ESTROGENIC MODULATION OF AUDITORY PROCESSING IN

FEMALE SONGBIRDS

by

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ABSTRACT OF THE THESIS

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In songbirds, male song is learned and is thought to be sexually selected for as a function of female mate choice. In this view, females must choose a conspecific mate whose song provides an honest signal of his quality. Male zebra finches copy an adult tutor's song during development, and a memory for this tutor song is stored in the brain auditory processing area NCM. Female zebra finches do not copy, but they do show a behavioral preference in adulthood for the tutor song that they heard during development. Surprisingly, female NCM does not appear to have a memory for the tutor-song like that in males. We hypothesized that the expression of this memory in female NCM can be modulated by gonadal hormones, specifically that physiological estrogen levels in breeding females may influence sexual behavior by inducing changes in NCM that allow the previously formed tutor-song memory to be expressed, enable conspecific sounds to be discriminated from less relevant sounds, and facilitate acquisition of new auditory memories - all processes which may contribute to successful mate choice and

ii

reproduction. We tested these hypotheses by rearing females (n=26) in a controlled environment and exposing them to an artificial song-tutor during the sensitive period. In adulthood, we recorded their electrophysiological responses to tutor-song playback during estrogen (n=10), anti-estrogen (n=8) or vehicle (n=8) treatment. We also tested the same animals for conspecific vs. heterospecific song discrimination and the acquisition of memories for recently heard songs. Our results did not show an effect of hormonal manipulations on the expression of the tutor-song memory and thus did not support our primary hypothesis. However, anti-estrogen treatment degraded conspecific song discrimination and the acquisition of a memory for recently heard songs, suggesting that these processes require normal estrogen levels for their expression. Our results are consistent with an important role for estrogen in auditory processing and memory acquisition, consistent with studies in other animal systems. Future work will address the mechanisms by which estrogen modulates these effects.

Table of Contents

List of Figures

Background

A. Song as a Sexually Selected Trait

 I. Overview of Sexual Selection

  When Darwin proposed his theory on evolution he suggested that the survival of a species depends on its members being able to adapt to the ever changing pressures of their environments. The ability of a species to survive also relies on its members having offspring to pass the more adaptive behaviors (and genes) on *to*. A major component of accomplishing that goal in sexually reproducing species necessitates that its members be able to recognize reproductive fitness, "sizing up" potential mates for the traits that will help them pass on their genes to future generations such as fertility and the ability to produce quality offspring. Darwin recognized this and suggested that a secondary form of selection, sexual selection, was also at work.

  In most species, the female sex invests more energy and time into reproduction. Therefore, the females of these species tend to be the more selective sex when choosing a mate. In order to produce viable offspring, she must select a mate that is able to father offspring that possess traits that will ensure their survival. Because potential suitors do not introduce themselves bearing transcripts of their genetic endowments, the female chooser needs some other way of distinguishing the good versus bad mates. Luckily, observable traits can serve as clues to the reproductive quality of the male. For example, in peacocks the male's long tail is thought to be metabolically expensive to produce and advertises that he is capable of surviving predation. He is capable of surviving even though his bright colors make him more noticeable and the long tail makes it more difficult to evade his predators. The female can rely on perceivable cues that reliably

predict mate quality. However, it is in the male's interest to present himself as fit as possible, so the female must rely on "honest" signals that males cannot fake.

II. Female songbirds size up suitors by their songs

Songbirds communicate with each other through calls (which are typically innate) and song (which must be learned). In most species, both males and females produce calls, which can provide an array of signals depending on the call emitted. For instance, the sounding of an alarm call can alert members of a colony that a predator is nearby. A female may emit a long call when separated from her mate in order to locate him. Hungry hatchlings may bombard their parents with begging calls in order to gain food. Calls serve as signals, providing the receivers with information about the environment. Though arguably in a different way, song serves the same role as a signal. In sexually dimorphic species (in which males sing and females do not), singing serves two purposes: 1) to defend one's territory and 2) to attract a mate (Zann, 1996). As in humans, the latter can be a very involved process, though in songbirds the courting process *literally* involves a song and dance. In an attempt to mate, an interested male will approach a female and perform a courtship dance as he serenades her with his song. The female will listen to her potential suitor's song and possibly signal her interest in mating with him. In response to songs that they find "sexy," female songbirds will perform a copulation solicitation display (CSD), a series of tail quivers and other movements. Past studies have used CSD assays and two-choice speaker paradigms in order to identify what features characterize a song as mate-worthy (reviewed in Nowicki et al, 2002). The results of those studies suggest that different birdsong species share common preferences

and that females distinguish among various songs and select a mate based on differences in three features: the length of song or the frequency with which it is sung, complexity, and locality of the dialect. According to female songbirds, the longer, the more complex, and the closer to her own dialect the song is, the better.

III. Songs Don't Lie: Females can rely on song for an honest signal

Why do females rely on song in order to select a good mate? Like any sexually selected trait, male song reveals details about a male's reproductive fitness. Nowicki and Searcy (2004) suggest that when a male woos his potential mate, he provides her with a "window" into his developmental history. According to the Developmental Stress Hypothesis (Nowicki and Searcy, 2004; Nowicki et al, 2002; Ritchie et al, 2008; Spencer et al, 2003), the ability of a male to sing well relies on his ability to fare well in the face of early stressors that may limit his physiological resources. Even though song-learning occurs later in development, the brain structures that enable that learning are being formed early in post-natal development and require energy to produce. A hatchling confronted with stressors such as limited food resources or parasitic attack may lose energy that could otherwise be invested in establishing song-learning dependent brain structures. However, a hatchling that *can* achieve proper brain development (and is therefore better equipped to learn song) in the face of such stressors carries more reproductive fitness, since he may pass his resiliency on to future offspring. Evidence for this theory comes from studies (reviewed in Nowicki and Searcy, 2004) that have examined the effects of nutritional challenges on song-learning. Hatchling songbirds that were hand-reared and subjected to food deprivation showed decreases in the volumes of

brain nuclei essential to song production, impaired song learning, growth stunting, and immune suppression (MacDonald et al, 2006; Nowicki et al, 2002). As adults, the food-deprived birds sang less often and sang more slowly than normally reared birds, effects that rendered their songs less attractive to females.

B. Female Preference

    I. Preference is Learned

If a male's song provides a signal of reproductive fitness, the female has access to the information she needs to select a quality mate, assuming she knows how to interpret the information being broadcast to her. Since the female invests much energy into reproduction and in some species (e.g., zebra finch) remains paired with her mate (Zann, 1996), she would be well-served if she could accurately distinguish a good song from a bad one. The ability to do so does not come naturally. Studies investigating female song preference have revealed that preferences depend on a female's experience with song. Her preference must be shaped through her own exposure to song throughout development. Experiments on the development of song preference have found that a young female requires auditory experience from an adult male to 1) Be able to distinguish the auditory qualities of what she hears (influence brain development to establish general perceptual abilities) and 2) Use features of what she hears to discriminate between good and poor quality songs. As in other sensory systems, the normal development of the auditory system is experience-dependent. Developing a preference for song, then, first requires that the auditory system is capable of discriminating among various qualities of song. Sturdy et al (2001) showed that in order for a female to develop a preference for quality song, she must be raised with exposure to male song. Females raised in female-only clutches were impaired in their performance on an operant task that required them to discriminate between different pairs of songs. In normal rearing conditions, young females will be exposed to the songs of their fathers and therefore develop the normal auditory processes that will allow them to discriminate among songs. Assuming

development of normal perceptual abilities and exposure to quality song as juveniles (Riebel, 2000; Lauay et al, 2004), the female will listen to and learn the characteristics that make up quality song as she grows. Later, she will use the knowledge she has gained about song quality to select a mate that sings a song with similar characteristics to the ones she heard from her male tutor during development.

II. Preferences for familiar song imply existence of a memory

When confronted with a male attempting to court her, a female must decide whether his song quality resembles that of early tutor-song. Female songbirds have been shown to prefer a familiar song over a novel one (Miller, 1979a and b; Clayton 1990). Miller (1979b) tested female zebra finches in a two-choice paradigm in which two speakers played the song of her father or a novel song and found that females spent significantly more time near the speaker playing her father's song. In a similar study (1979a), Miller used the same paradigm but using mate-song instead of the father-song. In that study, females spent significantly more time near the speaker airing the mate's song rather than the novel one. The results of these studies reveal that the female recognizes and prefers songs that are familiar to her rather than ones that she has never heard before. From such observations, one may infer that females develop a memory for the familiar songs. In selecting a mate, she may recall the memory of her father's song (or the male that fosters her) and compare its features with those of the male vying for her attention.

C. In Search of the Memory

I. The Male Counterpart

If female songbirds do develop a memory for their tutor's song, one of the easiest

places to start searching is in the place where it was found in the male songbird brain. As

the singing sex, young male songbirds develop a set of brain structures that make up the

"song-circuit," which enables the learning and production of song. The song-circuit

consists of one pathway important for song-learning (Anterior Forebrain Pathway: Area

X, DLm, LMAN) and a more caudal pathway necessary to song output and production

(Motor Pathway: HVC, RA, XIIts) (Figure 1). Because song is sexually dimorphic and

shares many features of human speech learning (Doupe and Kuhl, 1999), the majority of

experiments on songbirds have focused on the brain structures that underlie male song

production and perception in an attempt to identify possible similarities in the processes

of song and speech development. Those studies have contributed to a greater

understanding of the neural processes that allow a young male to mold his initial

vocalizations to represent those of his tutor. Song development progresses across three

stages (Figure 2). During the sensory phase (post-hatch day 30-50), male fledglings

listen to and memorize the song of their tutor and begin to produce vocalizations.

Juvenile males then progress into the sensorimotor period of song learning (ca. 45-90), in

which they practice singing and modify their output to match the song of their tutor. As

the males reach sexual maturity, song structure becomes stereotyped and closely

represents the tutor-song, though the final product is not an exact match. A male's

progression through this song-learning process depends on exposure to song during the

sensitive phase so that he may form a template. Recent studies suggest that this template
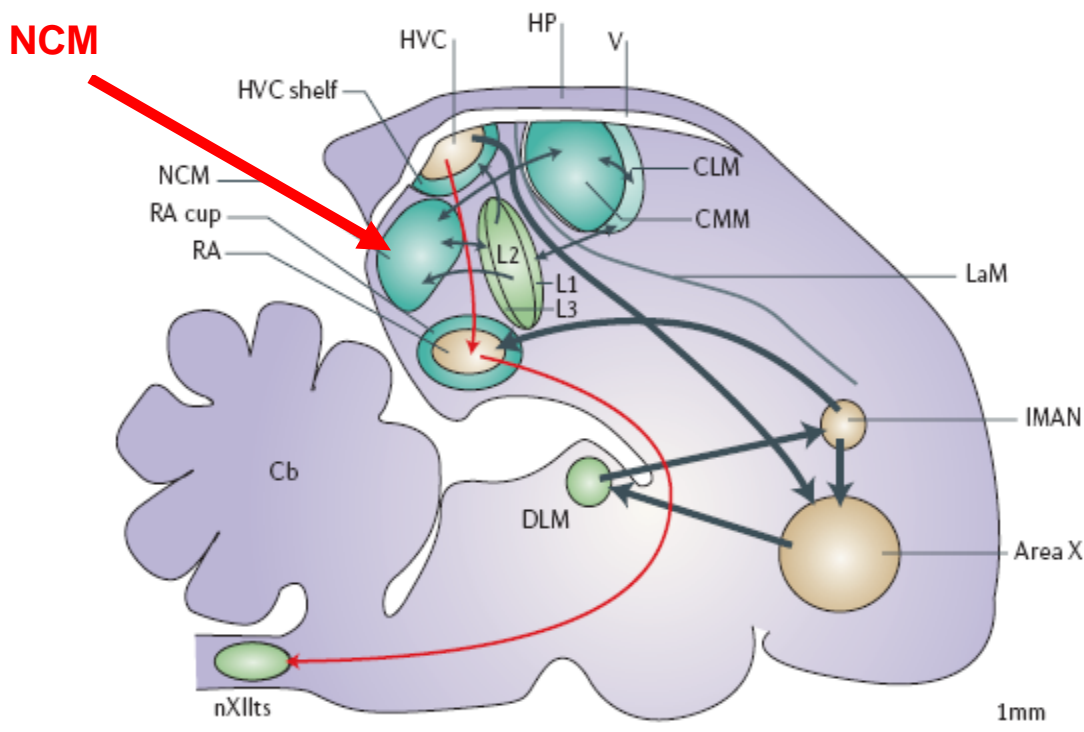
Fig. 1(top): Brain of a male songbird. HVC, RA, and nXIlts make up the motor pathway and are necessary for song production. IMAN, Area X, and DLM are structures important to song learning and are collectively referred to as the anterior forebrain pathway. Tutor-song memory is stored in NCM, an auditory association area that receives its input from the Field L complex, the thalamo-recipient forebrain area that is analogous to the primary auditory cortex of mammals. (Figure from Bolhuis and Gahr, 2006).

Fig. 2 (bottom): Trajectory of male song development in a closed-ended song learner. Song learning requires exposure to song during a sensory phase in which a template is formed, a plastic stage in which vocalizations are modified to match the tutor-song, and a crystallization stage in which song becomes stereotyped. (Figure from Johnson lab).
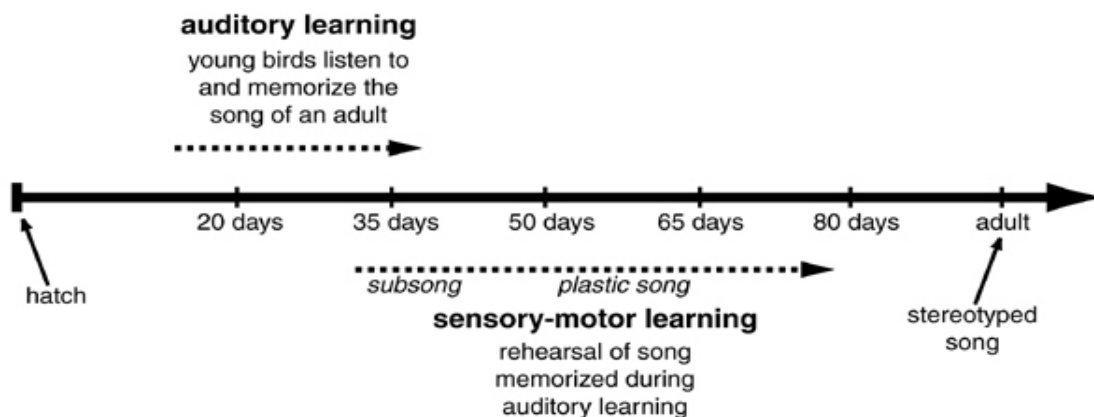
in male zebra finches is located in an auditory brain region of songbirds called the caudomedial nidopallium (NCM).

The NCM response patterns to auditory stimuli observed in electrophysiological studies share similar qualities with the pattern of ZENK induction observed in immediate early gene (IEG) expression studies. The IEG ZENK (an acronym for the names it was assigned by four labs that independently discovered it; aka zif-268) has been shown to be specifically expressed in male and female NCM in response to song stimuli rather than tones or silence and is more highly expressed in response to conspecific versus heterospecific song presentations (Mello et al, 1992). In parallel to the adaptation that occurs in electrophysiological recordings, ZENK expression in NCM also decreases in response to repeated presentations of a song stimulus, but is reinstated if a novel song is subsequently introduced (Mello et al, 1995). Together, the evidence from ZENK and electrophysiological studies indicates that NCM responses differ according to the nature of the auditory stimulus presented.

II. Caudomedial Nidopallium (NCM) and Memory

NCM is an auditory nucleus that lies outside the collection of song-circuit nuclei described above (Figure 1) and has been recognized as part of a system that enables a songbird to recognize individual calls and songs of other birds (because song imitation is inaccurate, a bird's song is similar to the tutor-song but unique to each bird) (Chew et al, 1995). It receives projections from primary auditory areas (Field L) and performs higher order processing of salient auditory stimuli. Electrophysiological recordings obtained from NCM in response to auditory stimuli indicate that its neurons initially respond robustly to song stimuli and then adapt rapidly with repeated presentations of a single

stimulus (Chew et al, 1995) (Figure 3). The adapted responses recover upon presentation of a novel stimulus. Furthermore, the adaptation rates exhibited in response to song are stimulus-specific, suggesting that NCM keeps an "independent neuronal record of each stimulus" (Chew et al, 1996, p. 1951). In other words, the response amplitude elicited by a single song will be maintained at a lower level even when a second stimulus is presented intermittently (Figure 4). These adapted responses are maintained for at least 48 hours for conspecific and 6-8 hours for heterospecific song after a session of repeated stimulus presentation and are thought to represent memory for specific auditory stimuli, in this case the songs of other individuals.

Fig. 3: Multiunit responses decrease following repeated presentations of the same conspecific song. Traces from top to bottom show the sonogram of a representative zebra finch song, the amplitude envelope of the song, and the rectified multiunit recording on the 1st, 20th, and 100th song presentations (Figure from Chew et al, 1995).
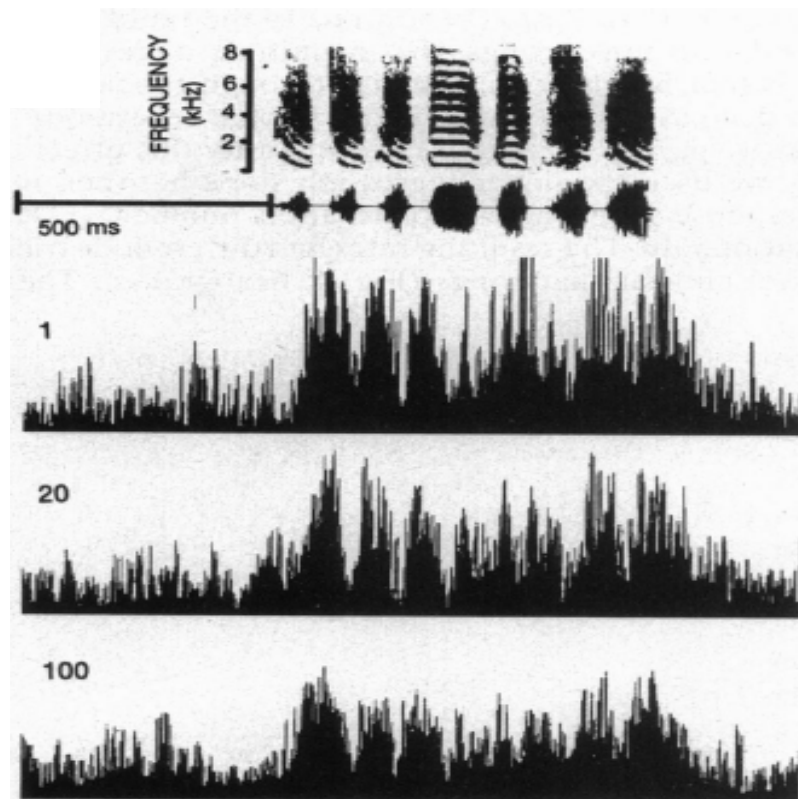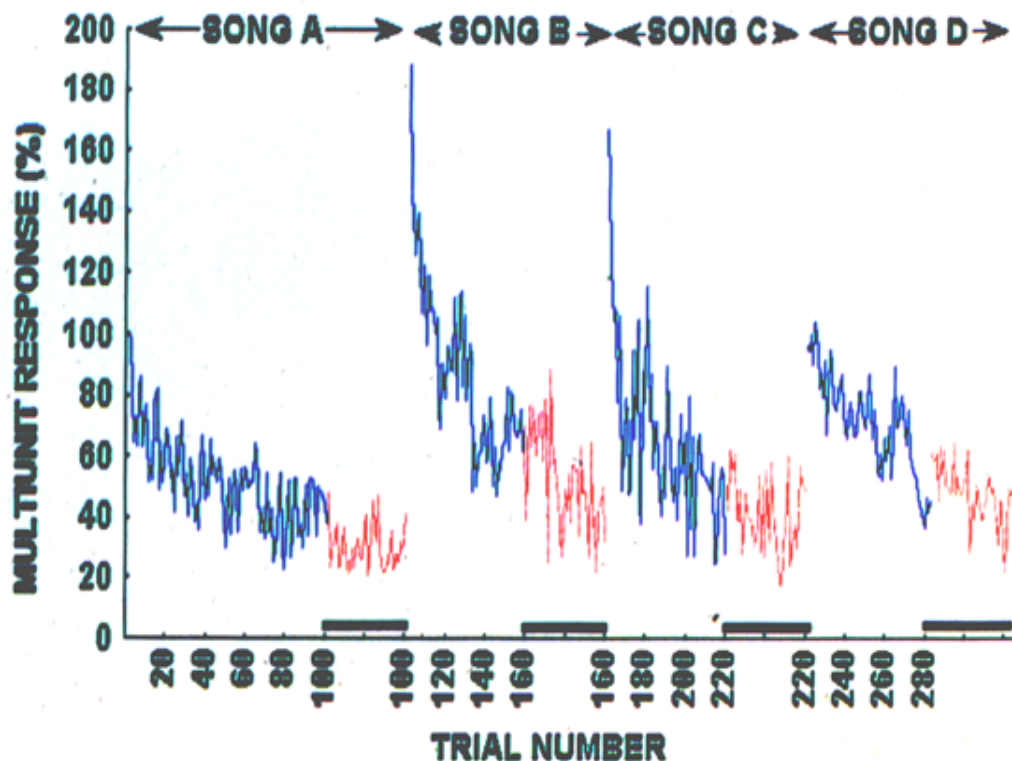
Fig. 4: MUA responses to four different songs presented sequentially at a single site. All responses have been normalized to the initial responses at song A. Different songs elicited different initial responses but all habituated during training. Immediately after training, songs A-D were tested on trials 281-320, 321-360, 361-400, and 401-440, respectively. The responses collected during testing (red) are plotted consecutively with responses collected during training (blue) to show that the habituated response level was maintained even after training with other songs. (Figure from Chew et al, 2005).

III. NCM and Tutor-Song Memory

Using the knowledge obtained about the behavior of NCM neurons from previous studies conducted in animals with a short-term exposure to song, Phan et al (2006) tested whether NCM also held the long-term memory for tutor-song. In this study, male and female zebra finches were removed from their natural parents and raised in sound-attenuating boxes either with an artificial tutor or in isolation. As adults, they were tested

for neural responses to various auditory stimuli including the song of their tutor and novel song. Since NCM adapts more rapidly to novel versus familiar stimuli, a familiarity index (FI= adaptation rate to novel / adaptation rate to "familiar" test song) was used to compare the familiarity of the tutor song in both tutored and untutored birds. When FI ~1.0, the test song is no different from novel; an FI > 1 indicates familiarity: a recognition memory. Males that had been raised with the tutor were found to have significantly higher FIs to tutor-song (mean FI =1.32, n=9) than birds raised in isolation song (mean FI= 0.959, n=5), evidence that the tutored males had a memory for their tutor's song. However, for females tested in the same paradigm, the difference in FI between tutored (mean FI= 1.008, n=7) and isolate (mean FI= 0.947, n=5) birds was not significant (M. Phan and D. Vicario, unpublished data).

IV. Memory in Females

In the early electrophysiological analyses of NCM responses to auditory stimuli (Chew et al, 1995, 1996), both males and females exhibited the same patterns of neural activity in this "neuronal record" keeper; no sex difference was found in the characteristics of NCM. Behavioral experiments have indicated that females recognize their father's song (Miller, 1979b). If females exhibit the same *general* response characteristics of NCM as males and display a behavioral preference for the tutor-song, then why do they fail to exhibit a memory for tutor-song?

As Cynx and Nottebohm (1992) point out, behavioral responses to stimuli depend on their salience, which in turn depends on factors such as motivation, attention, perception, and memory. For female songbirds, a male's song serves as an advertisement

of his reproductive fitness. The salience of a song to a female, therefore, may fluctuate with variables related to reproduction, such as the female's reproductive state. If the memory for the tutor-song in females is stored in NCM, as it is in males, the responses to song in this nucleus may vary depending on her reproductive state. This possibility is not unlikely since, as the studies described above illustrate, NCM is sensitive to changes in the behavioral relevance of auditory stimuli (e.g., conspecific vs. heterospecific song vs. tone stimuli elicit longer adaptation rates and greater ZENK induction, respectively). To a female in an active reproductive state, song may represent a more salient stimulus and NCM, as a nucleus that responds differentially to salient auditory stimuli may process it as one.

V. Hormonal State Modulates Female Receptivity to Song

Behavioral and cellular responses to song stimuli have been investigated in female songbirds treated with estrogen. In one study (Maney et al, 2006), female white throated sparrows were caught and maintained on short-day light cycle, a process that places seasonally breeding songbirds in a non-breeding state. Birds were then implanted with either estrogen-filled or blank Silastics and tested for behavioral and cellular (ZENK) responses to either male song or tone stimuli. Only the estrogen-treated females performed copulation solicitation displays (CSDs) in response to male song. In the same birds, ZENK expression was measured in NCM. In females that were implanted with blank Silastics, the levels of ZENK expression did not differ between birds that heard tones versus those that heard song. However, the estrogen treated females showed differential ZENK expression that depended on the stimulus played to them (See Figure
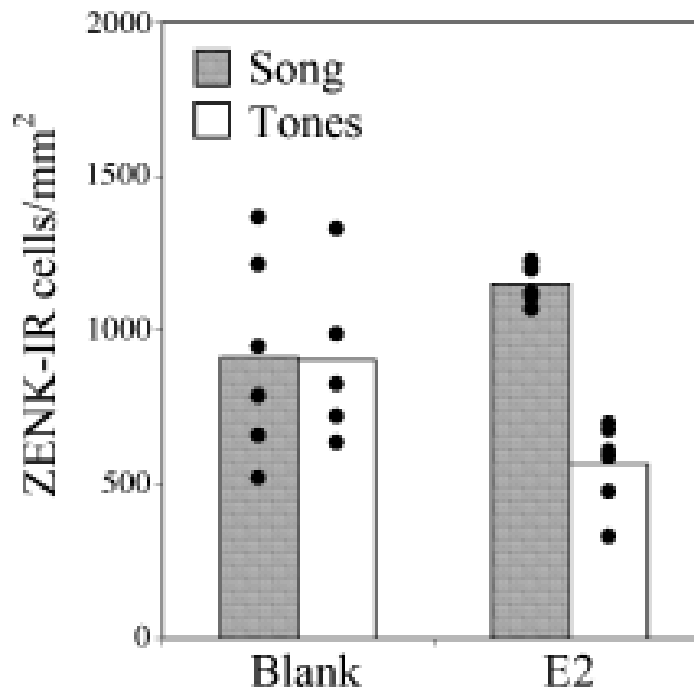
Fig. 5: ZENK-ir cells in NCM of blank-implanted and Estrogen implanted female white-throated sparrows listening to song (grey bars) or synthetic tones (white bars). Birds hearing song had more ZENK-ir cells than those hearing tones, but this effect was significant only in E2-treated birds. Among the birds hearing tones, E2-birds had significantly fewer ZENK-ir cells in NCM than blank-implanted birds. These results suggest that the hormonal state of the bird may modulate perception. (Figure from Maney et al, 2006)

5). Estrogen-treated birds hearing tones showed less gene expression than those hearing song. These results suggest that estrogen may enable discrimination abilities by modulating the perception of incoming auditory stimuli. A related study (Leboucher et al, 1998) employed Fadrozole, an aromatase inhibitor that prevents the synthesis of estrogen, to test the effects of low estrogen on behavioral responses to song in female canaries. Fadrozole-treated birds performed less CSDs in response to male song. A more recent study (Svec and Wade, 2009) has tested the effects of estrogen on song preference and ZENK expression in female zebra finches. Females implanted with estrogen-filled or

blank silastics were tested in a two-speaker choice paradigm. All birds regardless of treatment spent significantly more time near the speaker emitting a tutored-song than one emitting untutored song, results in line with other observations that females prefer high-quality song. However, when NCM ZENK expression was measured in the same animals, the level of ZENK mRNA expressed in NCM was significantly higher for estrogen-treated females versus untreated ones. The results of these studies indicate that the estrogen associated with the reproductive state of female songbirds alters both their behavioral and cellular responses to auditory stimuli.

VI. Estrogen Facilitates Memory Acquisition

In rodents, estrogen administration has been shown to improve performance for certain tasks (e.g., object recognition, Morris Water Maze, Radial Arm Maze) when it is administered at the time of training (reviews: Frick, 2009; Woolley, 2007; McEwen, 2001; see also Liu et al, 2008). The mechanisms by which estrogen induces these effects are not well understood, but it is becoming clearer that estrogen has the capacity to influence multiple molecular pathways (Malyala, Kelly and Ronnekleiv, 2005; Ronnekleiv and Kelly, 2005; Bodo and Rissman, 2006) that individually or collectively contribute to brain plasticity. Though many of these effects have primarily been observed in the hippocampus, a growing interest in the relationship between neurosteroids and behavior has produced observations in other brain regions, suggesting that estrogen may exert widespread influences throughout the nervous system. Previous work has shown that estrogen can increase the number of synaptic spines in the hippocampus (Shors et al, 2001) and hypothalamus (Schwartz et al, 2008), induce

changes in gene expression (Tremere et al, 2009; Maney et al, 2006; Pechenino and Frick, 2009) and neurotransmitter release (Remage-Healey et al, 2008; Nunez and McCarthy, 2009; Schwarz and McCarthy, 2008; Schultz et al, 2009) and increase membrane excitability (Schwarz and McCarthy, 2008; Nunez and McCarthy, 2009; Tremere et al, 2009). Based on these widespread effects observed in other species and in other brain areas, we investigate the hypothesis that estrogen, in addition to allowing NCM expression of a previously established auditory memory, may induce changes that also allow for better acquisition of newly formed memories. These changes may be observable in differences in the familiarity to training stimuli presented on the morning of electrophysiological testing. We expect that the Estrogen treatment group may acquire and therefore express a stronger memory for the training stimuli. If this hypothesis is supported by the data, further investigations into the mechanism by which these changes are induced may be possible in future studies.

D. Current Study

In the current study, I investigate the hypothesis that the neural responses of NCM, a nucleus involved in the processing and memory of salient auditory stimuli, are modulated by hormonal state in the female zebra finch. To do this, I exposed young female zebra finches to tutor songs during the sensitive period in development in an established paradigm (Tchernikovski et al, 1999; Phan et al, 2006). When these birds reached adulthood, I manipulated the level of circulating estrogen pharmacologically and then tested for neural responses to the tutor-song and other auditory stimuli in NCM, the locus for tutor-song memory in males of this species. In the estrogen-treated females, I expect to find a similar NCM response pattern to that which has been described: a selective familiarity to tutor-song versus novel conspecific songs that indicates a memory for tutor-song. In addition, I expect the calculated familiarity index to correlate with levels of circulating estradiol.

In addition to tutor-song memory, I also tested for memory for the songs that were played to the birds 6 hours prior to electrophysiological recording and re-played to them during the recording session. Based on findings from the literature reviewed above that suggests estrogen facilitates memory acquisition, I expect estrogen-treated females to have a higher FI to these conspecific training stimuli than the Fadrozole-treated group.

In this experiment, I also tested for group differences in the response strength to two classes of auditory stimuli: novel heterospecific and conspecific song. The findings of previous studies of female NCM and estrogen (Maney et al, 2006) show that the NCM of non-breeding females expresses non-discriminatory levels of ZENK in response to tones and song, whereas NCM of females in the breeding condition expressed

significantly less ZENK in response to tones. I expect to find similar results in the electrophysiological responses recorded from my animals: that the estrogen-treated group will respond differentially to conspecific versus heterospecific song, but that the Fadrozole group will not.

Methods

Subjects:

Pairs of adult male and female zebra finches from our aviary were placed in individual family cages to breed. After each clutch of eggs hatched, fathers were removed when birds in the clutch reached 10±2 days post-hatching. The mother and chicks were placed in a sound isolation box, consisting of an Igloo cooler lined with sound attenuating foam. At 25±2 days of age, female zebra finches were placed into individual isolation boxes, and the mothers were returned to the breeding population. Each box was equipped with an overhead fluorescent light set to a 12 hour light:dark cycle and an air-tube connected to a pump (Jemco, Inc.) to provide ventilation. The finches were supplied with seed (Abba 1900, Elizabeth NJ) and water *ad libitum* throughout the experiment, as well as with nestling food (Green 92, Abba, Elizabeth NJ) during early development to encourage healthy growth. Animals remained in these isolation boxes for the duration of the experiment, except when undergoing procedures performed in preparation for testing. All procedures were approved by the Rutgers University Animal Care and Use Committee.
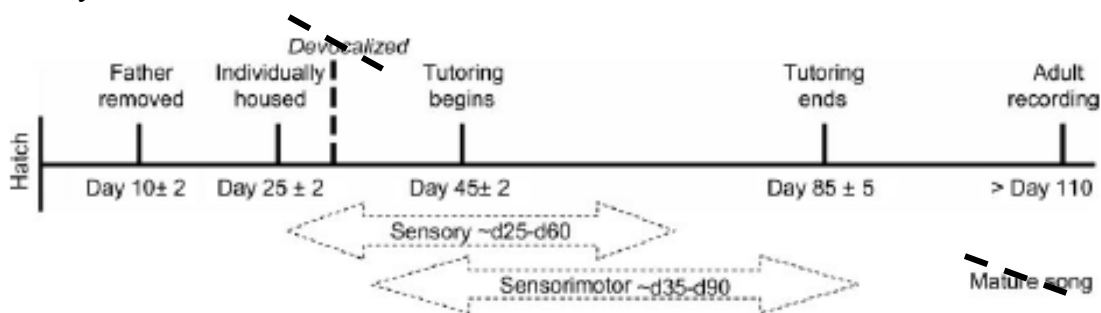
Fig. 6: Experimental paradigm modeled after Phan et al (2006). Females were isolated from their fathers and tutored during the sensitive period for song learning in males. In adulthood, these females were randomly assigned to receive blank, estrogen, or estrogen-inhibitor treatment and then tested for tutor-song memory.

Song-Playback/ Training:

On day 45, a song "tutor" (a plastic model bird secured atop a wooden perch placed in front of a speaker) was introduced into the isolation box and the sound playback equipment initialized. By pecking either of two keys (operated via a National Instruments NIDAQ PCI-6503 card) located on the rear wall of the cage, the young bird could elicit tutor song playback from the speaker a maximum of twenty times per day, 10 times in the morning, and 10 times in the evening. This paradigm has been shown to produce optimal song imitation in young males (Tchernikovski et al, 1999). During this training period, vocalizations were continuously recorded via a microphone (Audio-Technica) located within the cage. The training program was checked 1-2 days each week to ensure that key-pecks elicited song playback and that the computer software was running correctly. At day 90, the tutor-song playback was terminated. Thereafter, the bird remained in isolation until electrophysiological testing (d 110-120; see Figure 6).

Hormone Treatments:

Female finches were randomly assigned to receive either estrogen implants + Saline injections (n=10), blank implants + Fadrozole (Novartis Pharmaceuticals, Switzerland) injections (n=10), or blank implants + saline injections (n=10) on the 9th day prior to electrophysiological testing (Figure 7). Validated protocols for Fadrozole treatment in zebra finches require daily injections of 100ug dissolved in 10uL .75% NaCl and has been shown to decrease aromatase activity to 33% after six days of treatment (Wade et al, 1994). In female canaries, daily Fadrozole administration has also been shown to reduce plasma measures of estradiol by 60% and affected egg-laying and CSD

to male song (Leboucher et al, 1998). Validated protocols for estrogen treatment are based on subdermal implants (Harding, 1991), which consist of Silastic tubing (Dow-Corning, NY) filled with 17-beta-estradiol (Steraloids, Newport, RI). Estrogen from these implants may be detected in serum samples between four to five days after implantation and continues to be released for at least 80 days (Maney et al., 2006). On the day of implantation, birds were removed from their isolation boxes and treated with local anesthesia (Lidocaine Hydrochloride). A small incision was made on the dermis of the breast muscle, and the implant placed underneath the skin. The implantation process takes approximately five minutes. After implantation, the first dose of drug or saline was injected into the bird's breast muscle on the side opposite the implant. The bird was then returned to its isolation box and given soft nestling food.

Experimental	n=10	Estrogen + Saline
	n=10	Blank + Fadrozole
Control	n=10	Blank + Saline

Fig. 7: Treatment groups used in the experiment

Surgery:

Two days prior to electrophysiology, birds received partial craniotomies in order to 1) expose the under-skull for later removal prior to electrophysiology and 2)adhere a metal pin onto the rostral portion of the skull in order to stabilize the head during testing. During this procedure, animals are fasted for a half-hour (in order to prevent suffocation while the bird is under anaesthesia), anesthetized with .03-.05mL Nembutal (50mg/kg),

and placed in a stereotaxic apparatus. After partial craniotomy, the flat portion of a metal

pin is adhered to the bone using dental cement. The procedure takes 20-30 minutes to

perform. Immediately after surgery, the animal is removed from the apparatus and

placed in a cage under a heat lamp for a brief recovery period. Once the animal wakes, it

is returned to its isolation box and given soft nestling food. The bird remains in the cage

until the evening before testing.

Memory stimuli:

The evening before testing, animals were placed in the recording booth (Industrial

Acoustics, Bronx, NY) and allowed to acclimate to the testing environment. Placed in a

cage with *ad libitum* access to food and water, the bird was placed inside the booth,

directly across from a speaker that played all auditory stimuli throughout the experiment.

The next morning (7-8 a.m., 6-8 hours prior to neural recording), a set of auditory

"training" stimuli began to play to the bird. The training set included 2 conspecific

(zebra finch) and 2 heterospecific (canary) songs, each presented 200 times at an 8s

interstimulus interval (ISI). Heterospecific stimuli were partial canary songs similar in

duration to zebra finch songs. These four stimuli were presented again during neural

recording in order to test the short term memory of each animal for these songs. The time

frame between the initial stimuli presentation and testing lies well within the 48-hour

limit of the zebra finch memory capacity for conspecific song and within the 6-8 hour

limit for heterospecific song (Chew et al, 1995). Following training, the animal remained

in its cage within the sound booth with free access to food and water until the testing

procedure.

Electrophysiology:

The day of testing (10a.m.-12 p.m.), each bird had its head stabilized by clamping the pin in a special adapter on the stereotaxic apparatus and the brain was exposed by removing the remaining skull layer. A 30-gauge needle was used to cut through the dura, creating a small window above both brain hemispheres near the bifurcation of the mid-sagittal sinus, immediately rostral to the cerebellum. Using a Thomas Eckhorn microdrive, seven tungsten electrodes were positioned on the surface of the brain. Under computer control, electrodes were individually lowered into the brain to 500 uM. Electrodes were then lowered into NCM individually while playing a set of white noise stimuli that share the same response amplitude envelope of zebra finch song from a speaker located 0.5m in front of the bird. The physiological responses to these stimuli were amplified (Brownlee Precision, San Jose, CA) and filtered so that their frequencies fell within the range of 500Hz and 5kHz. After positioning all electrodes in NCM, the neural responses were recorded (Spike2 Software) as the bird listened to four sets of auditory stimuli. These stimulus sets consist of: 1) Memory Set is used to test the short term memory response as described in previous experiments (Chew et al, 1995, 1996): includes the 4 songs that the bird heard 6 hours prior and 6 novel songs (3 zebra finch songs and 3 canary songs, which are used to test differences in response to conspecific and heterospecific song) 2) Song Set: includes the "Samba" tutor-song and 9 novel songs) 3) Call Set: includes conspecific calls from aviary birds and can be used to test responses to bird's own vocalizations or those of birds that may be familiar to the test subject 4) Tone Set: includes tones of varying frequencies and can be used to test tuning of auditory areas. Recording spanned approximately two hours with a one-hour set-up

and search session. After recording, lesions were made at the tips of two electrodes in

each hemisphere. The locations of these lesions were recovered histologically and used

to reconstruct the anatomical locations of all the electrodes in the set.

Analysis of Electrophysiology Data:

Physiological responses obtained during the recording sessions were analyzed to

determine 1) the neuronal response during stimulus presentation relative to baseline

activity 2) the adaptation rate of these driven responses to each stimulus and 3) each

animal's relative familiarity to the tutor-song or training stimuli. Because overall

responsiveness of NCM neurons and baseline activity levels vary between electrodes, all

calculations prior to determining the final FI for each animal (aim 3 above) were

performed according to site instead of subject, thereby normalizing the responses

obtained from a given site against it's own activity level.

To determine the response to stimulus presentation relative to baseline activity,

the average response to each stimulus was calculated by subtracting the RMS (root mean

squared) during the Control window (500 milliseconds before the onset of the stimulus)

from the RMS during the Response window (duration of stimulus + 500 milliseconds of

silence). Thus, the Response-Control (R-C) was calculated for each trial of each stimulus

at each electrode.

To calculate the adaptation rate of NCM responses to each stimulus, a regression

was performed on the R-C for trials 6-25 (the linear portion of the adaptation function)

for each stimulus presented. The slope of this regression line represents the adaptation

rate of NCM neurons to a given stimulus, where a steeper slope (more negative)

represents responses that adapt more quickly and a shallower slope (less negative)

represents responses that adapt more slowly.

Since a novel stimulus elicits faster adaptation from NCM neurons compared to when it is familiar, the relative familiarity of a stimulus may be determined by comparing the adaptation rates of NCM neurons to it in comparison to novel stimuli. Therefore, to determine a site's relative familiarity to the tutor-song, "Samba", adaptation rates to the novel songs presented in the stimulus set were averaged (avg Novel) and then divided by the adaptation rate to Samba in order to obtain a Familiarity Index (FI; avg Novel/Samba) to tutor song. A similar procedure was used to calculate the familiarity for the training stimuli from the Memory set: the mean adaptation rate for novel songs at each site was divided by the adaptation rate for each training song to produce the FI (avg Novel/Training). Sites were excluded from these calculations if 1) they were not verified histologically within NCM (see below); or 2) responses to novel stimuli were not above baseline; or 3) if responses to novel stimuli did not have a negative adaptation slope; or 4) they did not have useable data from >= 2 novel stimuli.

Histology:

Approximately 24 hours following recording, animals had a blood sample collected (to be subjected to future analyses of steroids) and were killed by an overdose of Nembutal (0.15 cc) administered by intramuscular injection. They were then perfused by intracardial infusion of saline followed by .33% paraformaldehyde. Animals were decapitated and heads were placed in paraformaldehyde, refrigerated, for at least 3 days to allow for preservation of tissue. Fixed brains were removed from the skull and 50 uM

sagittal sections were cut and mounted on Fisher DuraFrost Slides (Fisher Scientific,

Pittsburgh PA). Dried sections were stained with Cresyl-Violet, coverslipped, and

visually analyzed for lesions under a microscope. Since NCM is bounded either by

ventricular space or by the rostrally-located strip of darkly-staining cells that constitute

Area L, recording sites that lay caudal to Area L were verified as NCM sites. Lesion sites

identified as outside of NCM were excluded from statistical analyses.

Statistical Analyses:

To test for statistical differences between groups, parametric statistics were used

when possible and non-parametric statistics were used when sample sizes were small.

When comparing the E2, FAD, and Blank treated groups, t-test or ANOVA was

performed followed by post-hoc Bonferroni. To draw comparisons between subsets of

the Blank group and another group, Mann-Whitney U Tests were performed.   For all

tests, the criterion for statistical significance was set at $p<0.05$, two-tailed.

Results

Effect of Hormonal Manipulations on Neuronal Memory for Tutor-Song:

We collected electrophysiological data in adulthood from a total of 26 tutored

females in the three treatment groups described in the Methods: 10 estrogen-implanted

females (E2), 8 Fadrozole-injected females (FAD), and 8 blank vehicle-treated females

(Blank). In order to assess the neuronal memory for tutor-song, we played back stimulus

sets that included the tutor-song and novel songs in a shuffled order. As described in the

Methods, electrophysiological responses were recorded from multiple sites in each bird

and processed to calculate absolute responses, adaptation rates and familiarity indices

(FIs) to all the stimuli. A mean FI to tutor-song was calculated for each treatment group

by averaging the FIs for each site according to treatment.

Unexpectedly, the mean FI to tutor-song was similar for E2 (1.23, SE=0.12; 39

sites in 10 birds) and FAD (1.26, SE=0.14; 27 sites in 8 birds) groups (see Figure 8). This

difference was not significant (t =-1.34, p=0.89). When compared with the experimental

groups, the mean FI for the Blank group (n=18 sites in 8 birds) was slightly higher (1.47,

SE=.16), but not significantly different from E2 (t=-1.18, p=.24) or FAD (t=-1.0,

p=0.31). However, it was observed at the time of tutoring that 4 females in the Blank

group were laying eggs, whereas the 4 others were not. Therefore, a post-hoc analysis

was conducted to determine whether FI to tutor-song differed among Blank Egg-layers

(BE) and Blank Non-layers (BN). A non-parametric Mann-Whitney U test on this small

sample revealed a significantly higher FI to tutor song in the BE (1.78, SE=.24; 9 sites in

4 birds) than in the BN (1.12, SE=.12; 9 sites in 4 birds) group (z= -2.117 p=0.034). The

FI for BE was also significantly higher than that of E2 (z=-2.311, p=.021) but not FAD
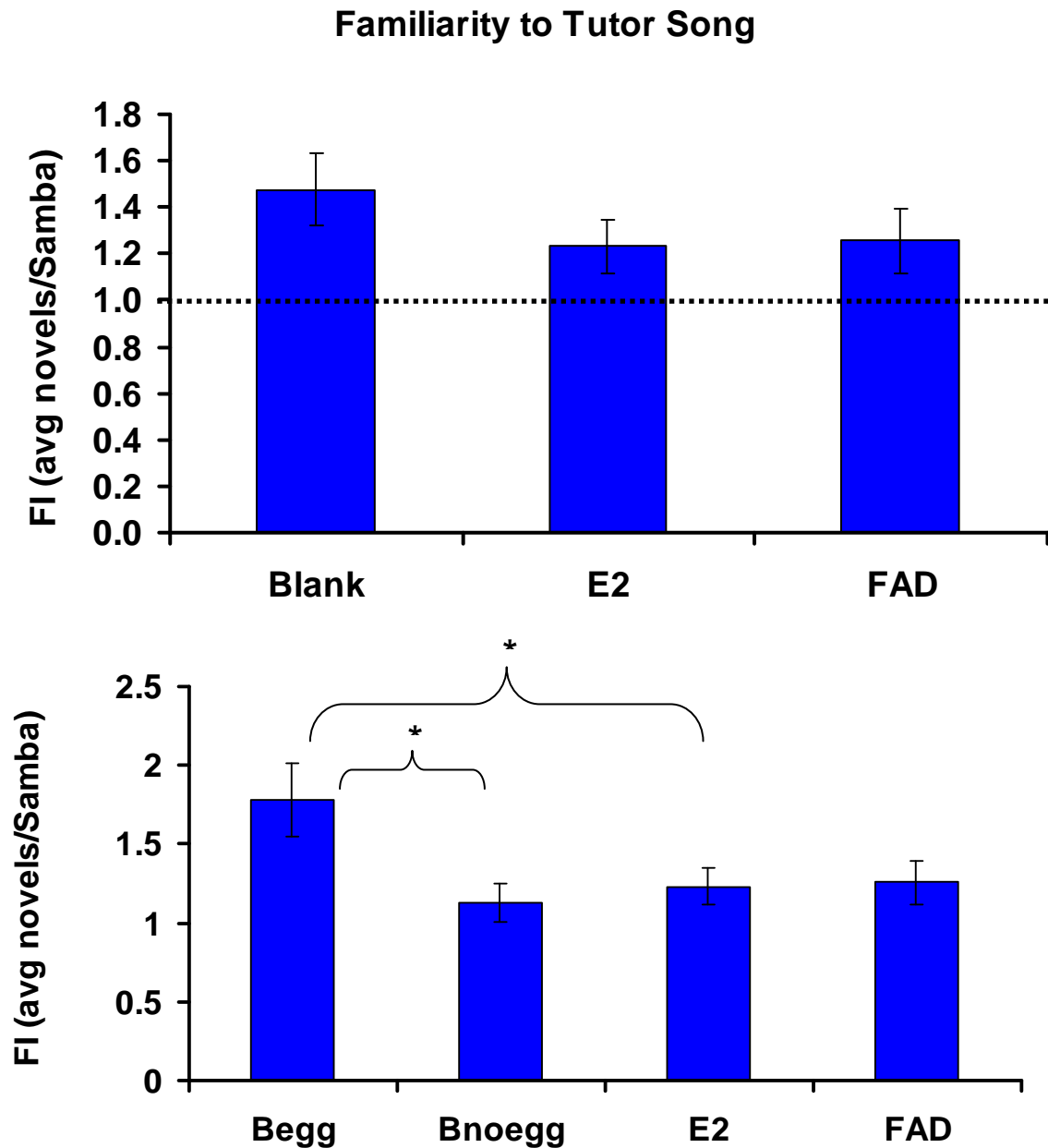
Familiarity to Tutor Song

Fig. 8: Familiarity Index to the tutor-song. No significant differences in FI to tutor-song were found among the treatment groups (top). Dashed line at 1 indicates no memory. However, post-hoc comparisons revealed significant differences between the subsets of the Blank treatment group (bottom).

(z=-1.849, p=0.064). The FI value for BN was not significantly different from that of either experimental group (BN-E2, z=-0.142, p=.887; BN-FAD, z=-0.447, p=0.0655).

In order to establish whether FI was correlated with circulating levels of estradiol on an individual basis, a tutor-song FI was calculated for each bird by averaging the FIs from the recording sites of an animal. These FI values allowed for comparison of variability in FI between individuals within a group, and were also to be later regressed against individual measures of estradiol obtained by RIA (however, see below). In the estrogen-treated group, 8 of the 10 animals tested had FIs above 1.0, showing a significant memory for the tutor-song. The remaining 2 animals had FIs of near or below 1.0 (the level indicating no memory). In the Fadrozole-treated group, half of the 8 animals tested had FIs near or below 1.0, while the other half had FI values above 1.0. Although these differences were suggestive, a 2x2 test of the number of animals in each treatment group showing versus not-showing a tutor-song memory was not significant ($X2 = 1.80$, p=0.18)

Technical Problems with Reliable Assessment of Plasma E2:

It was our intention to measure circulating E2 in all birds, and blood samples were collected at the conclusion of each experiment for this purpose. This would have enabled us to confirm the hormonal status of treated birds and measure the status of the Blank birds. However, due to technical problems involved with RIA, we were not able to measure the levels of estradiol from plasma samples obtained from birds that had been subjected to identical treatment paradigms as experimental birds. Therefore, we are unable to determine the relationship between plasma estradiol and FI to tutor-song at this time.

Effect of Hormonal Manipulations on Neuronal Memory for Recently Heard Song Stimuli:

In order to assess the effect of hormonal condition on the formation of song memories in our adult female birds, we presented training songs on the morning of each recording session and tested responses in NCM to those songs 6 h later (see Methods). We calculated a familiarity index (FI, as described for tutor song) for the training zebra finch songs in comparison to the novel songs presented in the same stimulus set. Results are represented in Figure 9. When the treatment groups were compared, the mean FI for training stimuli was significantly higher for E2 (1.25, SE=.09; 39 sites in 10 birds) than for FAD (FI=.97, SE=.06; 51 sites in 8 birds) (t =2.66, p=0.009). In the Blank birds as a group, FI (1.11, SE=.09) was not significantly different from E2 (t= 1.12, p=0.265) or FAD (t=-1.29, p=.20). Egg-layers showed a higher FI (BE: 1.28, SE=.16; 18 sites in 4 birds) than non-layers (BN: 0.92, SE=.08; 17 sites in 3 birds), but this difference was not significant, possibly due to high-variability in the egg-laying group (Mann-Whitney, z=-1.45, p=.146). Further post-hoc analyses revealed that E2 FI was not significantly different from BE (z=-0.061 p=.951) or BN (z= -1.949, p=.051), and that FAD FIs were not significantly different from BE (z=-1.49, p=.136) or BN (z=-.347, p=.729).

Hormonal Effects on Discrimination of Conspecific Auditory Stimuli:

Normally-reared zebra finches show higher responses to novel conspecific stimuli in NCM than to heterospecific stimuli (Chew et al, PNAS, 1996). In order to establish whether treatment with estradiol or Fadrozole affected the absolute response amplitudes to novel conspecific vs heterospecific song, average responses to novel songs (tested as
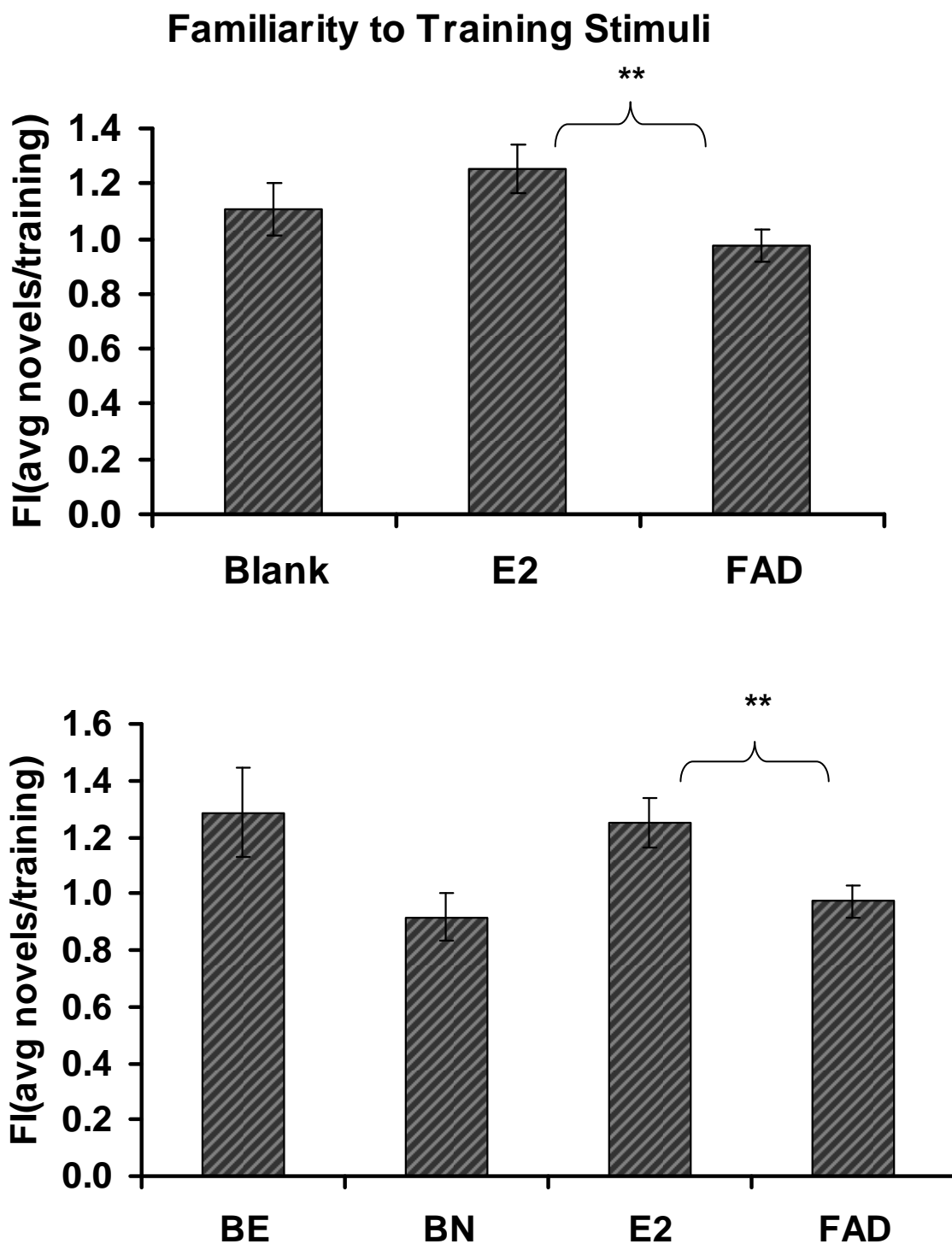
Fig. 9: Familiarity Index to Conspecific Training Stimuli. Significant differences in FI to training stimuli were found between the Estrogen and Fadrozole treatment groups (top). Post hoc comparisons revealed an additional difference between the Egg-Layers and Non-Layers in the Blank group (bottom).

part of the Memory set) were calculated for trials 6-25 (as described in the Methods). Results are represented in Figures 10 and 11. Repeated measures ANOVA yielded a significant main effect for treatment (F=6.96, p=0.0004), a main effect for song type (F=50.01, p=0.000) and an interaction between song type x treatment (F=10.28, p=0.0002). As observed in previous experiments conducted in untreated birds, both the E2 and Blank group exhibited higher absolute responses to conspecific (E2=47.05, SE=4.62; Blank=77.18, SE=8.37) than to heterospecific (E2=40.6, SE=4.36; Blank=59.62, SE=6.27) song (post-hoc Bonferroni, E2: p=0.046; Blank: p=0.000). However, the Fadrozole-treated group failed to show this differential response pattern to conspecific (mean=45.18, SE=4.65) vs heterospecific (mean=45.7, SE=4.82) song presentation. (Bonferroni, p>.05). Both subsets of the Blank group exhibited a similar response pattern to the E2 group. Egg-layers and Non-layers exhibited significantly higher responses to conspecific (BE: 65.16, SE=12.44; BN: 88.77, SE=11.01) than to heterospecific song (BE: 45.02, SE=7.62; BN: 73.7, SE=9.25) (Bonferroni, BE: p=.000; BN: p=.007).

A second set of post-hoc analyses was conducted to determine whether the absolute response strength to either song type was significantly higher in the Blank versus either experimental group. Responses to conspecific song were significantly higher in the Blank group (77.18, SE=8.37) than in E2 (47.05, SE=4.62, (t) =-3.15, p=0.002) or FAD (45.18, SE=4.65, t=-3.34, p=0.001). Responses to heterospecific song were also higher in the Blank group (59.62, SE=6.27) than in E2 (40.6, SE=4.36, t=-2.57, p=0.01) but not FAD (45.7, SE=4.8, t=-1.79, p=0.08).

NCM Response to Conspecific vs Heterospecific Song
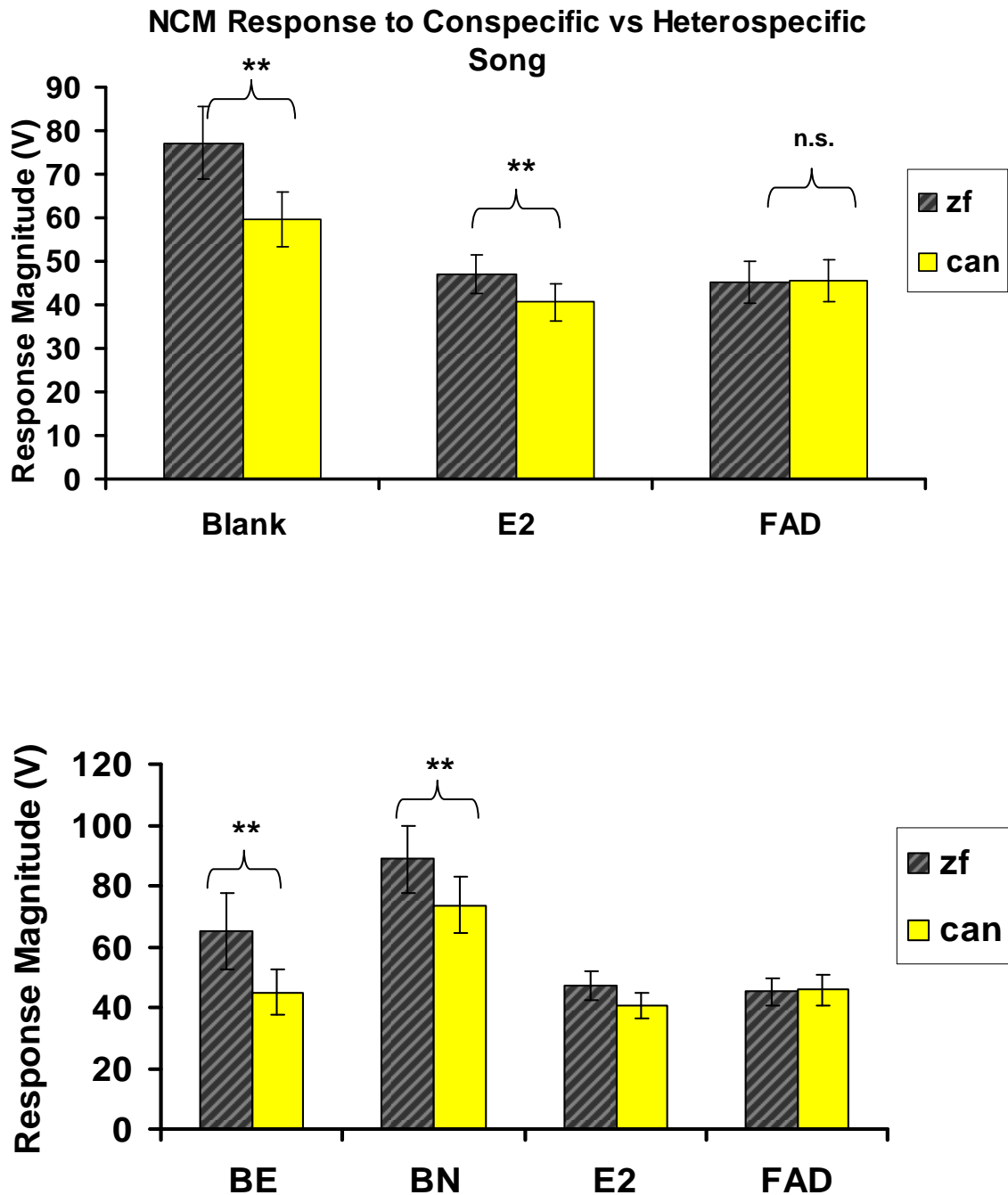
Fig. 10: Discrimination of Conspecific and Heterospecific Song. E2 and Blank groups exhibited higher responses to conspecific song, as is normally observed in untreated birds (top). Egg-layers and Non-layers exhibited the normal pattern of discrimination (bottom). Fadrozole treatment eliminated this discrimination.

NCM Responses to Conspecific vs Heterospecific Song

Fig. 11: Absolute responses to conspecific (ZF) song are higher than to heterospecific (CAN) song in the Estrogen-treated group. Fadrozole treatment eliminated this effect. The ANOVA shows a significant interaction between treatment and song type.

Discussion

Familiarity to Tutor-Song does not differ between Estrogen and Fadrozole Groups
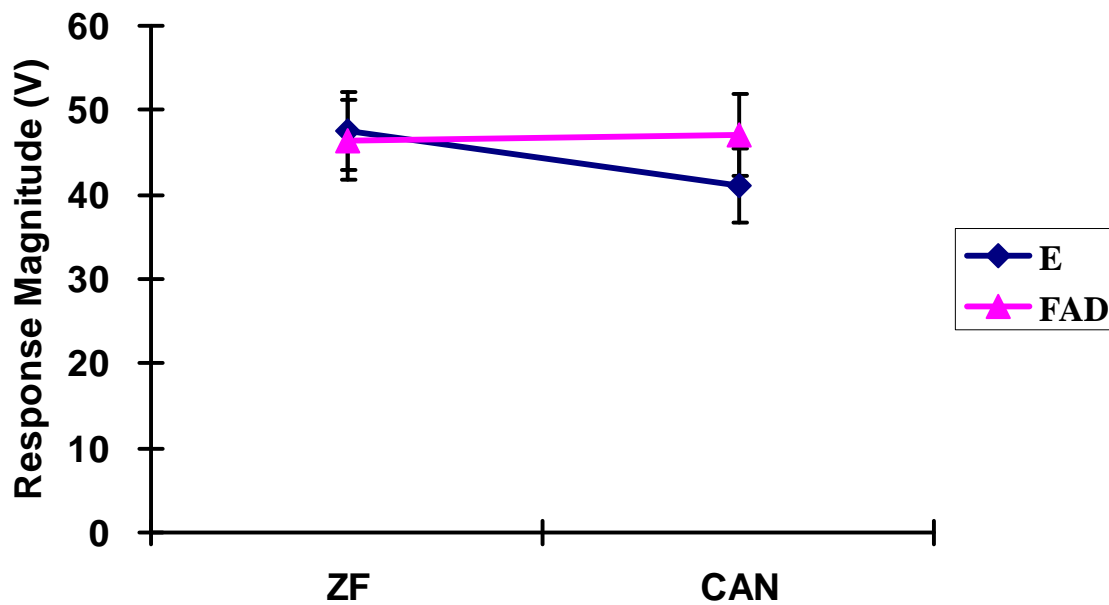
The primary hypothesis tested in this study was that estrogen treatment would induce changes in NCM responses such that a neuronal memory for tutor-song, similar to that documented in males (Phan et al, 2006), would be expressed. It was further hypothesized that tutor-song memory would be absent in FAD birds and might vary in Blank birds according to their individual hormonal condition (discussed below). Analyses of NCM electrophysiological responses to song stimuli revealed a slightly higher but non-significant FI to tutor-song in Estrogen-treated vs. Fadrozole-treated females. Therefore, the present results do not support the original hypothesis that estrogen-treated animals express more familiarity for tutor-song than Fadrozole-treated animals subjected to an identical training and testing paradigm.

Interestingly, the failure to observe differences between estrogen and Fadrozole treated groups was not because E2 *failed* to elicit a tutor-song memory (since the group mean showed significant memory and 8 out of 10 estrogen animals had FIs >1, implying that they had a memory for the tutor), but because half of the Fadrozole birds and half of the Blank birds *did* express a memory (4/8 FAD birds and 4/8 Egg-Layers had FIs>1) for the tutor-song. This finding that the majority of females in this study express a tutor-song memory is inconsistent with the results of previous experiments conducted in our lab on tutored untreated females, raised in the same conditions as part of the experiments that found tutor-song memory in males. The previous results showed a group mean FI= 1.008 (not different from 1.0) in tutored untreated females and only 2 out of 7 of the females tested earlier in this paradigm expressed a memory for the tutor-song (M. Phan and D.

Vicario, unpublished data). The results of the current study show that most (16 of 26) of the females tested do express the tutor-song memory. The origin of this difference in the datasets is unclear. The inconsistency between these sets of results could be due to various factors. It is unclear why the current blank birds showed more of a tutor-song memory than the earlier group. In addition, the fact that some Fadrozole birds had a memory and some did not raises the possibility that the Fadrozole administered to our animals was variably effective at inducing low levels of estradiol.

Unfortunately, after multiple attempts at setting up a valid, reproducible assay, due to unforeseen technical difficulties, we do not have independent confirmation of the actual hormonal status of these females at the time of testing, so it is possible that our treatments were ineffective at inducing high (estrogen implanted) or low (Fadrozole treated) levels of plasma estradiol, and therefore lead to no differences among treatment groups. Two ways to explore this issue are 1) by examining the variability in FI among individuals within a group, suggesting variability in the animals' hormonal condition at the time of testing (if FI is truly correlated with estradiol level) and 2) by examining the results of the two other hypotheses.

Because higher FIs were expected to be associated with higher estrogen levels, we may be able to infer whether a treatment was effective by examining the distribution of FIs within a treatment group. In the estrogen-treated group, eight of the ten animals tested had FIs well above 1.0 (indicating that they all had a memory for tutor-song). The remaining two animals had FIs of ≤ 0.85. It is possible that estrogen treatment was not effective in these two birds, since estrogen can, in rare cases, fails to permeate the membrane of the Silastic tubing and enter the circulating bloodstream due to drying of

the skin around the area of implantation. Precautions were taken to prevent drying from occurring (e.g., implants were soaked overnight prior to implantation and slightly adjusted after implantation daily to ensure proper placement) throughout the treatment period. In the Fadrozole-treated group, half of the eight animals tested had FIs near 1.0 while the other half had FI values above 1.0, indicating that either estrogen is not necessary for the expression of tutor-song memory in NCM or that Fadrozole treatment did not effectively induce low levels of circulating estrogen. The Fadrozole treatment administered to our animals, which prevents the enzyme aromatase from converting testosterone to estradiol, is widely used in other labs to inhibit behavioral or physiological responses known to be estrogen-dependent (Remage-Healey and Schlinger, 2008; Kim, Perlman, and Arnold, 2004; Adkins-Regan and Wade, 2001; Contreras and Wade, 1999) and has been shown to reduce aromatase levels to 33% of that measured in untreated animals (Wade and Schlinger, 1994). Therefore, although Fadrozole is very effective in inhibiting aromatase activity the remaining aromatase may be sufficient to synthesize adequate amounts of estrogen to influence neuronal processing.

Alternatively, the possibility exists that Fadrozole did effectively inhibit estrogen *synthesis* via the aromatase pathway, but exogenous sources of estrogen were being supplied to the animals in their diet. Numerous published papers (see Cederroth and Nef, 2009 for review) document the existence of phyto- and xeno-estrogens contained in food sources (e.g., soy, grains, fruit, vegetables), many of which are supplied to our animals on a daily basis. These plant-derived estrogens are similar in chemical structure to endogenous estrogen, bind to both estrogen receptors alpha and beta but with lower affinity, and have been shown to influence reproductive activity in several species,

exerting agonistic or antagonistic effects depending on the dose administered and baseline reproductive state of the animal. For example, genistein, a popularly studied phytoestrogen contained in soy products, has been shown to decrease endogenous estrogen levels in animals treated with estrogen implants but increase them in animals deprived of estrogen. In addition, compounds (e.g., 6-MBOA) naturally synthesized by plants that provide them with fungus resistance or that act as natural pesticides also have been shown to alter reproductive activity of animals that ingest them (Leopold et al, 1975; Sanders et al, 1981). Studies investigating the effects of these compounds suggest that, in climates where availability of food and water is scarce or unpredictable, their presence in local plants serves as a cue for animals that eat them that conditions are temporarily stable for breeding. Though no study has yet been conducted on dietary-induced changes in zebra finch reproductive cycles, the zebra finch is a prime candidate for one, as a species that evolved in arid areas of Australia and the Southeast and breeds only when conditions are favorable (see Zann, 1996). As a songbird that does not cycle on a seasonal basis, this type of study would help elucidate the conditions that modulate reproductive changes in an opportunistic breeder.

Though these alternative explanations for our results may be interesting pathways for investigation, the rejection of the primary hypothesis is the most parsimonious conclusion, pending estrogen assay results. It is unlikely that Fadrozole was ineffective, given results obtained in other labs and the results of secondary hypotheses described below. Since blood samples were drawn from all animals immediately after neural recording as well as 24 hours after their last injection, we hope to be able to measure estradiol and determine our treatments' efficacy when our developing method to assay

estradiol from zebra finch plasma can be validated and performed. Previous attempts to measure estradiol in our lab and in collaboration with other labs that routinely perform radioimmunoassay (RIA) have yielded problematic results, due to high concentrations of lipids in samples acquired from estrogen-implanted animals that must be removed prior to the assay procedure and/or to interfering substances that cross-react with the antibody to estradiol. When reliable estradiol readings are obtained for the animals tested in this study, these results will be revisited, and FI values will be correlated with estradiol levels. In line with the original hypothesis, we will test whether high FI values are associated with higher levels of circulating estradiol. Without available estradiol measures, it is not possible to completely reject the hypothesis that estrogen influences the expression of an auditory memory but the results obtained from the present comparison of Estrogen-versus Fadrozole treated females suggest that estrogen is *not* necessary for NCM responses to demonstrate a neuronal memory for the tutor-song.

Familiarity to Tutor-Song in the Blank Group: Correlation with Egg-laying

Based on previous observations of tutored, but unmanipulated female birds tested for tutor-song memory, it was expected that some of our Blank animals might show a memory for tutor-song but most would not. These animals received no hormonal treatment and were therefore free to vary in their reproductive status. Throughout the course of tutoring or shortly thereafter, it was observed and noted that some of these females were laying eggs and some were not. Therefore, post-hoc analyses were conducted to compare FI to tutor-song for Egg-Layers vs. Non-Layers. Surprisingly, a significantly higher FI to tutor-song was expressed in the group of animals that had lain eggs around the time of tutoring compared to those that had not. One consideration that

should be addressed with respect to these control groups is that egg-laying occurred at some point during the memory acquisition process rather than at the time of memory testing. The process of egg-laying requires the coordinated activity of structures that make up the Hypothalamo-Pituitary-Gonadal (HPG) Axis and the suite of hormones that regulate them (e.g., GnRH, FSH, LH, E2, P_4). As in the mammalian reproductive system, fluctuations in HPG hormones facilitate the growth of follicular tissue and ovulation in birds. In avian species, egg maturation and laying follows a hierarchical sequence in which maturing follicles are differentially responsive to FSH (less mature follicles and later in laying sequence) and LH (more mature follicles and earlier in laying sequence), and oscillations in estrogen concentrations at high levels modulate these effects (Jamieson, 2007;Murton and Westwood, 1977; Dawson and Chaturvedi, 2001).

In our egg-laying birds, therefore, high levels of estrogen (along with other gonadal hormones) were necessarily in circulation near the time of memory acquisition or consolidation for the tutor-song. Although the effect observed in Egg-layers may have been elicited by the action of either a single hormone or the concerted action of multiple ones during training, ample evidence from research in rodents suggests that estrogen itself can facilitate memory acquisition (Frick, 2009; McEwen, 2001). Although the explanations for the Control group results are limited to speculation since they reveal a correlation (rather than causation) between egg-laying and acquisition of the tutor-song memory, the differences observed in familiarity to training stimuli between the experimental groups suggest that estrogen present at the time of training may provide a plausible explanation for the difference in tutor-song FI observed between these subsets of control animals. A speculative possibility, that would require new experiments to

address, is that the birds who laid eggs when they reached sexual maturity were more stimulated by hearing the tutor song because they had formed a better memory of it at earlier stages of tutoring than the non-layers.

Treatment Effects on Memory for Training Stimuli

Prior to electrophysiological recording, animals were played a Memory training set of stimuli that consisted of hetero- and conspecific songs. During testing (6h later), the animals were played these training stimuli again, intermixed with novel hetero- and conspecific songs. A secondary hypothesis of this study was that estrogen-implanted females would have higher FIs to the training stimuli than Fadrozole-treated ones. The results support this hypothesis as well as lend insight into the rejection of the primary hypothesis (that FI to tutor-song would be higher in E2-treated animals). Since both E2 and FAD groups exhibited similar FIs to tutor-song but dissimilar FIs to the training set stimuli, it appears that estrogen may be important for memory acquisition rather than its expression; E2 and FAD birds had high and low estrogen levels, respectively, during short-term memory *acquisition* when they heard the training stimuli and also during testing the same day. In contrast, developmental song tutoring occurred without hormonal manipulation, which only began 9d before tutor-song memory *testing*.

The present finding that the Fadrozole-treated birds had significantly poorer memory for training songs than estrogen-implanted birds suggests an important, possibly essential, role for estrogen in the acquisition of this memory, consistent with evidence from studies conducted on rodents (Frick, 2009; McEwen, 2001). In addition, two recently published papers investigating zebra finch NCM suggest that estrogen is important for the discrimination of auditory stimuli and may be necessary for the

acquisition of auditory memories. Remage-Healey et al (2008) measured NCM estradiol levels via microdialysis and showed that endogenous estrogen is synthesized from testosterone in the NCM of male zebra finches during auditory playback of male song or aviary sounds, but not to white noise stimuli. The second study (Tremere et al, 2009) tested the effects of exogenous estradiol application to NCM and found that estradiol increased the firing rate of NCM neurons. The questions addressed in these and similar studies in other animal models of memory are beginning to elucidate the mechanisms by which estrogen may exert its influence on neuronal processing, and on the eventual formation of memory. Further evidence to the mechanism underlying such processing in NCM may be partially provided by the results of our analysis of NCM responses to novel conspecific vs heterospecific songs under different hormonal conditions.

Treatment Effects on Differential Responses to Conspecific vs Heterospecific Song

The results of previous electrophysiological studies from our lab (Chew et al, 1995,1996) and gene expression studies in other labs (Mello et al, 2004; Bolhuis et al, 2000) show that NCM responds differently to particular classes of auditory stimuli, such as novel vs familiar, song vs tone, and con- vs heterospecific song. In normal, aviary-reared zebra finches, NCM physiological responses are higher to conspecific than to heterospecific song. Driven by our primary hypothesis that estrogen influences NCM responses and previous results (Maney et al, 2006; Tremere et al, 2009) showing that NCM responds differently under estrogenic and non-estrogenic conditions, we hypothesized that estrogen-implanted birds would respond differently to conspecific vs heterospecific song compared to Fadrozole-treated animals. The data supported this

hypothesis, showing that Fadrozole treated animals lack the expected higher response to conspecific versus heterospecific songs found in the E2 and control group.   Therefore, estradiol appears to be essential to the normal NCM discrimination of conspecific vs heterospecific song, an important aspect of species recognition. Further investigation of NCM discrimination for other stimulus classes (e.g., tones vs. song or call) under estrogenic vs. low estrogen conditions may provide further information on the role of estrogen in NCM responses.

Possible Mechanisms of Estrogen Action

One question that arises from these results is how estrogen induces its effects. In our experiment, it is important to note that estrogen and Fadrozole were administered systemically, making it impossible to conclude anything about the direct, local effects of estrogen on NCM. In respect to our primary hypothesis related to mate-choice, the original design of our experiment was intended to alter the reproductive state of the females by altering *circulating* levels of estradiol rather than central concentrations in NCM. It is known that NCM neurons contain aromatase and could synthesize estrogen locally from a precursor, if available (Schlinger, 1997; Forlano, Schlinger, and Bass, 2006). Therefore, it is possible that the effects observed in NCM may be the result of feedback loops operating within the HPG axis (and/or NCM itself) and resulting hormones present during the nine days of treatment, acting concertedly to change the characteristics of NCM. Furthermore, studies of estrogen administration have focused on its "classical" (slower, genomic) versus its "non-classical" (faster, non-genomic) influences on neural activity and behavior. Estradiol, as a lipophilic molecule, is able to

traverse the plasma membrane, interact with the intracellular Estrogen Response Element (ERE), and initiate a signaling cascade that results in gene expression (including IEGs such as ZENK). Estradiol may also induce these effects by binding to estrogen receptors (e.g., ER alpha) that line the plasma membrane (Kelley and Ronnekliev, 2009; Schwarz and McCarthy, 2008; Woolley, 2006; McEwen, 2001). Clearly, to adequately assess the direct actions of estradiol on NCM properties, future experiments must be conducted by first administering estradiol directly to NCM neurons and secondly changing the parameters of the experiment in order to assess the mechanism of such effects (e.g., administer alpha/beta receptor blockers and estradiol concurrently; administer bovine serum albumin, a form of estradiol impermeable to plasma membranes, to determine whether the effect is induced by membrane vs intracellular binding).

Whether its effects are induced via binding to intracellular or membrane receptors, estradiol has been shown to interact with other neurotransmitters to increase neural excitability in multiple species. Two laboratories have begun to investigate the action of estradiol in songbird NCM. Recently, Remage-Healey et al (2008) reported increases in locally synthesized estradiol in male NCM measured via *in vivo* microdialysis following exposure to a nearby female, male song, or aviary sounds. In order to establish the influence of neurotransmitters on estradiol levels, they administered GABA, Glutamate, or NMDA via retrodialysis and found that glutamate suppressed estradiol without influencing testosterone levels, whereas GABA increased testosterone without influencing estradiol levels. In a separate *in vivo* study, Tremere et al (2009) administered estradiol to NCM and observed an increase in firing rate. *In vitro*, the same group addressed whether this effect was due to an excitatory or inhibitory influence of

estradiol by measuring miniPSPs in the presence of drugs that blocked excitatory (DNQX) or inhibitory (biciculline) activity. Reporting no change in mEPSPs but decreases in mIPSPs frequency in the presence of estradiol, their results suggest that estradiol increases the excitability of NCM neurons by inhibiting GABAergic activity. This finding lies in accordance with reports that estrogen receptors are colocalized with neurons expressing GAD, a marker for inhibitory neurons (see Woolley, 2007), in the dorsal hippocampus of the rat. Together, the results of these studies suggest a dynamic interaction between GABA, glutamate, and estradiol.

Investigations into these relationships may address not only the mechanism by which estradiol influences memory acquisition, but also larger questions of steroidal modulation of circuit balance (and complementary neurotransmitter modulation of steroid metabolism) within the central nervous system. As a nucleus that receives dense projections from GABAergic neurons (50% of NCM neurons are GABAergic and 90-95% of NCM neurons receive GABA projections (Pinaud et al, 2008), is sensitive to the effects of estradiol (is richly endowed with steroid receptors and aromatase), and is studied for its role in auditory discrimination and memory formation, NCM lends itself as an excellent model in which to investigate the relationship between memory formation and inhibition, and the neurosteroids that modulate this balance. Future studies may address the mechanisms by which estradiol acts at the molecular level to facilitate memory formation.

Conclusions

The results of our study provide positive evidence that adds to the growing body of literature suggesting that estrogen plays an important role in memory acquisition. Our results have shown that:

1) manipulating estrogen levels does not elicit differences in the expression of a previously formed auditory memory;

2) anti-estrogen treatment interferes with a recently-acquired auditory memory, suggesting a necessary role for estrogen in memory acquisition (future studies must dissociate estrogenic effects on acquisition + expression vs. acquisition alone);

3) anti-estrogen treatment interferes with normal discrimination between relevant classes of auditory stimuli, suggesting a necessary role for estrogen in auditory processing of communication signals.

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