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Hormonal Mechanisms Underlying Auditory Forebrain Selectivity in Female Songbirds

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Sara E. Sanford

B.A., Ripon College, 2006

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An abstract of

A thesis submitted to the Faculty of the Graduate School of Emory University

in partial fulfillment of the requirements for the degree of

Master of Arts in Psychology

2009

## Abstract

Hormonal Mechanisms Underlying Auditory Forebrain Selectivity in Female Songbirds

By Sara E. Sanford

Sex steroids facilitate dramatic behavioral changes related to reproduction in seasonally breeding vertebrates and are increasingly implicated in the modulation of sensory processing related to sociosexual cues. Female white-throated sparrows (*Zonotrichia albicollis*), for example, perform a copulation solicitation display in response to conspecific male song only during their mating season when plasma levels of estradiol (E2) are high. Using immediate early gene (IEG) transcription as a marker of cellular responses, Maney et al. (2006) demonstrated in female white-throated sparrows that (E2) affects the selectivity of the IEG response to hearing male song in the caudomedial nidopallium (NCM) of the auditory forebrain. Because NCM is a large heterogeneous area, we hypothesized that the effects of E2 on the expression of the IEG *zenk* are not uniform throughout NCM and sought to map the distribution of these effects. Non-breeding females with low endogenous levels of E2 were treated with E2 or a placebo and exposed to conspecific song, frequency-matched tones, or silence. We found that the effects of E2 on *zenk* induction in NCM are not uniform. In two rostral regions (NCMd and NCMv) the *zenk* response was selective for song over tones only in the E2-treated birds, whereas in the caudal region (NCMc) *zenk* expression was selective for song over tones regardless of hormone treatment. We also found that E2 treatment upregulated *zenk* expression independent of sound stimulus, which suggests that hormone treatment alone induces new gene transcription in the auditory forebrain. Our results suggest that specific regions of NCM are seasonally regulated and that basal levels of neuronal activity may be heightened in these regions during the reproductive season.

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Tables and Figures

For many species the process of selecting a mate (i.e. timing, preferences for traits) is strongly influenced by fluctuations in hormone levels. Accumulating evidence suggests that changing hormone levels affect mate choice by influencing sensory processing of social cues that are received via olfaction, vision, and audition (Schubert, Houk, Feldhoff, Feldhoff & Woodley, 2006; Gangestad & Thornhill, 1998; Penton-Voak et al., 1999; Gangestad, Simpson, Cousins, Garver-Apgar & Christensen, 2004; Feinberg et al., 2006; Maney, Cho & Goode, 2006). As gonadal steroid levels increase in preparation for reproduction, they may enhance the salience of relevant social stimuli.

In many songbirds, the female's behavioral response to male song depends on her level of gonadal steroids. Reproductively active females of many songbird species respond to the song of a conspecific male with a copulation solicitation display (CSD), which consists of a lifted tail and rapid flutter of the wings and can be accompanied by a vocalization (Searcy & Marler, 1981). This behavior is highly estrogen dependent such that it is performed only during the breeding season when estrogen levels are high. The estrogen dependence of the behavioral response to song suggests that, as has been demonstrated in a number of other species, gonadal steroids may affect behavioral responses in female songbirds by influencing sensory processing.

The present study focuses on the effects of estrogen on auditory responses to song in female white-throated sparrows. Electrophysiological studies have shown that at several levels of the auditory pathway, neurons fire selectively to auditory presentations of song (or the individual components of song) over less behaviorally relevant auditory stimuli (Theunissen, Amin, Shaevitz, Woolley, Fremouw & Hauber, 2004). Selectivity for particular songs or song types emerges at higher levels and culminates in areas

analogous to mammalian auditory cortex, including field L, the caudomedial mesopallium (CMM), and the caudomedial nidopallium (NCM) (Theunissen et al., 2004; Heil & Scheich, 1991). Of these higher levels, NCM is particularly well studied with respect to selectivity. For example, Stripling, Volman, and Clayton (1997) found that single cells within NCM responded to conspecific and heterospecific song with increased firing, and simple tones inhibited firing. Several studies also demonstrated that cells in NCM habituated for hours to repetitions of novel conspecific songs but habituated for shorter periods or not at all in response to less behaviorally relevant stimuli, such as heterospecific song and tones (Chew, Mello, Nottebohm, Jarvis & Vicario, 1995; Stripling et al., 1997; Chew, Vicario & Nottebohm, 1996). George, Cousillas, Richard, and Hausberger (2008) demonstrated that in European starlings (*Sturnus vulgaris*) the number of neurons responding to auditory stimuli in NCM, and the magnitude of those responses, increases as the social value of the auditory stimulus increases. Evidence from these electrophysiological studies suggests that NCM may play an important role in organizing and categorizing behaviorally relevant auditory stimuli.

In addition to electrophysiology, another method of measuring neuronal activity is the quantification of molecular indicators such as immediate early genes (IEGs). IEGs belong to a class of genes that are the first to be transcribed in response to neuronal activation and have a variety of functions downstream (Sheng & Greenberg, 1990; Davis, Bozon & Laroche, 2003; Kaufmann, Yamagata, Andreasson & Worley, 1994; Lanahan & Worley, 1998). Many years of research in songbirds have shown that IEGs in the auditory forebrain are expressed in proportion to the behavioral relevance of the auditory stimulus. For example, in NCM, the expression of the IEG *zenk*, also known as early

growth response protein 1 (egr-1), was greatest when birds heard conspecific song compared to when they heard heterospecific song or tones (Mello, Vicario & Clayton, 1992). *Zenk* expression in neurons within NCM also diminished during repeated exposure to the same song, which corresponds to habituation seen in electrophysiology studies (Mello, Nottebohm & Clayton, 1995). Gentner, Hulse, Duffy, and Ball (2001) exposed female European starlings to more attractive long-bout songs (Gentner & Hulse, 2000), and less attractive short-bout songs and saw greater expression of the protein product of *zenk* (ZENK) in NCM in those birds exposed to the long-bout songs even when the total duration of song heard was controlled (Gentner et al., 2001). In addition, female white-crowned sparrows exhibited greater ZENK expression in NCM when they listened to song from their local region than when they heard song recorded from a more distant region (Maney, MacDougall-Shackleton, MacDougall-Shackleton, Ball & Hahn, 2003). The correlation between the behavioral relevance of the stimulus and IEG expression in these regions of the auditory system suggests that IEG activity within distinct regions may facilitate discrimination and recognition of attractive song, thus playing a role in mate choice.

Maney et al. (2006) examined whether plasma E2 affects the selectivity of the song-induced *zenk* (*egr-1)* response in the auditory forebrain. Non-breeding, white-throated sparrow females were held on a winter-like photoperiod and implanted with silastic capsules containing either no hormone or E2. This manipulation resulted in females with high levels of E2 similar to those seen in the breeding season, and females with low, non-breeding levels. In E2-treated birds, hearing 42 min of conspecific song induced more immunoreactivity for the protein ZENK than did hearing frequency-

matched synthetic tones. This result was consistent with previous research conducted on females in breeding condition (e.g., Mello et al., 1992; Gentner et al., 2001; Maney et al., 2003). In control birds, however, the *zenk* response to song did not differ from that to tones. These results suggest that in females, *zenk* induction in the auditory system is selective for song only when plasma E2 exceeds non-breeding levels. E2-dependent plasticity of auditory pathways and processing centers may thus promote recognition of and attention to conspecific song during the breeding season.

Maney et al. (2006) reported E2-induced selectivity in NCM but did not investigate the anatomical patterns of selectivity within this area, which is not homogenous. As has been widely reported for many sensory systems across vertebrates, the auditory forebrain of songbirds is organized into subregions that in many cases resemble tonotopic or functional maps. Using IEG expression to map sound-induced neuronal activity, Ribeiro, Cecchi, Magnasco, and Mello (1998) found that neurons in the canary NCM respond to whole songs with a high density of activation in the rostral portion and more intense labeling around the periphery of NCM. Single syllables were mapped systematically in the rostral NCM as well: low frequencies mapped dorsally, high frequencies mapped ventrally. Synthetic whistles were mapped according to frequency in the rostral NCM as well but with less defined boundaries. Their analysis suggested that other features of natural song besides frequency are being mapped within the rostral NCM. Gentner et al. (2001) demonstrated that different patterns of ZENK were expressed in response to attractive, long-bout songs compared to less attractive, short-bout songs. Controlling for the total amount of song the birds heard, they found that attractive long-bout songs resulted in greater ZENK expression than short-bout songs

in the ventral portion of NCM (NCMv). Similarly, Maney et al. (2003) reported greater ZENK expression in dorsal NCM than in ventral NCM in response to hearing song of a local dialect compared to hearing song of a foreign dialect. These studies suggest that patterns of ZENK expression and selectivity in NCM are dependent on the behavioral relevance of the stimuli. Maney et al. (2006) found that ZENK expression and selectivity are also dependent on plasma levels of E2, suggesting that E2 treatment may influence the behavioral relevance of male song. The patterns of ZENK expression related to E2 treatment have not been investigated, however. The present study provides a closer examination of where and how E2 is modulating gene expression in response to auditory stimuli.

Studies that are designed specifically to reveal heterogeneous patterns of IEG expression in the songbird auditory forebrain involve sectioning the brains on the sagittal plane. Cutting on this plane reveals the functional neuroanatomy of these regions much more clearly than does sectioning in the coronal plane. Because at the time Maney et al. (2006) conducted their E2 manipulations they were primarily interested in hypothalamic, not auditory, regions, the brains were cut on the coronal plane. As such, neuroanatomical patterns of *zenk* induction in the auditory forebrain could not be examined easily. In the present study, I sectioned brains on the sagittal plane so that much more information on the anatomical distribution of *zenk* induction would be available. I also included a group of birds that heard silence as an additional control to investigate the effects of E2 on basal levels of ZENK expression. Because song-induced neuronal responses are known to map heterogeneously within NCM (Ribeiro et al., 1998; Gentner et al., 2001; Maney et al., 2003; Avey, Kanyo, Irwin, & Sturdy, 2008) and because we know the distribution of

estrogen receptors in this region (Gahr, 1996), mapping E2-induced selectivity allows us to investigate how estrogen may be involved in specific patterns of auditory processing related to mate choice. In future studies a map of E2-induced selectivity will allow us to compare the E2-dependent effects within the defined regions of NCM to the distribution of estrogen receptors in this species.

## Methods

### *Animals*

Forty-two female white-throated sparrows (*Zonotrichia albicollis*) were collected in mist-nets in Atlanta, GA on the campus of Emory University during October and November of 2006. The sex of each bird was confirmed using PCR analysis of blood samples (Griffiths, Double, Orr, and Dawson, 1998). The sparrows were housed in the Emory animal care facility in walk-in flight cages and supplied with food and water *ad libitum*. Day length was kept constant at 10:14 h light–dark, which simulates natural wintering conditions they would experience at the capture site. Before beginning the experiment, the birds were moved to individual cages (38 · 38 · 42 cm) inside large, identical walk-in sound-attenuated booths (Industrial Acoustics, Bronx, NY, USA) and were kept under the same light conditions (10:14 h light-dark) throughout the experiment to prevent the reproductive elevation of endogenous estrogen (Wolfson, 1958; Shank, 1959). All procedures involving animals were approved by the Emory University Institutional Animal Care and Use Committee.

### *Hormonal manipulation*

The birds underwent hormonal manipulation according to Maney et al. (2006). Briefly, each bird was implanted with one subcutaneous silastic tube (length 12 mm, ID

1.47 mm, OD 1.96 mm, Dow Corning, Midland, MI, USA): 21 birds receiving an empty implant and 21 receiving an implant packed with 17b-estradiol (Steraloids, Newport, RI, USA). All implants were sealed at the ends with A-100-S Type A medical adhesive (Factor 2, Lakeside, AZ, USA). E2-filled implants were expected to increase plasma E2 to physiological breeding levels, maintain breeding levels from day 2 for at least 80 days, as well as facilitate estrogen-dependent CSDs (e.g. Moore, 1983; Maney, Richardson & Wingfield, 1997; Maney et al., 2003; Maney & Wingfield, 1998). For six days after implantation birds were housed in individual cages in groups of four per sound-attenuation booth. The housing groups included birds from each hormone treatment.

*Sound stimuli*

Song presentations were the same as those used by Maney et al. (2006). Briefly, song stimuli consisted of the songs of 14 unique white-throated sparrow males and the presentation lasted 42 minutes. A unique male's song was repeated for exactly three minutes with 15 seconds of silence between each song. It was important to present a variety of male songs to avoid habituation to the stimulus (see Stripling, Volman & Clayton, 1997), and every three minutes during the presentation the identity of the singer changed to a novel male. Each female presented with the song stimulus heard a unique order of males with no single male's song preceding or following another's more than once. The order was determined by a Latin Square. Seven unique recordings were used, such that each female in the E2-treated group (n = 7) heard the same recording as one female in the blank-implanted group (n = 7).

Tone presentations were the same as those used by Maney et al. (2006). Each tone presentation was based on a specific song presentation. Fifteen seconds of silence

followed each tone sequence, and each sequence was repeated for three minutes with the full presentation lasting 42 minutes. The tone sequences matched individual songs in the average number of onsets and offsets as well as total sound energy at each frequency. As with the song stimuli, the tone sequences were assembled in an order determined by a balanced Latin Square. For birds hearing tones, the seven tone presentations matched the seven song presentations described above.

*Stimulus presentation*

Each bird was isolated in an empty sound-attenuation booth on the afternoon of the sixth day following implantation. Each booth was equipped with microphone, speaker, and video camera. One hour after the lights came on the following morning the stimulus playback was delivered via the speaker located inside the booth. One-third of the birds (E2-treated, n = 7; blank implanted, n = 7) heard song, one-third heard tones, and one-third were exposed to silence only. Birds hearing song or tone playback heard 18 min of silence following the end of the stimulus presentation. We recorded behavior that occurred before, during, and after the stimulus presentation.

*Tissue collection*

Sixty minutes after the onset of the stimulus birds were deeply anesthetized with isoflurane (Abbott Laboratories, North Chicago, IL, USA). They were then decapitated, and their brains were collected and fixed according to procedures used by Maney et al. (2003). A blood sample ($\geq$ 100 µl) was reserved for the quantification of E2 concentration using a direct radioimmunoassay; the antibody used was directed against 17b-estradiol (#1702, Arnel, New York, NY). Procedures were adapted from Wingfield and Farner (1975), and are further described by Williams, Kitaysky, and Vezina (2004).

The lower limit of detection was 0.015 ng/ml. A t-test was used to compare plasma E2 levels between blank implanted birds and E2 implanted birds to ensure the implants significantly elevated those levels in treated birds.

*Histology*

Two sets of 50-µm sagittal sections were collected from one hemisphere. The first set was immunolabeled for ZENK (*egr*-1) following procedures described in Maney et al. (2003). In short, sections were incubated in a primary antibody against the protein product of *zenk* (ZENK; anti-egr-1; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The sections were washed, incubated in a biotinylated secondary antibody, washed, and then incubated in an avidin-biotin complex (Vector, Burlingame, CA, USA), which amplified the signal. Labeling was visualized using diaminobenzidine (DAB) enhanced with nickel, which produced a color reaction (see Shu, Gong & Fan, 1998; Maney et al., 2003).

*Quantification of immunoreactive cells*

In order to map the effects of E2 in the caudomedial nidopallium (NCM), we divided it into distinct regions of interest. NCM is typically divided into two regions: a dorsal region (NCMd) and a ventral region (NCMv) (see Gentner, et al., 2001; Sockman et al., 2002; Maney et al., 2003, Lynch & Ball, 2008). We therefore quantified ZENK immunoreactivity within NCMd and NCMv as well as two other regions that we delineate here for the first time: a rostrodorsal portion (NCMr.d), which is located dorsal to field L; and a caudal portion (NCMc), which includes parts of NCMd and NCMv as previously defined (see Figure 1), as well as the area Woolley and Doupe (2008) described as NCMc. NCMc also may be a neurochemically-distinct region. Pinaud,

Fortes, Lovell, and Mello (2006) described a distribution of cells in the caudal portion of NCM that were immunolabeled for calbindin, and our lab has also observed this region to be heavily innervated by tyrosine hydroxylase-ir fibers (S. Sanford, unpublished). All regions were selected within four consecutive sections 100 μm apart that were between 500 and 800 μm from the midline (Gentner et al., 2001; Bolhuis, Zijlstra, den Boer-Visser, & Van der Zee, 2000; Maney et al., 2003; Phillmore, Bloomfield, & Weisman, 2003).

Images of NCM (46 MB) were collected using a Leica DC500 camera attached to a Zeiss Axioskop microscope and the light level was kept the same for each image. Each image was converted to an 8-bit gray scale image in Image J (National Institutes of Health), and each region of the medial NCM was selected using circles (NCMr.d, NCMd, NCMv) or freehand tool (NCMc) (see Figure 1b). For each region of interest encircled, the size of the circle was constant across birds within each medio-lateral level, or the categorical distance from the midline (see Table 1). The areas of the circles changed with the changing size and shape of NCM as we moved from medial to more lateral sections. The circle delineating NCMr.d was placed in the rostral- and dorsal-most portion of NCM without including field L. NCMc was traced such that its caudal boundary was defined by the caudal edge of the brain, and its rostral boundary was a constant distance from the caudal edge within each medio-lateral level. The distance of the rostral boundary from the caudal edge changed as we moved laterally (Table 1). NCMv was selected using the largest circle that would fit along the ventral boundary of NCM and the boundary of field L without extending into NCMc. NCMd was selected using the largest circle that would fit midway between NCMr.d and NCMv and midway between field L

and NCMc. Within all regions of NCM, ZENK-immunoreactive (ZENK-ir) cell nuclei were quantified according to methods described in Maney et al. (2006). In short, an optical density threshold was used to select objects that represented immunoreactive cell nuclei within each region. The number of labeled nuclei per unit area sampled was calculated for each region. Numbers from each region were then averaged across the four consecutive sections and analyzed using a multiple analysis of variance with hormone treatment and sound stimulus as between-subjects factors. Two-way ANOVAs tested for effects of hormone treatment and stimulus type within individual regions. Student's t-tests were used to test for effects of hormone treatment within stimulus group, and vice versa, when significant main effects or an interaction were found.

*Analysis of behavior*

Video recordings were scored for vocal behavior, including song and contact calls, and CSD behavior, including tail lifts, wing quivers, and trills. Each behavior was noted at the time it occurred in the video. A Kruskal-Wallis nonparametric ANOVA was used to test effects of auditory stimulus type on the performance of CSDs. In order to determine whether the birds' own vocal behavior (trills, contact calls, and song) could have affected our results by inducing ZENK expression in NCM, three Mann-Whitney *U*-tests were performed, as follows: to determine whether the birds vocalized more to a particular stimulus, we compared the number of vocalizations given in response to tones to the number given in response to song for each hormone treatment group. To test whether E2 treatment influenced the birds' propensity to vocalize, we compared the number of vocalizations given by E2-treated birds hearing silence to the number given by

blank-implanted birds hearing silence. Similar studies have shown that the birds' own vocal responses do not affect ZENK immunoreactivity (Maney et al., 2003; 2006).

## Results

### *Plasma estradiol levels*

At the time of tissue collection we noted that all of the females' ovaries were regressed, indicating that none of the blank-treated birds had been photostimulated. Plasma E2 levels of both the blank-implanted birds ($M = 0.38$, $SD = 0.67$ ng/mL) and the E2-treated birds ($M = 1.02$, $SD = 0.44$ ng/mL) fell within the physiological range (Moore, 1983), and a t-test revealed that they were significantly higher than plasma E2 levels of blank implanted birds ($p < 0.001$).

### *ZENK induction within regions of interest*

There was a significant effect of stimulus on the number of ZENK-ir cells in NCM, $F_{8, 66} = 10.419$, $p < 0.001$. There was neither an overall effect of hormone treatment on the number of labeled cells, $F_{4, 32} = 0.991$, $p = 0.427$, nor a significant interaction between stimulus and hormone treatment, $F_{8, 66} = 1.666$, $p = 0.123$. Two-way ANOVAs testing for effect of hormone treatment and stimulus type on the number of ZENK-ir cells within each region revealed significant main effects of stimulus type in several regions (NCMd, $F_{2, 35} = 35.214$, $p < 0.01$; NCMv, $F_{2, 35} = 10.046$, $p < 0.001$; NCMc, $F_{2, 35} = 84.941$, $p < 0.01$) as well as a significant interaction in NCMr.d, $F_{2, 35} = 4.804$, $p = 0.014$. There were no significant main effects of hormone treatment on the number of ZENK-ir cells in any region of NCM (NCMr.d, $F_{1, 35} = 2.074$, $p = 0.159$; NCMd, $F_{1, 35} = 2.778$, $p = 0.105$; NCMv, $F_{1, 35} = 2.112$, $p = 0.115$; NCMc, $F_{1, 35} = 4.021$, $p = 0.053$).

*Post-hoc* pairwise comparisons revealed distinct effects of stimulus and treatment throughout NCM. E2 treatment modulated ZENK expression in regions NCMd and NCMv so that the response was selective for song over tones. There was greater number of ZENK-ir cells in E2-treated birds hearing song compared to E2-treated birds hearing tones in both NCMd ($p = 0.011$, $d = 1.66$) and NCMv ($p = 0.043$, $d = 1.25$ ), whereas in blank implanted birds, song and tones induced similar levels of ZENK immunoreactivity (NCMd, $p = 0.170$, $d = 0.78$; NCMv, $p = 0.598$, $d = 0.29$) (Figure 2). In blank-implanted birds, hearing tones induced more ZENK immunoreactivity in both regions compared to hearing silence (NCMd, $p < 0.0001$, $d = 3.13$; NCMv $p = 0.004$, $d = 1.87$).

In NCMr.d, the number of ZENK-ir cells was greater for birds hearing song and birds hearing tones compared to the silence condition in blank-implanted birds ($p = 0.017$, $d = 1.48$, and $p = 0.005$, $d = 1.84$, respectively) (Figure 2). There was, however, no difference between the song and tones conditions among these birds ($p = 0.436$, $d = 0.43$). Unlike in NCMd and NCMv, E2 did not induce selectivity in NCMr.d. Rather, it eliminated differences in ZENK expression among stimulus groups. Among E2-treated birds, those hearing song and those hearing tones had similar levels of ZENK immunoreactivity ($p = 0.315$, $d = 0.58$); those hearing song and those hearing silence had similar levels ($p = 0.782$, $d = 0.16$); and those hearing tones and those hearing silence had similar levels ($p = 0.421$, $d = 0.45$).

In contrast with the other three regions, there was no effect of hormone treatment in NCMc. The ZENK response was selective for song over tones independent of hormone treatment (E2-treated, $p < 0.001$, $d = 2.75$; blank-implanted, $p < 0.0001$, $d = 3.08$) (Figure 2). In both E2-treated and blank-implanted birds, hearing tones induced

more ZENK immunoreactivity compared to hearing silence (E2-treated, $p = 0.009$, $d = 1.65$; blank-implanted, $p < 0.001$, $d = 2.88$).

We looked at the effects of E2 treatment on ZENK expression in the absence of an auditory stimulus by comparing the number of ZENK-ir cells in E2-treated birds that heard silence to the number of labeled cells in blank-implanted birds that heard silence. E2 did modulate basal levels of ZENK expression in several regions. In regions NCMr.d, NCMd, and NCMv, E2 treatment increased the number of ZENK-ir cells in birds that heard silence (NCMd, $p = 0.019$, $d = 1.44$; NCMv, $p = 0.035$, $d = 1.27$; NCMr.d, $p = 0.004$, $d = 1.90$). In NCMc, E2 treatment did not significantly increase ZENK expression in the silence condition ($p = 0.129$, $d = 0.87$), which coincides with the lack of an effect of E2 treatment on selectivity within this region. However, the lower $p$ value and large effect size might indicate a trend for E2 to increase ZENK expression in NCMc. A larger sample might expose an effect of E2 in this region. Overall, our results suggest that E2 treatment does not modulate ZENK expression in NCMc.

*Behavioral responses*

Only E2-treated birds hearing song performed CSDs (Kruskal-Wallis H = 6.790, $p = 0.034$). In order to determine whether hearing a particular auditory stimulus may have affected the number of vocalizations produced and thus the auditory ZENK response, we tested whether the number of vocalizations differed between the birds hearing song and those hearing tones. There was no effect of auditory stimulus in either the blank-implanted birds ($p = 0.710$) or the E2-treated birds ($p = 0.234$). Taken together, these results suggest E2 induces selectivity in the ZENK response by a mechanism other than affecting vocal responses to auditory stimuli. When we compared the number of

vocalizations given by E2-treated birds hearing silence to the number given by blank-implanted birds hearing silence, we found no difference ($p = 0.165$). This result suggests that the increased ZENK expression in E2-treated birds was unlikely to be caused by E2-induced increases in vocalization behavior.

## Discussion

In the caudomedial nidopallium of songbirds, immediate early genes are expressed in a quantity relative to the behavioral significance of the auditory stimulus being perceived (Gentner et al., 2001; Maney et al., 2003, Mello et al., 1992; Mello et al., 1995). The patterns of these genomic responses are also dependent on the qualities and contextual information of the stimulus as well as differences among the species that perceive the stimulus (Ribeiro et al., 1998; Gentner et al., 2001; Maney et al., 2003; Avey, Kanyo, Irwin, & Sturdy, 2008). In addition, our lab has previously demonstrated that the reproductive state of a seasonally breeding female influences the selectivity of the ZENK response, such that the response is selective for song over tones when birds are treated with E2 (Maney et al., 2006). The present study demonstrates that the effects of E2 on selectivity are not uniform throughout NCM and that the ZENK response within distinct regions may be modulated seasonally. We also demonstrate that breeding levels of E2 alone increase ZENK expression within specific regions of NCM, which suggests that neuronal activity in this auditory area may be heightened when seasonally breeding females are searching for a mate.

### *E2 and selectivity within subregions of NCM*

NCM was divided into subregions based on previous literature as well as known distributions of neurochemicals and receptors. The regions we defined as NCMd and

NCMv overlap regions that have been demarcated in previous literature and are known to exhibit selective ZENK responses to auditory stimuli (Gentner et al., 2001; Maney et al., 2003). In the present study we sought to investigate the effects of E2 on selectivity within NCMd and NCMv and found effects that were similar to the E2-induced selectivity described by Maney et al. (2006). In E2-treated birds, hearing song induced significantly more ZENK-ir than hearing tones. In blank-implanted birds, however, song and tones induced similar levels of ZENK-ir. These results suggest that regions NCMd and NCMv are selective for song only when plasma E2 is at breeding levels.

Maney et al. (2006) reported a significant decline in ZENK immunoreactivity in E2-treated birds that heard tones compared to blank-implanted birds that heard tones. This decrease in labeled cells in response to tones stimuli suggests that E2 plays a role in inhibiting responses to stimuli that are not behaviorally relevant and increasing auditory discrimination. In our study, however, there was no clear inhibition of the ZENK response to tones due to E2 treatment. The discrepancy between the results of our study and results described by Maney et al. (2006) might be due to differences in the location of the ZENK immunoreactivity that was quantified in each study. Maney et al. (2006) quantified ZENK expression within a lateral portion of NCM (~2.0-2.5mm from the midline), whereas we investigated more medial sections (500-800 μm from the midline). Quantifying ZENK immunoreactivity in sections lateral to our selected area and comparable to the region of NCM Maney et al. (2006) analyzed will provide an additional and more thorough illustration of the effects of E2 on selectivity throughout NCM. It is possible that different mechanisms allow E2 to influence ZENK expression in multiple ways across the lateral expanse of NCM.

It was necessary to consider as an alternative explanation for our results the idea that the birds' own vocalizations might have induced ZENK expression. We saw an effect of E2 treatment on selectivity that may be explained by an E2-induced increase in the number of vocalizations produced. We found no effect of E2 treatment on the number of vocalizations, however, which suggests that E2 treatment did not affect the birds' propensity to respond to a particular stimulus more than the other. In addition, because there was no difference in the number of vocalizations produced, the differences we see in ZENK expression must be attributable to E2-induced changes in gene expression in response to the auditory stimuli and not the birds' own vocalizations. Similarly, Maney et al. (2003) reported that the birds' own vocalizations did not influence ZENK expression within NCM of white-crowned sparrows, and Maney et al. (2006) reported that the E2-induced selective response for song was not due to the number vocalizations birds produced in response to song.

NCMr.d was selected because it is a region of NCM that is not often discussed in previous literature. This region of NCM has often been ignored or lumped together with other regions of NCM (e.g. Chew et al., 1995; Stripling et al., 1997; Ribeiro et al., 1998; Gentner et al., 2001; Maney et al., 2003; Phillmore, Bloomfield & Weisman, 2003; Pinaud et al., 2006; Terleph, Mello & Vicario, 2006; Tomaszycki, Sluzas, Sundberg, Newman & DeVoogd, 2006; Avey et al., 2008). Our results suggest that NCMr.d is an interesting region of NCM that behaves differently from NCMd and NCMv. In response to sound and silence, ZENK is expressed differentially in NCMr.d only when plasma E2 is at low (non-breeding) levels. In blank-implanted birds, hearing song or tones induced significantly more ZENK immunoreactivity than hearing silence, whereas E2- treated

birds had similar levels of ZENK immunoreactivity in all stimulus conditions. Whereas in NCMd and NCMv E2 appears to tune the ZENK response to the more behaviorally relevant stimulus (song), in NCMr.d E2 appears to have the opposite effect in that ZENK is expressed in response to auditory stimuli and silence equally in the E2-treated birds. ZENK expression is often associated with membrane depolarization (reviewed by Mello, 2004), and because ZENK expression is similar across stimulus groups with E2 treatment in NCMr.d, our results could indicate that E2 eliminates differential firing to auditory stimuli in this region. However, depolarization is not always associated with ZENK expression. ZENK expression has been linked to neuronal plasticity in the absence of depolarization, and the effects seen in NCMr.d could be indicative of long-term, downstream changes (reviewed by Mello, 2004). The lack of a ZENK response to sound in NCMr.d across stimulus groups may also reflect a ceiling effect. E2-treatment appeared to elevate ZENK expression in the silence group to the peak of the potential labeling within NCMr.d, and sound was unable to induce a detectable ZENK response.

In this study, the caudalmost region of NCM, NCMc, was not affected by E2 treatment. Several studies suggest that NCMc may be a neurochemically distinct region of NCM. A subset of GABAergic neurons labeled for calbindin has been located along the caudal region of NCM and may play a regulatory role in this region (Pinaud et al., 2006). In our own lab we have observed a dense collection of fibers immunopositive for tyrosine hydroxylase (TH), a rate-limiting enzyme involved in the synthesis of catecholamines, in this region (S. Sanford, unpublished). Catecholamines are often implicated in attentional and auditory processing in songbirds (Appeltants, Ball, & Balthazart, 2001; Appeltants, Del Negro, & Balthazart, 2002; Appeltants, Ball,

Balthazart, 2004; Lynch & Ball, 2008). Because we also know that E2 treatment increases the density of TH labeling in the auditory forebrain (LeBlanc, Goode, MacDougall-Shackleton, & Maney, 2007), we suspected that the effects of E2 on catecholamines within NCM might be one mechanism for E2-induced changes in auditory processing and that NCMc might be a prime region where these effects occur. Estrogen receptors have been located along the caudal edge of NCM (Gahr, 1996), and we hypothesized that E2 might be having direct effects on neurons in this region. Unlike the other regions we investigated, however, NCMc was not affected by E2 treatment. E2 treatment did not appear to modulate sound-induced ZENK immunoreactivity in this region, because the ZENK response in NCMc was selective for song in both the blank-implanted and E2-treated birds. These results were surprising given the distribution of estrogen receptors, previous results from our lab demonstrating that E2 increases the density of TH labeling in NCM (LeBlanc et al., 2007), and that in our lab we see the densest TH staining in the caudal edge of NCM. Nonetheless, the fact that NCMc responds differently than the other regions we investigated further supports the idea that this region is neurochemically distinct and possibly even functionally distinct. Because E2 did not modulate ZENK immunoreactivity in NCMc, this region may exhibit less seasonal plasticity than the more rostral regions.

*E2 and basal levels of ZENK expression*

In addition to mapping the effects of E2 on selectivity within NCM, we also wanted to test whether E2 influenced basal levels of ZENK expression. Whereas E2 had no effects on ZENK expression in NCMc, E2 treatment increased basal levels of ZENK expression in the three rostral regions (NCMd, NCMv, NCMr.d). In the absence of

auditory stimulation ZENK expression within NCM is low (Mello & Clayton, 1994), and this low level of expression may be the result of various factors, including responses to background noise or natural activity within neurons (Worley, Christy, Nakabeppu, Bhat, Cole, & Baraban, 1991). Elevated basal levels of ZENK expression are known to occur during the song acquisition period in juvenile zebra finches independent of the birds' own vocalizations, and may reflect an increase in the efficiency of synaptic modifications related to consolidation and learning (Jin & Clayton, 1997). By enhancing neuronal activity, E2 effects downstream changes in the structural or functional properties of synapses. Because we do see an increase in ZENK expression in birds that heard silence, our results suggest that neurons in rostral regions of NCM may be more active in synthesizing proteins and more plastic in general during the breeding season.

Although E2 treatment elevated basal levels of ZENK expression, E2 treatment did not increase ZENK expression in either the tones or song stimuli groups. Similar studies in songbirds have shown that elevated plasma E2 levels do not increase ZENK expression in birds hearing auditory stimuli such as tones and conspecific song (Maney et al., 2006; Duffy et al., 1999). Lynch and Wilczynski (2008) looked at the effects of elevated E2 levels and male chorus playback on *zenk* (egr-1) expression in the auditory midbrain of an anuran. They found that both elevated E2 levels alone and male chorus playback alone did not increase *zenk* expression above baseline (saline treated frogs listening to silence). However, when these two conditions were combined there was a significant increase in *zenk* expression above baseline suggesting an additive effect of elevated E2 levels and hearing a conspecific signal on *zenk* expression. Based on these results in a frog we might have expected E2 treatment to enhance expression in the two

stimuli groups in our birds given that E2 treatment alone enhances IEG activity. We did not see this enhancement of IEG expression in the stimuli groups but did see it in the silence condition, which suggests that in our birds E2 and sound are affecting ZENK expression in a non-additive manner.

In order to verify that E2 treatment did not lead to an increase in the birds' own vocalizations and thereby an increase in ZENK expression, we compared the number of vocalizations produced by E2-treated birds hearing silence to the number produced by blank-implanted birds hearing silence. We found no difference between these treatment groups in the number of vocalizations, which suggests that E2 treatment did not increase the birds' vocal activity. Therefore, our results suggest E2 treatment increased basal levels of activity in NCMr.d independent of the birds' own vocalizations.

*Conclusion*

We saw effects of E2 on ZENK immunoreactivity only in the rostral regions NCMd, NCMv, and NCMr.d, which suggests that these regions of NCM are seasonally regulated whereas the caudalmost region, NCMc, is not. We demonstrate that E2 influences selectivity in NCMd and NCMv and appears to heighten neuronal activity in all three rostral regions during the reproductive season. ZENK expression within NCMc, however, was not modulated by E2, and NCMc may continually process song selectively irrespective of the reproductive state of the bird. NCM is large heterogeneous area, and patterns of ZENK expression throughout NCM vary depending on the behavioral relevance and qualities of the auditory stimuli the bird is hearing (Ribeiro et al., 1998; Mello et al., 1992; Mello et al., 1995; Gentner et al., 2001; Maney et al., 2003; Avey et al., 2008). We also see patterns of ZENK expression that are dependent on hormone

treatment, which suggests that E2 may be influencing the behavioral relevance of the auditory stimuli our birds heard. Outside the breeding season the female is not receptive to males, and when the male sings she does not perform the copulation solicitation display (CSD) that is indicative of her readiness to mate. During the breeding season, however, the behavioral relevance of conspecific male song changes for the female, and this change is evidenced in a change in behavior where the female demonstrates her receptivity by performing the CSD. Our results, in addition to the results of Maney et al. (2006), suggest that the change in behavioral relevance is reflected in auditory processing. Seasonal changes that occur in auditory processing are not likely related to changes in discrimination abilities (Reeves, Beecher & Brenowitz, 2003). Rather, the influx of E2 during the breeding season appears to set up an internal context within NCM for mating, and as a result, tunes the response of neurons in specific regions to song. This tuning may allow neurons to be more responsive to relevant stimuli (or less responsive to less relevant stimuli) and may increase the potential for categorization or memorization of male songs during the mating season (Ribeiro et al., 1998; McKenzie, Hernandez, & MacDougall-Shackleton, 2006). The pattern of the effects of E2 within NCM, as revealed in our regions, appears to reflect this tuning process. Our results were similar to results of previous studies in that there was no effect of E2 treatment on the absolute level of ZENK immunoreactivity induced by hearing song (Duffy et al., 1999; Maney et al., 2006). Rather, tuning appears to result from the differences in neuronal responses to dissimilar stimuli, with these differences in responses being induced by seasonal changes in reproductive state (Stripling et al., 2001; Maney et al., 2006). The next issue to explore is the mechanism behind this E2-induced tuning within NCM. Investigation into

the effects of E2 on the neurochemical makeup of NCMd, NCMv, NCMr.d, and NCMc will help characterize potential functional domains within NCM and the role they play in the processing and interpretation of auditory stimuli.

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Table 1

*Dimensions for Regions of Interest within NCM*

| | Area ($mm^2$) | | | Distance from caudal boundary of NCM (mm) |
|---|---|---|---|---|
| Distance from the midline (μm) | NCMd | NCMv | NCMr.d | NCMc |
| 500 | 0.208 | 0.238 | 0.103 | 0.264 |
| 600 | 0.259 | 0.259 | 0.085 | 0.251 |
| 700 | 0.262 | 0.297 | 0.107 | 0.281 |
| 800 | 0.317 | 0.317 | 0.115 | 0.284 |

## Figure Captions

*Figure 1.* Sagittal views of our regions of interest within NCM. (A) Schematic representation of the auditory forebrain, (B) Image of NCM showing how our regions of interest were selected in photomicrographs. CMM, caudomedial mesopallium; HP, Hippocampus; L, field L; NCMc, caudal region of the caudomedial nidopallium; NCMd, dorsal region of the caudomedial nidopallium; NCMr.d, rostrodorsal region of the caudomedial nidopallium; NCMv, ventral region of the caudomedial nidopallium.

*Figure 2.* The number of ZENK-ir cells per $mm^2$ (± S.E.) within regions of NCM in blank implanted and E2-treated females listening to song, tones or silence. NCMc, caudal region of the caudomedial nidopallium; NCMd, dorsal region of the caudomedial nidopallium; NCMr.d, rostrodorsal region of the caudomedial nidopallium; NCMv, ventral region of the caudomedial nidopallium. *Significantly greater than silence, $p < 0.05$; ** Significantly greater than tones and silence, $p < 0.05$; † Significant effect of E2 in the silence condition, $p < 0.05$.

Figure 1. Top

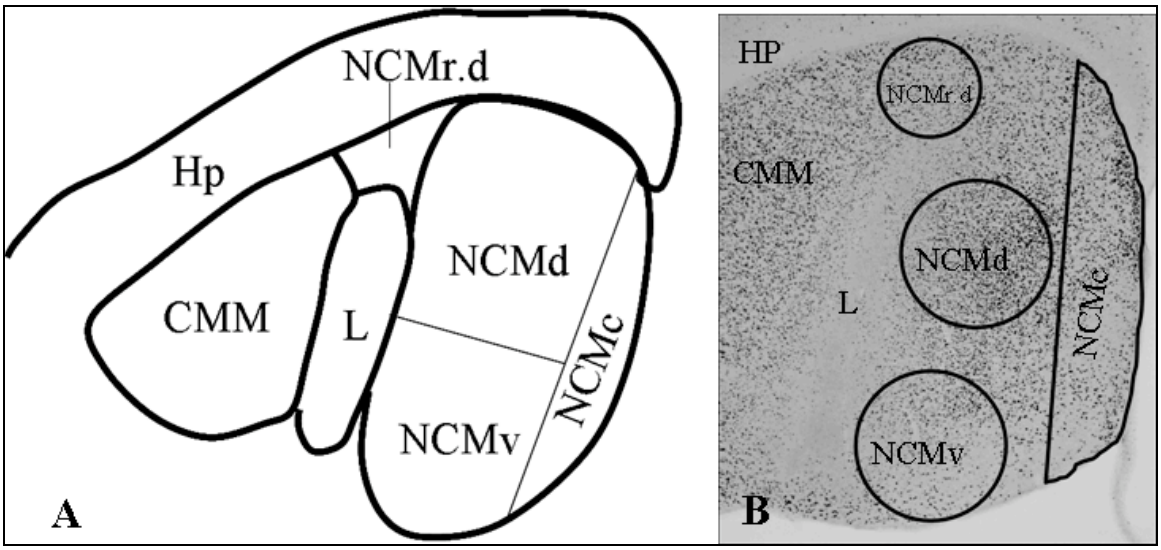
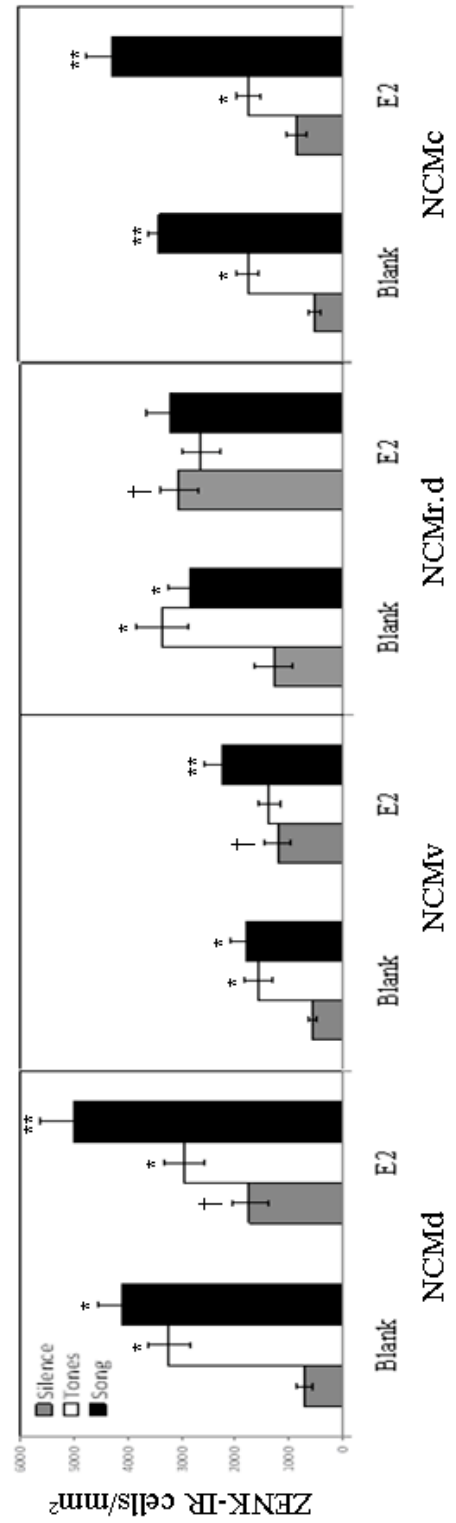


Figure 2.

Top


ZENK-IR cells/mm²
6000
5000
4000
3000
2000
1000
0
Silence
Tones
Song
NCMd
NCMv
NCMr.d
NCMc
Blank
E2