Response Biases in Auditory Forebrain Regions of Female Songbirds Following Exposure to Sexually Relevant Variation in Male Song

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Received 7 August 2000; accepted 18 September 2000

ABSTRACT: In many species of songbirds, individual variation between the songs of competing males is correlated with female behavioral preferences. The neural mechanisms of song based female preference in songbirds are not known. Working with female European starlings (*Sturnus vulgaris*), we used immunocytochemistry for *ZENK* protein to localize forebrain regions that respond to sexually relevant variation in conspecific male song. The number of *ZENK*-ir cells in ventral caudo-medial neostriatum [NCMv] was significantly higher in females exposed to longer songs than in those exposed to shorter songs, whereas variation in the total duration of song exposure yielded no significant differences in *ZENK* expression. *ZENK* expression in caudo-medial ventral hyperstriatum [cmHV] was uniformly high in all subjects, and did not vary significantly among the three groups. These results suggest that subregions of NCM in female starlings are tuned to variation in male song length, or to song features correlated therewith. Female starlings exhibit robust behavioral preferences for longer over shorter male songs (Gentner and Hulse; *Anim Behav 59:443–458, 2000*). Therefore, the results of this study strongly implicate NCM in at least a portion of the perceptual processes underlying the complex natural behavior of female choice. © 2000 John Wiley & Sons, Inc. J Neurobiol 46: 48–58, 2001

Keywords: ZENK*; auditory perception; birdsong; female choice mechanisms; European starling; *Sturnus vulgaris

The songs of male oscine birds (songbirds) are among the most phonologically complex of all nonhuman vocal communication signals, and there is a rich body of literature describing the ecological and adaptive functions that this acoustic variation serves. Male songbirds typically produce the majority of songs, and these are often directed at female conspecifics (i.e., the same species). This is especially true in mating contexts, where male songs can act as a female attractant and/or a conspicuous cue for mating decisions made by the female. Under both of these conditions, female behavior is closely linked to phonological variation in male song. Females in many species of songbirds show behavioral preferences that are based on variation in male song repertoire sizes, song bout lengths, and/or song output rates (Searcy and Yasukawa, 1996 for review). Although little is known about the neural basis of female song preferences (or about the neural basis of conspecific song perception in general), the close correspondence between the acoustic variation in male song and female behavior implies a set of perceptual mechanisms that are sensitive to relevant variation in song. We confirm this in the present study, by identifying forebrain regions sensitive to behaviorally relevant variation in conspecific male song.

Among European starlings (*Sturnus vulgaris*), in

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Contract grant sponsor: NSF; contract grant number: IBN 9317868 (S.H.H.).

Contract grant sponsor: NIH; contract grant number: NS 35467 (G.F.B.).

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particular, there is a great deal of evidence from the field suggesting that individual variation in male song plays an important role in female mating decisions. Males direct their songs primarily at females (Eens et al., 1993), and variation in male song bout length and repertoire size (but not morphology) are correlated directly with increased mating success (Mountjoy and Lemon, 1996; Eens, 1997) and female choice (Eens et al., 1991). Recent laboratory findings have extended the knowledge of female choice in starlings by demonstrating that, in contexts mimicking natural mate choice situations, female starlings attend closely to variation in song length, such that strong preferences are observed for longer compared to shorter male song bouts (Gentner and Hulse, 2000a).

In songbirds, auditory information ascends from the level of the thalamus to several sites in the telencephalon, including the major subdivisions of field L and the caudo-medial neostriatum (NCM). NCM also receives projections from field L, and both NCM and field L possess extensive reciprocal connections with medial and lateral portions of the caudal ventral hyperstriatum (cmHV and clHV, respectively) (Vates et al., 1996). All of these auditory telencephalic regions exhibit electrophysiological responses to conspecific song (Leppelsack and Vogt, 1976; Müller and Leppelsack, 1985; Rübsamen and Dorrscheidt, 1986; Scheich, 1991; Theurich et al., 1984). In NCM and cHV, the presentation of conspecific song also elicits rapid and transient expression of the immediate early gene (IEG) *ZENK* (*ZENK* is an acronym for *zif-268;* Mello et al., 1992; Christy et al., 1988; *egr-1,* Sukhatme et al., 1988; *ngf-Ia,* Milbrandt, 1987; and *krox-24,* LeMaire et al., 1988). The *ZENK* mRNA response to conspecific song is stronger than the response to heterospecific song and other species atypical sounds (Mello et al., 1992), and the topography of *ZENK* responses in NCM is related to variation in different song syllables in a manner that cannot be accounted for by simple frequency tuning maps (Ribeiro et al., 1998). Consistent with a role in higher level auditory processing, neurons in NCM also show song-specific long-term modulation in firing rate following the repeated presentation of a single song (Chew et al., 1995, 1996; Stripling et al., 1997). This earlier work suggests that NCM and cmHV provide a spatially distributed representation of functionally relevant conspecific song features. In the present experiment, we examine responses in these regions by measuring *ZENK*-protein expression in female starlings exposed to conspecific male bouts of varying length.

MATERIALS AND METHODS

Subjects

Twenty-seven female European starlings served as the subjects for this experiment. All of the subjects were wild caught on a farm 30 miles north of Baltimore, Maryland. Prior to testing, subjects were housed in large flight cages with two to four other females in a mixed sex aviary containing 10 to 20 other European starlings. Fifteen of the subjects had previous experience with one of two different operant apparatuses (Gentner and Hulse, 1998, 2000a), but had not participated in any testing procedures for at least 3 months prior to use in this experiment. These 15 birds were maintained in the aviary on a light/dark schedule synchronized to the natural photoperiod in Baltimore, and were tested in June of 1997. The remaining 12 birds were captured in the spring of 1998 and housed on an 8L/16D photoperiod to maintain photosensitivity until testing the following August. These 12 subjects were naive to all testing procedures. Animals from each of the two main groups were distributed evenly across the three experimental conditions, and there was no systematic variation between the results reported here and whether or not a bird came from the first or second group of subjects. Food and water were available *ad libitum* throughout the experiment.

Stimuli

To construct the stimulus sets used in this experiment, we recorded a large library of complete song bouts from a single male starling. During recording, the male was housed in a large ($2 \times 2 \times 1.5$ m) sound attenuation chamber where he had visual and auditory access to a female starling. Complete details of the recording procedure are given elsewhere (Gentner and Hulse, 1998). For the present experiment, we selected 12 exemplars from the library of recorded songs. Each exemplar was a complete (i.e., uninterrupted) bout of singing, the length of which varied between exemplars. Based on song bout length we divided the 12 exemplars into two sets of six: a long set for which the mean bout length was 55.6 s (range: 53–68 s), and a short set for which the mean bout length was 25.4 s (range: 3.5–40 s). These two sets of songs were identical to two of the stimulus sets used to demonstrate female preferences for longer song bouts in a previous behavioral study (Gentner and Hulse, 2000a). To create the playback stimuli used here, exemplars in each set were concatenated into a 30-min sound file using a computer (Macintosh, Quadra 650, Cupertino, CA) running SoundDesignerII software (Digidesign, Menlo Park, CA), and downloaded to digital audio tape at a 48 kHz sample rate or compact disc at 44.1 kHz, in both cases with 16-bit resolution.

The six bouts in the long set were used to create the *long-song* playback stimulus. We first appended 6.8 s of silence to the end of each bout, concatenated the six bouts, and then repeated this six-bout phrase four times, so that the entire stimulus set lasted exactly 30 min from the start of the

Table 1 Stimulus Set Parameters

Stimulus Set	Total Duration of Song (s)	Total Duration of Silence (s)	Number of Bout Repetitions	Mean Song Bout Length (s)	Total Number of Unique Motifs	Total Number of Repeated Motifs
Long	1650.0	150.0	4.0	55.6	105.0	39.0
Short	1648.0	152.0	13.0	26.0	74.0	11.0
Low	381.0	1419.0	3.0	26.0	74.0	11.0

The three stimulus sets used in the present experiment along with several relevant parameters associated with each set. The "total duration of song" and the "total duration of silence" indicate the amount of conspecific song and silence, respectively, presented to an animal over the course of 30 min. Each stimulus comprised six separate song bouts. The "number of repetitions" gives the number of times that each of those six song bouts was repeated during the playback period. The "mean song bout length" gives the mean length of all the bouts in a given set. The "total number of unique motifs" gives the number of different motifs in all the bouts of a given set, and is analogous to repertoire size. The "total number of repeated motifs" gives the balance of the motifs composing the bouts in each set. The sum of the last two columns is equal to the total number of motifs in all the bouts of a given stimulus set.

first bout to the end of the last bout. Using the six short song bouts and a similar strategy, we created the *short-song* playback stimulus. However, in the short-song stimulus, each bout was separated by 1.9 s of silence, and the run of six bouts was repeated 13 times. The long-song and the short-song stimuli each contained a total of 27.5 min of song, but differed in the total number of song bouts, the length of the silence between each bout, and of course, the actual bouts comprising each stimulus. We also created a third playback stimulus, the *low-exposure* stimulus, that was made up of the same six song bouts used in the short-song stimulus, and was also 30 min long. However, for the low-exposure stimulus, each bout was separated by 83.5 s of silence and repeated only three times, yielding a total song duration of 6.4 min. Thus, the low-exposure stimulus contained a much lower total amount of song than either the long-song or short-song stimuli, but the repetition rate was roughly equivalent to that of the long-song stimulus. Table 1 shows several measures for each of the three stimulus sets used in this experiment.

Procedure

Playback. Each bird was acoustically isolated in a sound attenuating chamber (IAC model # AC-3, Bronx, NY) for at least 44 h prior to the start of stimulus presentation. Each isolation/playback chamber (three total) was equipped with a speaker mounted in the upper corner, and a house-light for which the on-off schedule was synchronized to the natural photoperiodic light/dark schedule. There was a small microphone mounted on the wall of each chamber that allowed monitoring of the acoustic environment inside the chamber. During stimulus presentation, most of the subjects did not vocalize, and among those that did, there was no systematic relationship between the total duration of vocal output during stimulus presentation and the results reported here. Food and water were available *ad libitum* while the subjects were in isolation.

Following the initial isolation period, each subject was presented with one of the three playback stimuli. Sound levels inside each chamber were set to a peak loudness of 78 dB SPL. Each playback lasted exactly 30 min, followed immediately by 30 min of silence, during which time the animal remained in the isolation chamber. On a given day, we conducted one playback with each of the three stimulus sets, and the order of stimulus set presentation was counterbalanced across subjects. All playbacks occurred between 8 and 11 A.M. Each subject heard only one stimulus set. Each of the three playback stimuli was presented to nine different birds.

Tissue Preparation. Sixty minutes after the start of a given playback, the subject was administered a lethal dose (80 mg/kg) of secobarbitol (50 mg/mL) via intramuscular injection. Once deeply anesthetized, we perfused the brain tissue, via the carotid artery, with approximately 50 mL of heparinized (150 IU/10 mL) 0.9% saline, followed by 300–400 mL of 4% paraformaldehyde in a 0.1 *M* phosphate buffer (pH 8.5). Following this initial fixation, the brain was removed from the skull case and postfixed in 4% paraformaldehyde for 24 h, and then cryoprotected in 30% sucrose solution [in 0.1 *M* phosphate-buffered saline (PBS), pH 7.5] until saturated (2–4 days). The brains were then frozen on dry ice and stored at −70°C until immunocytochemistry (ICC) for *Egr-1* protein (*ZENK*) was performed.

Immunocytochemistry. The brain tissue was sectioned at 40 μm in the sagittal plane using a cryostat, and collected into PBS (pH 7.5) for processing as free-floating tissue. Following two washes in 0.1 *M* PBS, the tissue was incubated at room temperature (RT) in 0.5% hydrogen peroxide for 15 min to reduce endogenous peroxidase activity, washed again (three times in 0.1 *M* PBS), and then incubated in 10% normal goat serum (Vector Laboratories) at RT for 1 h. We then incubated the tissue in a commercially available *Egr-1* antibody (Santa Cruz Biotechnology, catalog # sc-189) diluted at 1:100,000 in 0.1 *M* PBS containing 0.3% Triton X-100 (PBS/T; Sigma) for approximately 40 h at 4°C. The polyclonal *Egr-1* antibody that we used is raised in rabbit against the carboxy-terminus of the mouse *Egr-1*, a highly conserved region in the canary *ZENK* homologue

(Mello and Ribeiro, 1998). Following incubation in the *Egr-1* antibody and another wash in 0.1% PBS/T, the tissue was incubated in biotinylated goat anti-rabbit IgG (dilution 1:250; Vector Laboratories) for 1 h at RT, then washed three times in 0.1% PBS/T. Next, we incubated the tissue in an avidin-biotin horseradish peroxidase complex (Vectastain ABC, Elite kit; dilution 1:200) for 1 h at RT, and then washed it twice in 0.1% PBS/T and once in 0.1 *M* PBS. We visualized the avidin-biotin complex in a solution of 0.08% diaminodenzadine tetrachloride (DAB; Sigma) and 0.1% hydrogen peroxide in distilled water. Reaction time in the DAB was held constant across all the tissue in a given ICC run. In all, six separate runs were necessary to process all of the tissue used in the present study. Equal numbers of subjects from each experimental group were included in each run, and no systematic variation across the separate ICC runs was observed. Following visualization, the sections were mounted on gelatin covered slides, dehydrated, and coverslipped with Permount (Sigma).

Quantification and Statistical Analyses. We quantified the level of *ZENK* expression in each subject by sampling from three regions of the auditory telencephalon that exhibit high levels of expression in response to conspecific song: the cmHV, the dorsal portion of the caudo-medial neostriatum (NCMd), and the ventral portion of the NCM (NCMv). For each subject, we took 30 samples from each of these three regions—one sample per region from each of the 15 most-medial tissue sections in each hemisphere (provided that that section contained the region of interest). This strategy yielded samples from each region, concentrated within $\sim$ 700 μm of the midline, from which we mapped the relative levels of *ZENK* expression following stimulation with the three types of playback stimuli. The samples themselves were 640 $\times$ 480 pixel 8-bit grayscale images of the expression in each region, captured via a video camera mounted on top of a microscope (20X objective, 10X eyepiece). The actual size of each sample was 992 $\times$ 770 μm. The strategy for placing a sampling window within a given region was as follows. In cmHV the sampling window was placed as far caudal as possible such that it was within the dorsal boundary created by the lateral ventricle and the caudo-ventral boundary of the lamina hyperstriatica (LH). The dorsal, ventral, and caudal borders of the NCM were defined throughout by the lateral ventricle, whereas the rostral border was defined in medial sections by the LH and in more lateral sections by the band of very light *ZENK* expression characteristic of field L [Fig. 1(E)]. For the purpose of our sampling regime, NCM was arbitrarily divided into a dorsal and ventral half, and within each subregion our sampling window was placed so that its entire area was within that region. This appeared to capture a realistic estimate of the local expression. In all cases, the sampling windows were placed by an individual blind to the experimental condition of each animal. Due to variations between animals, planes of sectioning, and positioning of the tissues on the slides, the exact position of the sampling window within each region was somewhat random, within the boundaries defined above. We obtained at least 90 images/animal (30 images/region/animal).

We counted the number of *ZENK* immunoreactive (*ZENK*-ir) cells in each digitized image using an automated NIH image routine that was written explicitly for this task (NIH image v1.62, developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). The only subjective portion of the counting routine involved setting the initial parameters that the computer used to identify *ZENK*-ir cells. The only one of these parameters not held constant across all subjects was the "threshold." The threshold specified the optical density value (in grayscale decimal) above which a given cluster of pixels was considered signal, and below which it was considered noise and discarded. For images in which the signal-to-noise ratio was approximately equal, the threshold was standardized across images by setting it at a given distance from the mean background optical density obtained from regions of tissue exhibiting no *ZENK* expression. However, when the signal-to-noise ratio was either particularly high or extremely low, this method of setting the threshold yielded very inaccurate counts. Therefore, the threshold value for each of these animals was set by selecting a random subsample of all the images from that subject and then adjusting the threshold for each image until an accurate count of *ZENK*-ir cells (verified manually) was achieved for each image in the subsample. The average threshold value for this subsample of images was calculated, and that average threshold was then used for the automated counting of all the images from that subject. In all cases, the person setting the threshold was blind to the experimental condition of each subject. The accuracy of the cell counts obtained with the automated procedure were verified by manual counts on a subset of the sample tissue. In addition, we estimated the number of *ZENK*-ir cells in the entire region of interest (all of the cmHV or NCM) using computer controlled stereological techniques (Stereo Investigaor software, MicroBrightField, Inc.). The stereological estimates yielded results qualitatively similar to those reported here, but with higher variance than in the subregion counts.

Differences between regions and groups were examined using a repeated measures analysis of variance (ANOVA) with the level of significance set at $p = .05$, two-tailed. Where appropriate, differences between means were tested using Fisher's protected least squared difference (PLSD) with the level of significance set at $p = .05$. The raw cell counts were log transformed prior to statistical analysis to meet assumptions of the ANOVA model. Analyses identical to those reported here, but using the raw cell counts, yielded qualitatively similar results. All means were expressed along with their corresponding standard errors ($\pm$ SEM). In three cases, one or the other hemisphere from a subject could not be analyzed because of a problem during the sectioning of that tissue. A portion of these data have appeared in abstract form (Gentner et al., 1998).

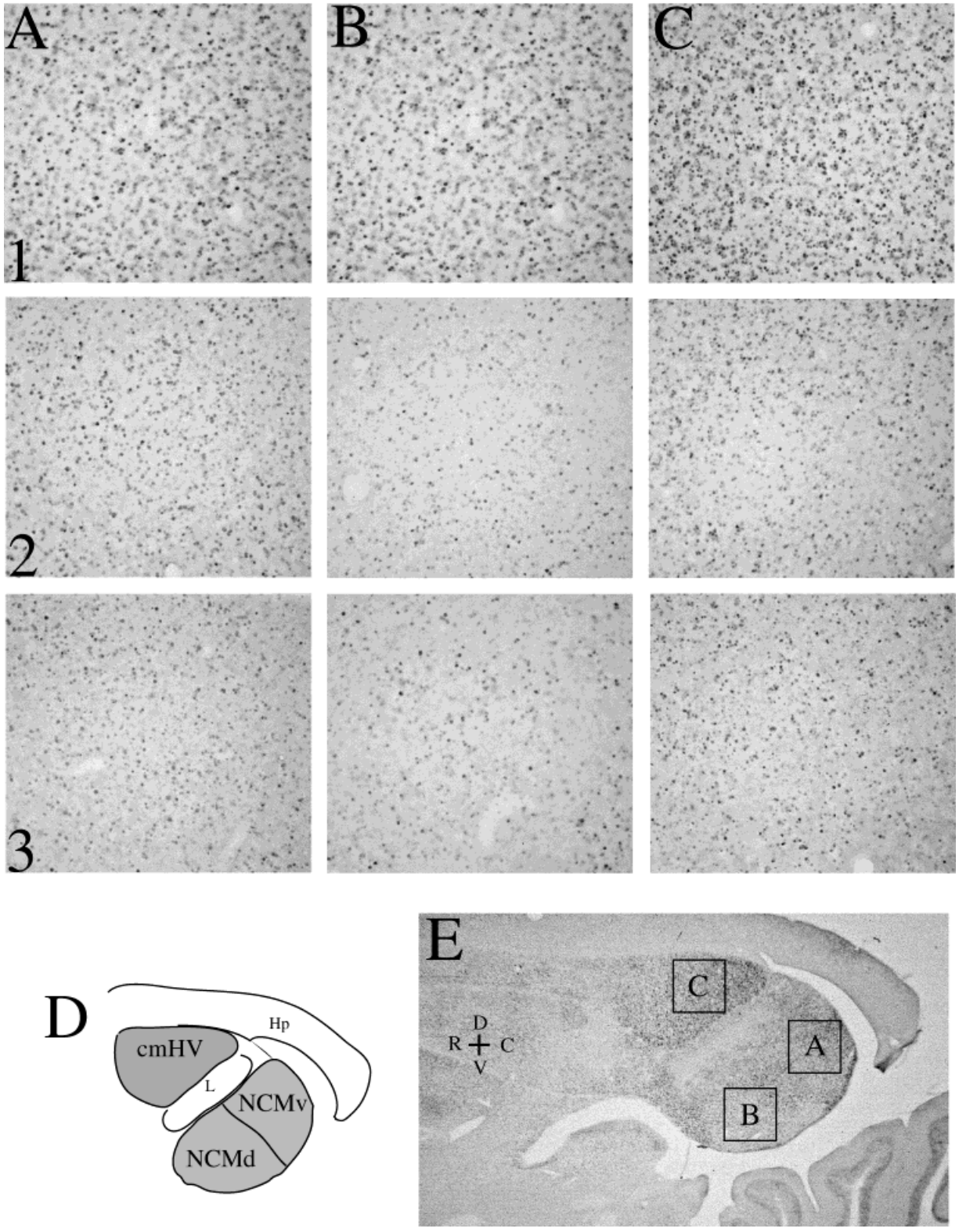

Figure 1 Examples of *ZENK* protein immunoresponsive cells in ventral NCM, dorsal NCM, and cmHV (columns A, B, and C, respectively) in female starlings exposed to 28 min of either male long-song bouts (row 1) or male short-song bouts (row 2), or 6.4 min of male short-song bouts (row 3) presented over the course of 0.5 h. For reference, a para-sagittal plane of section through the telencephalon is shown in (E) giving the approximate location of the sampling window in each region at this plane (see Materials and Methods). The drawing in panel (D) shows the boundaries of the regions from which the samples were taken. The images used for quantification were captured at higher resolution, and a slightly different size (see Materials and Methods).

RESULTS

Spatial Distribution of *ZENK* Expression

We observed robust staining of *ZENK*-ir cells throughout cmHV, NCMd, and NCMv in response to the presentation of all three sets of stimuli (see Fig. 1), and the overall level of *ZENK* expression varied among each of the three sampling regions. Overall, when the data for all three stimulus types are combined, expression was highest in the cmHV, lower in the dorsal portion of NCM, and lowest in the ventral portion of NCM. The differences among all three regions were statistically significant [ANOVA: $F(2, 40) = 76.198$, $p = .0001$, main effect of region], as were posthoc comparisons between the mean expression levels in all three regions [Fischer's PLSD, $p < .0001$ for all comparisons between cmHV, NCMd, and NCMv]. In addition to the overall differences between regions, expression levels also varied according to the position of the sample in the para-sagittal plane. In general, expression density was greatest near the midline and gradually decreased as the sample location moved laterally [ANOVA: $F(14, 280) = 5.764$, $p = .0001$, main effect of lateral-medial sample location]. This analysis also revealed a significant interaction between the region and section position [ANOVA: $F(28, 560) = 1.981$, $p < .01$], which suggests that the variation in the para-sagittal plane was not the same within each region. We examined this possibility with separate analyses of the data from each region. These analyses showed significant lateral-medial sampling position effects in both NCMd [ANOVA: $F(14, 294) = 4.548$, $p < .0001$] and NCMv [$F(14, 294) = 5.121$, $p < .0001$], but not in cmHV [$F(14, 266) = 2.574$, NS, see Fig. 2]. There were no remarkable differences between expression patterns observed in the right and left hemispheres [ANOVA: $F(14, 280) = 0.883$, NS, interaction between hemisphere and sample location].

Song Elicited Variation in *ZENK* Expression

Along with the spatial variation across sample sections and regions, the mean number of *ZENK*-ir cells was also affected by variation between the three sets of playback stimuli. These effects were strongest in ventral NCM, where the long-song stimuli elicited greater *ZENK* expression than either the short-song or low-exposure stimuli [ANOVA: $F(2, 21) = 3.853$, $p < .05$, main effect of playback stimulus in NCMv, see Fig. 2(b)]. Consistent with this, posthoc analyses showed that the long-song stimulus elicited higher *ZENK* expression in NCMv than did either the short-song stimulus (PLSD mean diff. $= 0.224$, $p < .05$) or the low-exposure stimulus (PLSD mean diff. $= 0.167$, $p < .05$). Moreover, the difference in expression density between subjects exposed to the short-song and low-exposure stimuli was not reliable (PLSD mean diff. $= 0.057$, NS), suggesting that song bout length, but not total duration of song exposure, has a direct effect on *ZENK* expression in ventral NCM.

In contrast to the robust effects of song exposure observed in NCMv, variation among the stimulus sets appeared to have less effect on *ZENK* expression density in NCMd and no effect in cmHV. We observed no significant differences among the mean number of *ZENK*-ir cells elicited by the different stimulus sets in cmHV [ANOVA: $F(2, 20) = 0.447$, NS, main effect of stimulus set in cmHV, see Fig. 2(c)], and there were no significant interactions involving the three groups of subjects. In dorsal NCM, however, although the overall mean expression densities did not differ significantly among the three groups of subjects [ANOVA: $F(2, 21) = 1.295$, NS, main effect of stimulus set in NCMd], there was a significant interaction between the lateral-medial change in expression and that elicited by the different stimuli [ANOVA: $F(28, 294) = 1.592$, $p < .05$, interaction between sample position and stimulus set]. This effect may be due, in part, to significant differences in *ZENK* expression among the three groups in the most medial portion of NCMd. When the analysis was restricted to the five most medial sections of NCMd [Fig. 2(a), sections 11–15], the mean expression elicited by the long songs was significantly higher than that for both the short songs and the low-exposure stimuli (t test, two-tailed, $p < .001$ both cases), whereas the mean level of expression for the short and low-exposure groups did not differ significantly over this same interval. This differs from the expression pattern in NCMv, where long-song elicited expression was elevated above that for the other groups across the entire lateral-medial sampling range [see Fig. 2(b)], and from that in cmHV, where expression for all song stimuli was elevated across the entire sampling region [see Fig. 2(c)]. Although the song related effect in NCMd was more subtle than that observed in NCMv, it is reliable, and it suggests that behaviorally relevant dimensions of acoustic variation in male song are represented in both the dorsal-ventral and the medial-lateral planes of NCM.

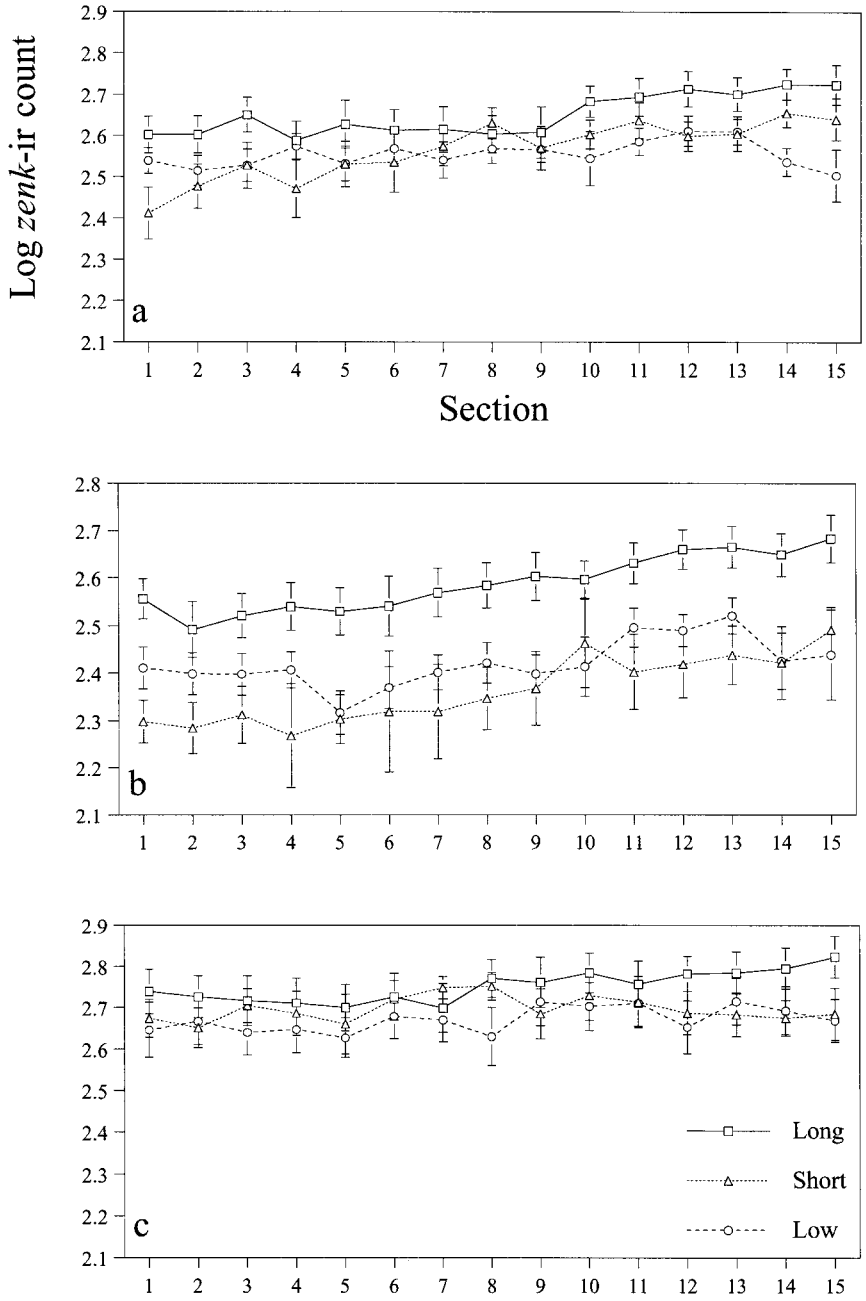

Figure 2 Distribution of *ZENK*-ir cells in the para-sagittal plane through (a) dorsal and (b) ventral portions of NCM, and (c) cmHV, in female starlings exposed to 28 min of either long bouts of conspecific male song (□) or short bouts of song from the same male (△), or 6.4 min of short song bouts (○). One (1) denotes the most lateral section and 15 the most medial section through each region. The data from right and left hemispheres are combined.

DISCUSSION

The results of the present experiment are straightforward: variation in the bout length of male songs elicits variation in the level of *ZENK* expression in subregions of the female starling auditory telencephalon. Exposing females to longer male song bouts leads to significantly higher numbers of *ZENK*-ir cells throughout the dorsal and ventro-medial portions of NCM as compared to exposure to shorter songs, independent of the total amount of song that females hear. Variation in male song bout length also elicits

differential behavior in female starlings (Gentner and Hulse, 2000a). Like most natural behaviors, female choice in songbirds can be viewed as a suite of many behavioral processes, including the detection, discrimination, and recognition of conspecific song, as well as processes for decision making and the execution of well-coordinated motor responses. The results of this study suggest a functional localization for at least a portion of the perceptual processes that underlie this complex behavior.

Integrating the Behavioral and Neural Mechanisms of Female Choice

The songs of male European starlings function as a primary cue for female mating decisions (Eens and Pinxten, 1996; Eens, 1997; Mountjoy and Lemon, 1991, 1996; see also Searcy and Yasukawa, 1996). Moreover, both preference and choice behavior in female starlings can be closely controlled by variation in male song-bout length. When given control over access to male song, female starlings will spend significantly more time listening to the presentation of longer song bouts than to shorter song bouts, and will preferentially track the location of long songs as they shift between different sources (Gentner and Hulse, 2000a). In addition, this preference for longer songs requires a period of song exposure before emerging. That is, females will initially respond at high levels to both long and short songs, but over time, responding to longer songs presented from one location gradually increases, while responding to shorter songs presented from another location gradually decreases. After roughly 30 min of song exposure preferences are readily observed, suggesting an underlying habituation mechanism that is specific to both the song and the source location (Gentner and Hulse, 2000a). Such a mechanism is consistent with the more general habituation mechanisms proposed for female preferences based on variation in male repertoire size (Searcy, 1992).

The behavioral data on female preference just described find a compelling neurobiological parallel in the differential habituation of responses observed for NCM neurons. As we have already noted, the *ZENK* IEG response exhibited by cells in both NCM and cmHV is closely tied to the nature of the acoustic stimulus. Exposure to conspecific song elicits significantly higher levels of *ZENK* mRNA expression compared to that in acoustically isolated songbirds (Mello and Clayton, 1994; Nastiuk et al., 1994; Duffy et al., 1999), and to that in birds exposed to either heterospecific song or pure tone stimuli (Mello et al., 1992). However, repeated exposure to the same song leads to an eventual habituation of the *ZENK* mRNA and protein response. Expression elicited by a single song peaks after approximately 30 repetitions over the course of 30–40 min (Mello and Clayton, 1994), and then declines to pre-exposure levels (Mello et al., 1995). Although physiological data from NCM neurons are limited, they also indicate a song-specific long-term habituation in the response of NCM cells to conspecific song (Chew et al., 1996; Stripling et al., 1997). This pattern of results, along with the probable role of differential habituation mechanisms in female behavioral preference, suggest the involvement of NCM in the perceptual processing that underlies preference behavior in female songbirds (Ryan, 1998). Our data support this conclusion by demonstrating that behaviorally relevant variation in male song elicits differential activation of a subpopulation of cells in NCM. In addition, we note that the duration of song exposure required for the emergence of behavioral preferences in starlings (Gentner and Hulse, 2000a) closely parallels the duration of song exposure resulting in maximum *ZENK* expression in NCM (Mello and Clayton, 1994). It remains unclear, however, whether the patterns of *ZENK* expression observed in the present study are due to differential rates of habituation associated with long and short songs, or to selective tuning properties of cells in NCM or its afferents. Thus, while the growing body of evidence continues to implicate NCM in the processing of conspecific song, its computational role remains unknown.

In light of the fact that differential *ZENK* responses in female NCM are elicited by exposure to behaviorally relevant variation in male songs, it is important to consider more precisely the acoustic features driving this response. Earlier results suggested that *ZENK* mRNA expression density was a function of the duration of song exposure (Mello and Clayton, 1994), with a period of dishabituation following the presentation of a novel song (Mello et al., 1995). Results of the present study suggest that, in more acoustically diverse environments where multiple conspecific songs are presented nearly simultaneously, the level of *ZENK* expression in NCM is not a simple function of either the duration of song exposure or the rate of whole song repetition. If the total duration of song exposure controlled the level of *ZENK* expression in NCM, then the females exposed to the shorter songs should have exhibited levels of expression equal to that in females exposed to the longer songs. Likewise, if the rate of song repetition was the only important variable in controlling the level of *ZENK* expression, then the females presented with the low-exposure stimulus should have exhibited levels of expression

equal to that for the long-song group. Neither conclusion is supported by the data. Instead, the most straightforward interpretation of the present results is that *ZENK* expression density in NCM is controlled by song bout length. Consistent with the importance of this feature, the volume of song control nuclei HVc and the robust nucleus of the archistriatum (RA) in male starlings are positively correlated with variation in song bout length but not repertoire size (Bernard et al., 1996).

On the basis of the present results, however, we cannot exclude the possibility that song bout duration and repetition rate interact to elicit higher levels of *ZENK* expression, or that expression is tuned to a feature of song that is itself closely correlated with variation in bout length. This may be true, particularly, if one considers repetition rate as a function of the number of different motifs in a song bout. Among the stimuli used in the present study, the number of unique motifs associated with the set of long songs was substantially larger than that for the short songs (see Table 1), and these songs elicited the highest levels of *ZENK* expression in NCM. Thus, it may be that longer songs are more effective stimuli than shorter songs because they maximize the duration of song exposure while minimizing habituation to specific acoustic features (i.e., motifs). This interpretation fits well with all of the available data on *ZENK* expression in songbirds, including the recent demonstration that *ZENK* mRNA expression patterns in NCM of canaries correspond to acoustic variation among different song syllables (Ribeiro et al., 1998). It also fits with the electrophysiological data from NCM in zebra finches, showing song specific habituation (Chew et al., 1995, 1996; Stripling et al., 1997), and with behavioral data showing that starlings can easily parse songs into their constituent motifs (Gentner and Hulse, 2000b). The notion that *ZENK* expression is tuned to the intermotif variation in male song is also consistent with Searcy's (1992) repertoire habituation hypothesis, and deserves further attention. Finally, in the túngara frog (*Physalaemus pustulosus*), female preferences for lower-frequency chucks appear to derive from the tuning of the peripheral hearing organ, which is maximally sensitive to frequencies slightly below the mean dominant frequency of male calls (Ryan et al., 1990). Therefore, we note that the effects described here for NCM may be the result of tuning at structures that are more peripheral. However, if the source of this differential signal lies in the degree of acoustic variability between motifs (as perhaps for starlings), or in the recognition of specific motifs (as perhaps for canaries), then this level of processing may well require the representational capacities of forebrain structures such as NCM.

Other structures in the forebrain have also been implicated in neural basis of song mediated preferences. In female canaries, lesions to the song control nucleus HVc affect the number of copulation solicitations given to heterospecific and conspecific song (Brenowitz, 1991). Although these lesions were very large and may have included portions of cHV and lateral NCM, subsequent studies using ibotenic acid lesions in HVc showed similar effects (Del Negro et al., 1998). In addition, more recent results indicate that HVc auditory responses in female canaries are affected by the presence or absence of sexually attractive syllables in male song (Del Negro et al., 2000). In contrast, however, other studies have failed to find any effect of HVc lesions on female preferences in zebra finches (*Taeniopygia guttata*), but do report effects for lesions to cHV (MacDougall-Shackleton et al., 1998). Varying results from lesion studies are notoriously difficult to interpret, and while these differences may reflect significant variation in song processing between species, they may also reflect the choice of stimuli, the difficulty in separating perceptual effects from those involved in decision making, or motor components of this complex behavior. In starlings performing song-based operant discriminations, HVc lesions affect song-based associative processes, but not straightforward song discrimination (Gentner et al., 2000). The results of the present study suggest that the central auditory system is tuned to behaviorally relevant variation in conspecific song as early as NCM. Thus, to the extent that HVc is involved in some manner of conspecific song processing, any auditory signal it receives is likely to be strongly biased.

Very few studies have investigated perceptual processes in songbirds using stimuli with known behavioral relevance, but the data that are available suggest a consistent role for NCM. In adult male zebra finches, *ZENK* expression in NCM appears to be a function of early song exposure, such that songs presented to juveniles and copied with high fidelity elicit higher expression than those copied with lower fidelity (Bolhuis et al., 2000). Similarly, in canaries, songs paired with an aversive stimulus using a Pavlovian procedure elicit higher *ZENK* expression in NCM than unpaired songs (Jarvis et al., 1995). Together with the data presented here, these studies strongly suggest that NCM is involved in the processing of behaviorally relevant variation among conspecific songs, and that it does so in variety of different behavioral contexts. It may be that NCM serves as a common source for behaviorally relevant distinctions

among conspecific song features, which are then extracted by different higher processing areas, depending on the perceptual task at hand. Anatomically, NCM is well positioned for such a role as a functional pattern recognizer in that it lies afferent to HVc and shares heavy reciprocal connections with cHV (Vates et al., 1996). Little is known, however, about the response properties of cHV. Consistent with the results reported here, Bolhuis et al. (2000) reported that *ZENK* expression in cHV is high, but not differentially related to song variation, and although cells in cHV show electrophysiological responses to conspecific song (Janata and Margoliash, 1999), their selectivity has not been studied. Investigating the relationship between NCM and other auditory processing regions will require complex experimental designs that maintain simultaneous control over both functionally relevant behavior and behaviorally relevant stimulus variation.

Undergraduate research assistants Paul Kaloudis, Will Rankin, Evan Ellis, Ed Gometz, Kristy Korzic, and Anneliese Eichel helped with data collection and analysis.

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