphere having greater response to a bird's own song and familiar songs, and the left hemisphere having greater response to unfamiliar songs [10]. Thus, lesion studies, electrophysiology, and now immediate-early gene expression all indicate lateralization in the processing of conspecific song by songbirds.

Acknowledgments

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