Immediate Early Gene (ZENK) Responses to Song in Juvenile Female and Male Zebra Finches: Effects of Rearing Environment

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ABSTRACT: **Accurate song perception is likely to be as important for female songbirds as it is for male songbirds. Male zebra finches (*Taeniopygia guttata)* show differential ZENK expression to conspecific and heterospecific songs by day 30 posthatch in auditory perceptual brain regions such as the caudomedial nidopallium (NCM) and the caudomedial mesopallium (CMM). The current study examined ZENK expression in response to songs of different qualities at day 45 posthatch in both sexes. Normally reared juvenile zebra finches showed higher densities of immunopositive nuclei in both the dorsal and ventral areas of NCM and CMM (formerly cmHV), but not HA, a visual area, in response to normal song over untutored song or silence. Male and female patterns of ZENK expression did not differ. We next compared responses of birds reared without exposure to normal song (untutored) to those of normally reared birds. Untutored birds did not show higher responses to normal song than to untutored song in the three song perception areas. Furthermore, untutored birds of both sexes showed lower densities of immunopositive nuclei in all four areas than did normally reared birds. In addition, ZENK expression was greater in untutored females than in males in the dorsal portion of NCM and in CMM. Our findings suggest that at least some neural mechanisms of song perception are in place in socially reared female and male finches at an early age. Furthermore, early exposure to song tutors affects responses to song stimuli.** © 2006 Wiley Periodicals, Inc. J Neurobiol 66: 1175–1182, 2006

Keywords: **immediate early genes; ZENK; zebra finches; song learning; sex differences**

INTRODUCTION

For both male and female zebra finches (*Taeniopygia guttata*), early exposure to song is important for normal adult behavior. Although only males sing, females must learn about song in order to develop preferences for particular songs as adults. Females raised without fathers do not show the species-typical preference for normal song over the impoverished song produced by males reared without hearing song (Lauay et al., 2004).

Both males (Marler and Peters, 1982) and females (Miller, 1979; Riebel et al., 2002) remember songs heard at an early age. Activation of immediate early genes (IEGs), such as ZENK, is linked to synaptic plasticity and may be part of the song memory consolidation process (reviewed in Mello et al., 2004). Certain structures, such as the caudal medial nidopallium (NCM) and the caudomedial mesopallium (CMM, previously cmHV, see Reiner et al., 2004) are involved in conspecific song perception (Mello and Clayton, 1994).

Effects of song on ZENK expression have been well studied in male songbirds. In adult male zebra

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finches, the number of cells expressing ZENK after hearing conspecific song is twice as high in CMM and NCM as after hearing canary song (Mello et al., 1992). These responses are apparent at a young age. Hearing song induces ZENK expression in NCM in males at day 30 posthatch but not at day 20 (Jin and Clayton, 1997). Social isolation affects patterns of IEG expression in response to song. Jin and Clayton (1997) found that clutch isolates (animals reared with conspecifics from the same clutch and their mother, but without fathers) at posthatch day 30 showed higher ZENK responses to normal song just as did socially reared birds. However, birds reared in solo isolation (with mother only) differed from socially reared birds in that they showed only baseline level responses to song.

Males also respond to differences in conspecific song characteristics. When males are re-exposed as adults to the songs of their tutor, there is a correlation between the number of immunopositive cell nuclei in NCM and the number of song elements that the male copied from the tutor's song (Bolhuis et al., 2000; Terpstra et al., 2004). Furthermore, repeated exposure to a particular conspecific song leads to a marked decrease in ZENK expression (Mello et al., 1995).

Although much is known about how and when males process song stimuli, less is known about song perception in females. Adult female white-crowned sparrows show higher rates of ZENK expression in NCM and CMM in response to their hatch dialect than to foreign dialects (Maney et al., 2003). ZENK expression in NCM is higher in response to conspecific song relative to songs from unrelated species in adult female zebra finches (Bailey et al., 2002). Adult female starlings show greater ZENK responses to long song (which is attractive to females) than to short song (Sockman et al., 2002). Adult female canaries similarly show higher ZENK responses in CMM, but not NCM, to ''sexy'' syllables (more complex syllables) than to less complex syllables (Leitner et al., 2005).

Although it is clear that females distinguish between songs on the basis of quality, much less is known about the development of song perception in females of any avian species. Normally reared female zebra finches show differential ZENK responses to conspecific song and song from unrelated species at posthatch day 45 (Bailey and Wade, 2005), but not at day 30 (Bailey and Wade, 2003). There is some evidence that, in adulthood, female zebra finches do not prefer songs they heard before 25 days posthatch, but further characteristics of the sensitive period for song learning in females still remain unclear (reviewed in Riebel, 2003).

The present study examines ZENK expression at posthatch day 45 in males and females that had been reared in two conditions. In normally reared male zebra finches, 45 days is near the end of the phase of acquiring an auditory model for song and is early in the phase of practicing song (Tchernichovski et al., 2001). We compared ZENK responses in both sexes between socially reared zebra finches and finches reared without exposure to song (untutored). We focus on ZENK expression in two regions of NCM and in CMM in response to normal zebra finch song and untutored song. The number of ZENK-labeled cells in the ventral portion of HA, a visual area, was quantified as a control. Untutored song is less complex than normal zebra finch song in that it contains fewer syllable types, has longer intersyllable intervals, and is higher in frequency than normal song (Price, 1979; Lauay et al., 2004). We predicted that socially reared animals would show higher ZENK responses to social song stimuli in comparison to untutored song or silence. In addition, we predicted that normal males and females would not differ in patterns of ZENK expression in each of the three auditory perception areas, because auditory song learning is equally important for males and females. We also predicted that untutored males and females would show lower responses than socially reared animals, due to impoverished learning during development. Finally, we predicted that untutored animals would not differ in their responses to social and untutored song.

METHODS

Subjects

A total of 82 juvenile zebra finches (47 females and 35 males) were included in the study. Subjects were obtained from breeding colonies at Cornell University. Forty-four subjects (27 females and 17 males) were reared in social aviaries. These social aviaries consisted of 8–10 paired adults and their young. Thirty-eight subjects (20 females and 18 males) were reared in the untutored condition. These aviaries were the same size as the social aviaries, but all adult males were removed when the oldest nestling reached 8 days posthatch. To minimize variation in hatch dates, breeding was synchronized by removing nest boxes for several months and then reintroducing the nest boxes along with nesting materials. Thus, clutches in each aviary hatched out within a week of each other. This same synchronization process was carried out in both rearing conditions. All aviaries were maintained on a 14:10 light cycle. Seed and water were provided ad libitum. Additionally, animals were supplemented three times per week with egg and alfalfa sprouts. At the time of testing, subjects were

between the ages of 43 and 52 days. They were randomly assigned to one of three stimulus categories: normal zebra finch song ($n = 32$), untutored zebra finch song ($n = 30$), or silence ($n = 20$). When possible, all three conditions were conducted each testing day.

Stimulus Procedure

Subjects were removed from their aviaries the evening before testing and isolated overnight. All stimulus presentations were conducted during the first 5 h of the light portion of the cycle. All subjects within a particular condition were exposed to the same auditory stimulus. Stimulus tapes created using songs of three different unfamiliar males from our colony were used for both the normal and the untutored song conditions. Thus, each individual within a treatment condition heard the same stimuli. Tape recorders were kept at a consistent volume and distance from the testing cage throughout the study to control for differences in song amplitude. The song stimuli consisted of 15 s of one song followed by 45 s of silence. Then, 15 s of the second song was played, followed by another 45 s of silence. The third song was played in the same manner, and the entire presentation was repeated for 45 min. After the stimulus presentation, birds were kept in silence for 45 min. Birds in the silence condition received 90 min of silence.

Tissue Collection and Processing

At the end of the testing period, birds were given an overdose of Chloropent and were perfused transcardially with 0.9% saline phosphate buffer (NaPB) followed by 4% paraformaldehyde in NaPB. The brain was immediately removed, postfixed for 1 h in the same fixative, and stored in 30% sucrose in potassium phosphate-buffered saline (KPBS) until sectioning. Single hemispheres were frozen with dry ice and sectioned in the sagittal plane at 40 μm with a sliding microtome. Sections were stored in 0.2 *M* KPBS with 0.1% sodium azide until being processed for immunocytochemistry.

ZENK Immunocytochemistry

Every fourth 40 μm section was immunolabeled for ZENK (also known as *zif*-268, *egr*-1, NGFI-A, or *krox*-24). Adjacent sections were stained with cresyl violet to aid in locating major brain subdivisions. Sections were washed for 3×10 min in KPBS and then incubated for 1 h at room temperature in a blocking solution of 5% normal goat serum (Vector, Burlingame, CA) and 0.15% Triton X-100. The tissue was washed again (3×10 min in KPBS) and incubated for approximately 72 h at 4°C using a rabbit polyclonal antibody raised against EGR-1 (Santa Cruz Biotechnology, Santa Cruz, CA) diluted at 1:1000 in KPBS with 2% normal goat serum at 0.15% Triton X-100. Following this incubation in the primary antibody, the tissue was washed and incubated for 1 h at room temperature in a goat antirabbit secondary (Vector) diluted at 1:500. Following another wash, the tissue was incubated for an hour at room temperature in avidin-biotin horseradish peroxidase complex (Vectastain ABC elite kit, diluted at 1:200; Vector). Sections were then placed in diaminobenzidine (DAB; Sigma Corp.) enhanced with nickel chloride to visualize immunolabeled cells. Sections were mounted onto gelatin-coated slides and coverslipped.

Quantification of ZENK

ZENK-immunopositive nuclei were quantified in three regions selected on the basis of findings in previous research: dorsal portion of NCM, ventral portion of NCM, and CMM (Vates et al., 1996; Gentner et al., 2001; Maney et al., 2003). The ventral portion of HA, a nearby telencephalic area involved in visual processing (Jarvis et al., 2005), was quantified as a control. Although we did not use any specific scientific procedure to discriminate between neurons and glia, we avoided counting small nuclei, because glia typically have a nucleus that is smaller in diameter than neurons. All immunopositive nuclei within a 250×250 μm sampling area were counted in each of the areas of interest. Three to four sampling areas from separate sections (mean = 3.5) were quantified in each region for each subject. Counts from individual sampling areas were averaged within each individual. All observers were blind to the experimental condition of the brain tissue they assessed. A total of three observers quantified the data. Observers were trained together until a high degree of interobserver reliability (>95%) was reached. Then, two observers each quantified a single area (HA and NCMv). The primary observer (M.T.) quantified the rest of the data and verified the data from a random sample of quantifications made by the other two observers.

Quantification of Cell Densities

Differences in number of ZENK-immunopositive nuclei between experimental groups could result from differential responses of cells within an area, or from differences in overall cell densities in each of the four regions without a difference in proportional ZENK response. To address this possibility, every fourth 40 μm section was stained with cresyl violet. Using the same criteria as above for identifying brain areas, we chose one section from each individual and quantified the densities of cells in each of the four areas in a 800×800 μm sampling area. These data were quantified by the primary observer (M.T.).

Data Analysis

Cell density data were analyzed in a sex $\times$ rearing condition $\times$ stimulus ANOVA with brain area as the within-subjects variable. ZENK data were analyzed using a sex $\times$ rearing condition $\times$ stimulus ANOVA with brain area as the within-subjects variable and cell densities in the four brain regions as covariates. Effects significant at the 0.05 level were further examined using Bonferroni posthoc anal-

Table 1 Density of Cells in the Ventral Portion of the Caudal Medial Nidopallium (NCMv), the Dorsal Portion of the NCM (NCMd), the Caudomedial Mesopallium (CMM), and Hyperpallium Accessorium (HA) in 45-Day-Old Socially Reared or Untutored Male and Female Zebra Finches

	Cell Density (Mean ± SE)			
	NCMv	NCMd	CMM	HA
Socially reared female	50.48 ± 2.67	46.70 ± 3.25	44.83 ± 2.26	30.61 ± 1.71
Untutored female	48.28 ± 3.89	43.39 ± 3.09	46.50 ± 2.31	33.06 ± 3.83
Socially reared male	56.31 ± 3.60	55.94 ± 3.34	59.81 ± 4.67	37.31 ± 3.34
Untutored male	46.29 ± 1.98	43.43 ± 2.72	46.79 ± 2.48	39.00 ± 1.29

yses or paired *t* tests (for within-subjects variables). The significance level was set at 0.05 for all analyses. Data in all figures represent actual means and standard errors, despite the inclusion of covariates in the analysis.

RESULTS

Cell Densities in Socially Reared and Untutored Zebra Finches

Brains in which visualization of cell nuclei was difficult were excluded from the analysis. A total of 71 brains were included in the analysis. Overall, there were significant difference in cell densities across brain areas [Wilk's Lamda; $F(3, 65) = 53.09$; $p < 0.05$]. Further analyses revealed that NCMv (50.41 ± 1.61) contained more cells per unit volume than did NCMd (47.30 ± 1.68; $t_{70} = 2.58$, $p < 0.05$). The HA (34.39 ± 1.40) contained fewer cells per unit volume than all other brain areas, including CMM (49.01 ± 1.62; NCMv vs. HA, $t_{70} = 10.78$, $p < 0.05$; NCMd vs. HA, $t_{70} = 8.31$, $p < 0.05$; CMM vs. HA, $t_{70} = 9.71$, $p < 0.05$).

Overall, there was a significant effect of sex on cell densities across all brain areas [$F(1, 67) = 4.21$; $p < 0.05$]. Females (45.56 ± 2.25) had fewer cells in CMM than did males [51.633 ± 2.29; $F(1, 69) = 6.72$; $p < 0.05$]. Females (31.68 ± 1.92) also had fewer cells in the HA than did males [38.10 ± 1.86; $F(1, 69) = 5.45$; $p < 0.05$].

There was no significant effect of rearing environment on cell densities [$F(1, 67) = 3.10$; $p > 0.05$; see Table 1 for means]. There was also no significant interaction between sex and rearing condition on cell densities across the four brain areas [$F(1, 69) = 2.64$; $p > 0.05$; see Table 1].

Overall Patterns of ZENK Expression in Juvenile Zebra Finches

There was no significant effect of brain area on ZENK expression [Wilk's Lamda; $F(3, 53) = 1.09$; $p > 0.05$]. Levels of ZENK expression were highest in NCMv (19.09 ± 2.31) and HA (18.41 ± 2.24), lower in NCMd (17.53 ± 2.34), and lowest in CMM (14.22 ± 1.98).

There was no effect of sex on patterns of ZENK expression [$F(1, 55) = 1.93$; $p > 0.05$]. However, there was a main effect of rearing condition [$F(1, 55) = 12.31$; $p < 0.05$]. There was also an overall effect of stimulus condition on patterns of ZENK expression [$F(2, 55) = 6.20$; $p < 0.05$]. There were no significant interactions between sex, rearing environment, and stimulus condition.

ZENK Expression in Socially Reared Zebra Finches

In socially reared birds, there was a significant interaction between stimulus condition and brain area

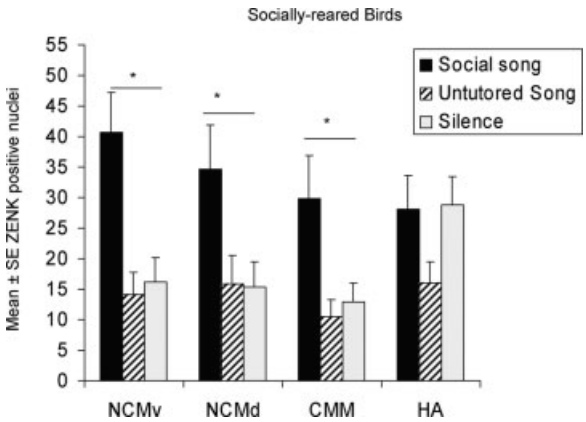

Figure 1 Density of ZENK-positive immunostaining in the ventral portion of the caudal medial nidopallium (NCMv), the dorsal portion of the NCM (NCMd), the caudomedial mesopallium (CMM), and hyperpallium accessorium (HA) in 45-day-old socially reared male and female zebra finches exposed to normal male zebra finch song, untutored zebra finch song, or silence. Social song stimulus, $n = 18$; untutored song stimulus, $n = 17$; silence stimulus, $n = 9$. Asterisk indicates significant difference from social song stimulus at the 0.05 level.

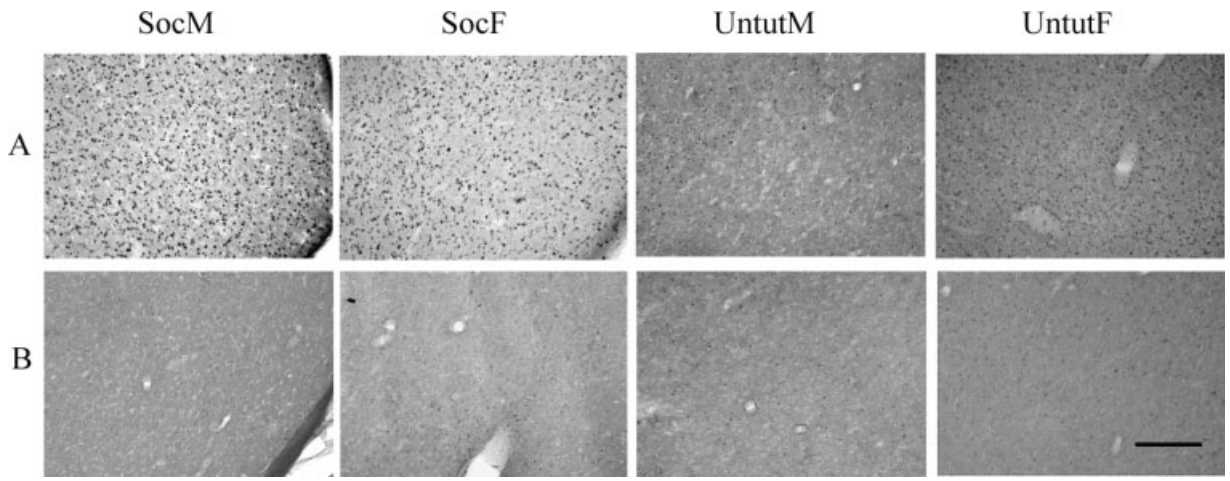

Figure 2 Examples of ZENK-positive immunostaining in the ventral portion of the caudal medial nidopallium (NCMv) in 45-day-old zebra finches exposed to (A) normal male zebra finch song and (B) untutored male song. SocM, socially reared male; SocF, socially reared female; UntutM, untutored male; UntutF, untutored female. Densities of ZENK staining are similar in socially reared males and females, but lower in untutored animals. Socially reared animals of both sexes showed higher responses to normal zebra finch song than to untutored zebra finch song, whereas untutored animals did not. Scale bar = 200 μm.

[Wilk's Lamda; $F(6, 54) = 3.68$; $p < 0.05$]. Posthoc analyses revealed that hearing normal song produced greater IEG expression than untutored song or silence (Figs. 1 and 2) within NCMv, NCMd, and CMM, but not HA.

There were no significant sex differences in patterns of ZENK expression (see Fig. 3). Thus, males and females do not differ in patterns of ZENK expression at 45 days of age in response to songs of different complexities.

Comparison of Untutored and Socially Reared Male and Female Zebra Finches

There was a main effect of rearing condition [$F(1, 55) = 12.31$; $p < 0.05$]. Posthoc analyses revealed that socially reared birds showed higher levels of IEG expression in the three auditory regions and HA than did untutored birds (see Fig. 4). We next examined responses by untutored birds to the different stimulus types. Like socially reared birds, untutored zebra finches showed an overall significant effect of stimulus type [$F(2, 22) = 4.84$; $p < 0.05$]. This was true for both regions of NCM [NCMv: $F(2, 22) = 5.36$; $p < 0.05$; NCMd: $F(2, 22) = 4.05$; $p < 0.05$; see Fig. 5]. Follow-up analyses revealed that levels of IEG expression in response to social song were significantly higher than responses to silence, but responses to normal song and untutored song were similar. Unlike socially reared animals, IEG expression in CMM did not significantly differ across stimulus conditions in untutored animals [$F(2, 22) < 1$]. Furthermore, expression in HA differed across stimulus conditions [HA: $F(2, 22) = 3.62$; $p < 0.05$]. Responses to normal song were higher than responses to untutored song, but neither song condition significantly differed from the control condition. Thus, untutored animals did not show selective IEG responses to song in any of the three song perception areas.

ZENK Expression in Untutored Male and Female Zebra Finches

We then examined patterns of IEG expression in untutored male and female zebra finches. Overall, there was no significant sex $\times$ stimulus interaction [$F(2, 22) = 2.20$; $p > 0.05$]. There was, however, a significant effect of sex on patterns of IEG expression in untutored animals [$F(1, 22) = 5.45$; $p < 0.05$]. Further analyses revealed that untutored females showed higher IEG responses than did untutored males in NCMd and CMM (see Fig. 6). IEG responses were similar in NCMv and HA.

DISCUSSION

We found that normally reared juveniles of both sexes exposed to normal (social) song showed greater ZENK expression in three auditory perceptual brain

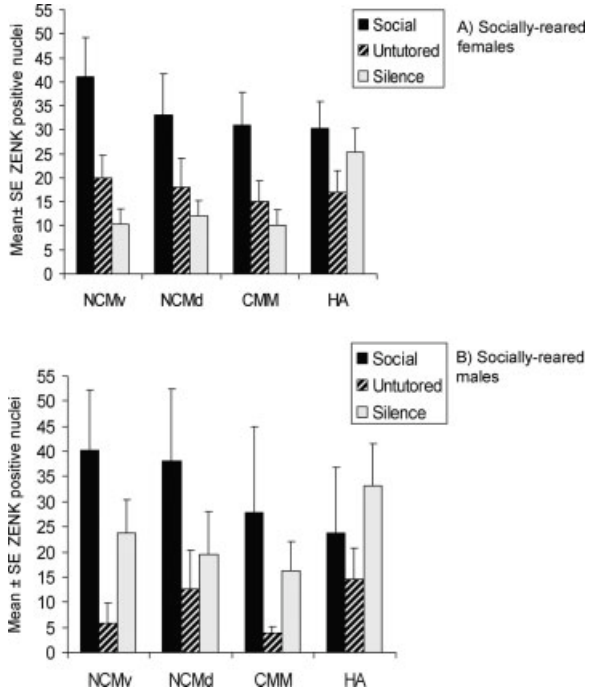

Figure 3 Comparison of 45-day-old socially reared female (A) and male (B) zebra finches in the density of ZENK-positive immunostaining in the ventral portion of the caudal medial nidopallium (NCMv), the dorsal portion of the NCM (NCMd), the caudomedial mesopallium (CMM), and hyperpallium accessorium (HA). They were exposed to normal male zebra finch song, untutored zebra finch song, or silence. Females: social song stimulus, $n = 12$; untutored song stimulus, $n = 10$; silence stimulus, $n = 5$. Males: social song stimulus, $n = 6$; untutored song stimulus, $n = 7$; silence stimulus, $n = 4$.

areas than juveniles exposed to either untutored song or silence. These results were consistent across the three areas involved in song perception and were similar in the two sexes. This suggests that at least some of the neural mechanisms that allow birds to perceive differences in the complexities of songs are similar in male and female zebra finches.

Much more is known of the timing and characteristics of song learning in males than in females. By 45 days of age, male zebra finches have learned song features from a tutor and are beginning to practice their own songs. We report here that IEG expression in three high perceptual regions is modulated by the quality of songs presented to males at this age.

In females, less is known about the sensitive period for song learning. Recently, we found that adult females raised without exposure to song have fewer dendritic spines (reflecting fewer spine synapses) in NCM than normally reared females (Lauay et al., 2005). Additionally, adult females reared without

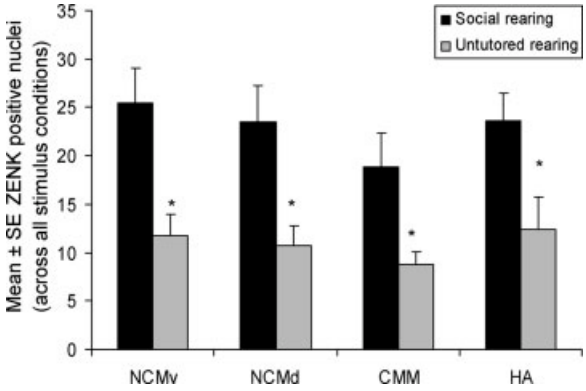

Figure 4 Overall (across the three stimulus conditions), the density of ZENK-positive immunostaining is greater in the ventral portion of the caudal medial nidopallium (NCMv), the dorsal portion of the NCM (NCMd), the caudomedial mesopallium (CMM), and the hyperpallium accessorium (HA) in 45-day-old zebra finches reared socially than in birds reared in the untutored condition (reared without adult males). Social rearing condition, $n = 44$; untutored rearing condition, $n = 38$. Asterisk indicates significance at the 0.05 level.

fathers show no preference for social song over untutored song in behavioral tests (Lauay et al., 2004). Adult females also do not prefer songs they heard before day 25 posthatch, though later exposure periods have yielded conflicting results (reviewed in Riebel, 2003). We now find that, by day 45, normally

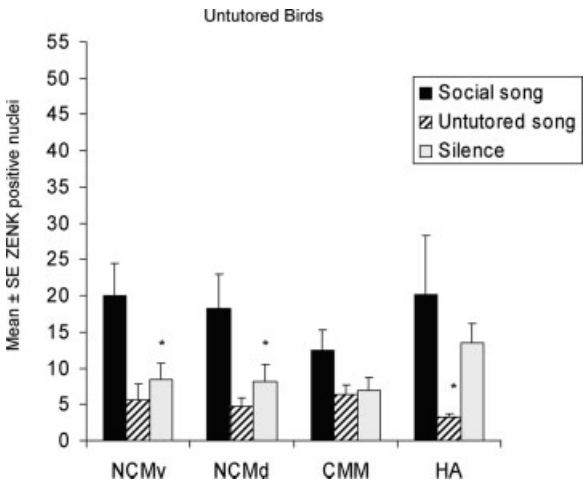

Figure 5 Density of ZENK-positive immunostaining in the ventral portion of the caudal medial nidopallium (NCMv), the dorsal portion of the NCM (NCMd), the caudomedial mesopallium (CMM), and the hyperpallium accessorium (HA) in 45-day- old untutored zebra finches exposed to either normal male zebra finch song, untutored zebra finch song, or silence. Social song stimulus, $n = 14$; untutored song stimulus, $n = 13$; silence stimulus, $n = 11$. Asterisk indicates significance at the 0.05 level.

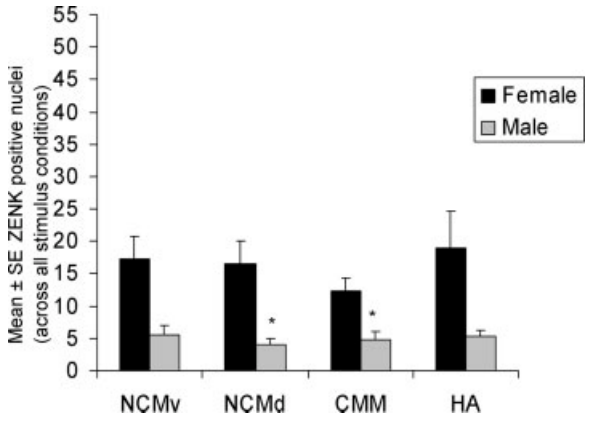

Figure 6 Density of ZENK-positive immunostaining in the ventral portion of the caudal medial nidopallium (NCMv), the dorsal portion of the NCM (NCMd), the caudomedial mesopallium (CMM), and the hyperpallium accessorium (HA) in 45-day- old male and female untutored zebra finches. Untutored female, $n = 20$; untutored male, $n = 18$. Asterisk indicates significance at the 0.05 level.

reared females, like males, show higher ZENK responses to conspecific songs of higher quality.

Bailey and Wade (2003) reported that females at day 30 showed similar ZENK responses to conspecific and heterospecific song. By day 45, females were similar to males in patterns of ZENK expression in both CMM and NCM (Bailey and Wade, 2005). The current study extends these results to differences in responses to conspecific song quality, and suggests that females may develop this differential ZENK expression between day 30 and day 45. Furthermore, these mechanisms are in place prior to forming a pair bond with males. At the time of pair formation (approximately 90 days posthatch), females choose male mates, in part, on the basis of song quality (Tomaszycki and Adkins-Regan, 2005).

Our observation that zebra finch juveniles of both sexes showed basal levels of ZENK expression to untutored song is somewhat surprising. One might expect that untutored song would result in greater ZENK induction than the absence of auditory stimuli (silence condition). However, earlier research suggests that juvenile zebra finches have higher baseline levels of ZENK expression in NCM in comparison to adults (Jin and Clayton, 1997), and our results (which also found high baseline levels of ZENK induction in response to silence) may replicate these findings. On the other hand, these responses may, in part, be induced by hearing their own vocalizations, although previous research has found no relationship between singing and ZENK induction in NCM in males (Mello et al., 1992), and females do not sing. Little is known about ZENK expression in auditory perception regions when hearing nonsong vocalizations (such as contact calls), and we did not quantify vocal behavior during our stimulus presentations.

Overall, untutored birds of both sexes had lower densities of ZENK immunopositive staining than did socially reared zebra finches (Fig. 4). This suggests a potential difference between socially reared and untutored zebra finches in the processing of auditory information. Because there were also differences between socially reared and untutored animals in IEG expression in HA, the control area, the possibility that isolate rearing causes generalized, nonauditory specific deficits in ZENK expression cannot be ruled out. One possibility is that the rearing environment could have affected overall developmental rates, because juvenile ZENK activity is higher than it is in adults.

We also found that untutored females had higher ZENK responses than untutored males in the NCMd and CMM, but not NCMv or HA, suggesting the possibility that fatherless rearing might have more profound effects on IEG expression in males than in females. This finding is paradoxical and further research is needed to elucidate potential differences in learning and patterns of IEG expression between males and females.

Untutored birds also differed from socially reared birds in responses to songs of differing quality: responses to normal song and untutored song were similar in both in the three auditory perception areas (Fig. 5). Previous research from our laboratory has found that females reared without fathers show no behavioral preference for social song over untutored song (Lauay et al., 2004), and our research parallels these findings. Jin and Clayton (1997) found that clutch isolates (similar to our untutored condition) were similar to normally reared birds, in that they had higher ZENK responses to conspecific than heterospecific song. However, untutored song is simply low quality conspecific song, and is therefore more similar to normal conspecific song than is heterospecific song. Our results show that untutored birds may differ from socially reared birds in their ability to distinguish between conspecific songs of differing quality.

Future studies should also examine patterns of ZENK expression in response to finer distinctions in zebra song, such as differences in song length, quality, or complexity. Adult female starlings, for example, show greater ZENK responses to long song (which is attractive to females) than to short song (Sockman et al., 2002). It will be important to test whether male and female songbirds of other species can make similar behavioral distinctions, to deter-

mine whether these abilities are in place at an early age, and whether IEG expression in auditory perception areas reflects these distinctions.

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