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Sex differences and rapid estrogen signaling: a look at songbird audition

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Abstract

The actions of estrogens have been associated with brain differentiation and sexual dimorphism in a wide range of vertebrates. Here we consider the actions of brain-derived ‘neuroestrogens’ in the forebrain and the accompanying differences and similarities observed between males and females in a variety of species. We summarize recent evidence showing that baseline and fluctuating levels of neuroestrogens within the auditory forebrain of male and female zebra finches are largely similar, and that neuroestrogens enhance auditory representations in both sexes. With a comparative perspective we review evidence that non-genomic mechanisms of neuroestrogen actions are sexually differentiated, and we propose a working model for nonclassical estrogen signaling via the MAPK intracellular signaling cascade in the songbird auditory forebrain that is informed by the way sex differences may be compensated. This view may lead to a more comprehensive understanding of how sex influences estradiol-dependent modulation of sensorimotor representations.

Introduction

The recent forceful call to balance the study of both sexes in biomedical research (Clayton & Collins, 2014) reflects a resurgent interest in the biological understanding of sex differences. Sex differences in brain structure and function have been intimately linked to the synthesis and actions of estrogens in the central nervous system (CNS). A fundamental role for estrogens in shaping the differentiation of forebrain structures in particular is evident across vertebrates. Accumulating evidence shows that the nonclassical ‘acute’ actions of estrogens (~ 30 min) are different between the sexes and that the underlying mechanisms for acute actions may in fact themselves be differentially organized during development. Here, we consider these themes as they relate to the role of brain-derived estrogens in the regulation of the songbird brain, with a particular focus on sex differences in auditory function. The work synthesized here illustrates four broad themes. First, although the songbird brain is potently sensitive to the masculinizing effects of estrogens during development, brain estrogen levels (within the auditory forebrain) are not detectably different between males

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and females during the critical masculinization window. Second, neuroestrogen fluctuations occur in response to socially-relevant stimuli in the auditory forebrain of both males and females. Third, the acute, modulatory actions of estrogens on auditory representations in the songbird auditory forebrain are also similar in males and females. These findings indicate a broad conservation of mechanism between the sexes for the control of auditory representations by neuroestrogens. However, there is still evidence that auditory circuitry in the songbird is influenced by sex-specific mechanisms that are driven by neuroestrogens. We suggest that when considering the rapid ‘nonclassical’ signal transduction pathway(s), sex is likely an important factor that influences how cells respond to estrogens, drawing upon work in other model organisms and the parallels in songbirds. Taking into account the predominantly peripheral vs. central source of estrogens in zebra finches (females vs. males, respectively), acute estrogen signaling in the auditory forebrain and the molecular signaling pathways recruited are also likely to reflect mechanisms of compensation for (rather than further derivations of) sex differences. Below, we propose a working model for a nonclassical estrogen-dependent MAPK (mitogen-activated protein kinase) signaling pathway in the songbird auditory forebrain and how it can be used to test the mechanisms of compensation.

1. Sex differences in estrogen actions in vertebrates

Sexual differentiation of the brain has been intimately tied to the aromatization of androgens and the local actions of estradiol (E2) in neural circuits. Pioneering work in rodents established that exposure to pre and post-natal surges of testosterone masculinized sexual behavior (Phoenix *et al.*, 1959) through the aromatization of testosterone into estradiol (Naftolin & Ryan, 1975). Following this proposed model for sexual differentiation, many other neural and behavioral sex differences have been attributed to estradiol’s permanent or organizational effects early in development as well as the transient or activational role estradiol plays in adulthood. While these organizational effects can be explained in part by long-term genomic actions of estradiol interacting with nuclear estrogen receptors, a more unified view of sexual differentiation proposes that genetic differences attributable to sex chromosome complement interact with hormonal and environmental factors to direct masculine vs. feminine development (Arnold *et al.*, 2004, McCarthy & Arnold, 2011). Previous reviews have considered how organizational effects of testosterone and estradiol direct sexual differentiation during critical periods in mammals (Forger & de Vries, 2010, McCarthy, 2010) as well as birds and lizards (Ball & Wade, 2013, Balthazart *et al.*, 1996). Here, we consider how neuroestrogens may shape auditory processing differently in male vs. female songbirds, which relies on this foundational framework.

As noted by McCarthy and Konkle (2005), while the organizational/activational hypothesis has been a useful model to understand sex differences, the simplicity of the aromatization story for the reproductive diencephalon of the mammalian brain does not always hold true for other non-reproductive regions such as the hippocampus and cortex. In particular, observed differences between males and females may not be due to sex differences in the traditional, organizational sense, but rather molecular compensatory mechanisms of hormone signaling that contribute to sex “sameness”. Here, we draw upon this perspective to consider how neuroestrogens are controlled both independently and in conjunction with

gonadal steroids, and consider how downstream estradiol signaling mechanisms can inform our understanding of acute neuromodulation in sensory and sensorimotor cortex. This perspective keeps us cognizant of alternative molecular strategies between the sexes and how they may arrive at similar neuro-behavioral endpoints. This conceptual framework for the actions of estrogens can be considered part of a larger, growing appreciation for a sub-category of differentiated mechanisms that may compensate for sex differences in brain morphology and function to achieve similar behavioral ends in males and females (De Vries, 2004, McCarthy *et al.*, 2012). Below, we review recent work on sexually dimorphic molecular mechanisms for the rapid actions of estradiol signaling and the control of auditory representations in the songbird brain.

2. Sex differences in acute effects of estrogens in the brain

Acute effects of estrogens in peripheral tissues have been well documented since the experiments of Szego and Davis (1967) on rat uteri. Kelly *et al.* (1976) first documented rapid estrogen effects in the hypothalamus of cycling females, demonstrating that acute estrogenic actions also occur in the brain. The acute effects of estradiol have been observed at multiple levels of biological organization, and it is therefore difficult to reach consensus for what classifies as an ‘acute’ effect. The initial observed acute effects in the brain were noted immediately after estradiol application (seconds) to the electrophysiological recording site (Kelly *et al.*, 1976). Changes in kinase activity and phosphorylation occur over the time course of several minutes (Abraham *et al.*, 2004, Boulware *et al.*, 2007, Boulware *et al.*, 2005, Heimovics *et al.*, 2012) and behavioral changes have been described in as little as fifteen minutes to an hour (Cornil *et al.*, 2006, Cross & Roselli, 1999, Fernandez *et al.*, 2008, Phan *et al.*, 2012, Taziaux *et al.*, 2004, Trainor *et al.*, 2008). For the purposes of this essay, we refer to acute events as changes in cellular physiology, signal transduction, or genetic expression that occur within 60 minutes, which is shorter than the canonical long-term effects initiated by nuclear estrogen receptors that can range from several hours to days after estradiol treatment (O'Malley & Means, 1974). It has been hypothesized that acute effects are initiated through estrogen interactions with extra-nuclear and/or membrane receptors (Blaustein *et al.*, 1992, Milner *et al.*, 2001, Revankar *et al.*, 2005, Toran-Allerand *et al.*, 2002), and there is ample evidence to support that membrane receptors can mediate acute effects (Filardo *et al.*, 2000, Revankar *et al.*, 2005, Srivastava & Evans, 2013). Here, we will focus on effects that are observable within a maximum time course of one hour and/or those that have been explicitly characterized by membrane associated mechanisms.

2.1 Electrophysiology in rodents

Estradiol can initiate cellular responses via membrane-associated actions in a variety of brain regions (Luine & Frankfurt, 2013, Meitzen & Mermelstein, 2011, Roepke *et al.*, 2011, Srivastava *et al.*, 2011), and sex differences have been reported since the very beginning of this literature. The initial findings in the hypothalamus were shown to fluctuate depending on the stage of estrus of female rats (Kelly *et al.*, 1976), and slices from males and females exhibited different firing responses to testosterone and estradiol, depending on hormonal state (Teyler *et al.*, 1980). The acute effects of estrogens have been extensively studied in the context of long-term potentiation (LTP) in the hippocampus, but few comparisons have

been made between the sexes (as reviewed by McCarthy & Konkle, 2005). One exception is the observation that estradiol-induced LTP is more pronounced in intact females as compared to ovariectomized females and intact males (Vierk *et al.*, 2012). However, the majority of experiments exploring these questions in vitro test either one sex or a mix of tissues without explicit comparisons between the sexes.

One major issue that has received recent attention is that many studies examine only one sex (primarily males), usually for the sake of simplicity. However, adding in both sexes to a research design can change the scope of the question as well as gain unforeseen insight to how these mechanisms are understood. An example of this is the ‘instant classic’ work of Huang and Woolley (2012) in which estradiol-dependent suppression of inhibitory hippocampal neurons was determined to be sex-specific. In this case, the acute effects of estrogens occur through a membrane version of the estrogen receptor (ER α) that is associated with a metabotropic glutamate receptor. This mechanism is in turn coupled to retrograde signaling of the endocannabinoid anandamide to ultimately suppress GABAergic inhibitory currents. After identifying this mechanism in slices from female hippocampus, Huang and Woolley then observed that E2 had no effect in gonadally intact or castrated males. Beyond the intriguing signaling mechanism for rapid E2 effects, this study is important because it illustrates the importance of including both males and females in a study design. If this study had focused on either sex exclusively, an important E2-dependent effect on inhibitory synapses would have gone unnoticed or the mechanism may have been assumed to be ubiquitous for E2-dependent changes in the hippocampus, which is a conclusion often drawn in single sex studies. Therefore, the necessity of continuing to focus our attention on potential sex differences in the acute effects of steroids like estrogens has become ever more apparent.

2.2 Intracellular estradiol-dependent effects

Some rapid intracellular signaling events initiated by estradiol actions at the cellular membrane are also sex specific in the female hippocampus (Meitzen *et al.*, 2012). Specifically, actions at a membrane estrogen receptor have been shown to regulate cAMP response element binding protein (CREB) phosphorylation in female but not male hippocampus. Interestingly, this sex difference of rapid estradiol signaling is organized within the first few days of life. Females exposed to testosterone or estradiol only did not develop the estradiol sensitive pCREB expression, indicating masculinization; however, females that were given dihydrotestosterone developed the female-typical signaling via CREB. This illustrates that conversion of the testosterone metabolite into estradiol is essential for this non-classical hormone action. What is truly remarkable about this work is that it shows that rapid estradiol mechanisms may also be under the control of sexually differentiated mechanisms early in development and these actions can be permanent.

Rapid estradiol signaling has also been reported to affect behaviors that differ greatly between males and females such as aggression, copulation, and learning (as reviewed by Laredo *et al.*, 2014). While there has been extensive study of how these behaviors differ between males and females (Adkins-Regan & Leung, 2006, McCarthy *et al.*, 2012, Rhen & Crews, 2000, Riebel *et al.*, 2002), and while these behaviors have been linked to rapid

estradiol signaling, few studies address how rapid estradiol signaling mechanisms might differ between males and females to mediate or modulate these behaviors. One example of this is the recent work examining rapid mechanisms underlying estradiol-dependent signaling that influences object recognition memory in the hippocampus (Boulware *et al.*, 2013, Fan *et al.*, 2010, Fortress *et al.*, 2013, Luine & Frankfurt, 2013). Despite the importance of this literature, to date, most of this work has been conducted in females and to our knowledge similar relationships in males have not yet been tested. Because there are sex differences reported in how males and females perform in objection recognition tasks (Frick & Gresack, 2003), it is possible that this behavioral difference is in part due to changes in molecular mechanisms of estradiol signaling in the hippocampus. This hypothesis has been partially supported by differential ERK (extracellular-signaling regulated kinase) phosphorylation (a target of membrane estradiol effects) in the ventral hippocampus in males and females after fear conditioning (Gresack *et al.*, 2009). Future experiments exploring these mechanisms of estradiol-mediated effects through ERK signaling in both sexes will give a more thorough picture of how downstream mechanisms maybe be utilized in males and females to serve similar or dissimilar behavioral endpoints.

Behavior can also change the production of brain-derived estradiol rapidly, which could be associated with sex differences in downstream mechanisms. For example, one study has reported rapid changes in estradiol content and aromatase activity in the hypothalamus that differs between male and female Japanese quail depending on behavioral context (Dickens *et al.*, 2014). Specifically, these authors examined how exposure to copulation or restraint stress changes rapid E2 content and aromatase activity in hypothalamic regions, reporting that males have more aromatase activity and estradiol than females in medial preoptic nucleus and bed nucleus of the stria terminalis (POM/BNST), and that only males exhibited changes in estradiol synthesis following behavioral manipulations whereas females remained unchanged. In summary, there are promising avenues for understanding sex differences in how estradiol signaling is modulated rapidly within the brain in response to different environmental cues or behaviors. Of particular interest is the need to fully resolve the relationship between peripheral and brain-derived estrogens and their combined impact on rapid neuroestrogen fluctuations and downstream intracellular signaling pathways.

3. The zebra finch model system

Zebra finches have a long history serving as an animal model for vocal learning, including behavioral, molecular, neural circuitry, and hormonal perspectives. For example, the vocal learning period in songbirds has direct parallels to the sensitive period of human language development (Jarvis, 2004). Discoveries about the molecular mechanisms essential for song development in the song circuit have led to insights into genes involved in human developmental language disorders (as reviewed by Enard *et al.*, 2002, Wohlgemuth *et al.*, 2014) as well as intriguing homologies with the language structures of the human brain (Pfenning *et al.*, 2014). Songbirds therefore have translational power in comparison to other model organisms that do not exhibit vocal learning. Intensive work on the neurobiology of the zebra finch has yielded a detailed map of the interconnected network of discrete nuclei involved in auditory function, sensorimotor integration and motor patterning of vocal communication signals and vocal

learning (Bolhuis & Gahr, 2006, Brainard & Doupe, 2000, Hahnloser & Kotowicz, 2010, Jarvis, 2004, Mooney, 2009). Steroid hormones have been shown to be essential for development and masculinization of the song circuit (Holloway & Clayton, 2001) as well as playing an important neuromodulatory role in auditory perception and discrimination in adults (Maney & Pinaud, 2011, Pawlisch & Remage-Healey, 2015, Pinaud & Tremere, 2012). A unique advantage of the zebra finch model is that it provides a vocal learner that breeds well in lab settings and exhibits pronounced neuronal steroidogenesis, leading to the direct examination of the relationship between neuronal steroids and vocal learning (Mello, 2014). Because the song-circuit has been well studied, the zebra finch is an ideal model for studying the influence of neuronal steroids on sensory and motor aspects of song. In songbird species like the zebra finch, song itself is a sexually-dimorphic behavior; only males learn songs for use in mate attraction and females learn to discriminate among potential mates via their songs (Zann, 1996). This behavioral sex difference led to the discovery of profound sex differences in brain regions essential to song production in the songbird brain (Nottebohm & Arnold, 1976), which has itself led to the recent exploration of the role of steroid production in the brain in directing sexual differentiation in neural circuits and behavior.

3.1 Steroidogenesis in the zebra finch

Steroidogenesis in the brain has been well-established (Corpechot *et al.*, 1981) and is highly conserved in the forebrain of vertebrates such as fish, reptiles, amphibians, and birds (Callard *et al.*, 1978). Steroidogenesis in the central nervous system has been implicated in the sexual dimorphisms found in the zebra finch brain. While early elevation in gonadal testosterone that is aromatized into estradiol plays an essential role in sexually dimorphic brain development in mammals, this mechanism does not fit perfectly with the development of the song circuit in male songbirds. Many motor nuclei of the zebra finch song circuit are sexually dimorphic, such that males have large nuclei devoted to the output of song and these nuclei are either much smaller or nonexistent in females. Estradiol plays a critical role in the masculinization of this circuit (Grisham *et al.*, 2002, Nordeen *et al.*, 1986), in particular estradiol made in the brain independent of gonadal steroids.

One proximate explanation for sexual dimorphism in the zebra finch is the sex differences driven by chromosome complement. Many sex differences in gene expression can be attributed to Z-linked genes (Naurin *et al.*, 2011), and there is little dosage compensation of sex-linked genes on the Z and W chromosomes (Naurin *et al.*, 2012), in which males have two copies of Z genes that are readily expressed. The steroid synthesis enzyme 17 β -hydroxysteroid dehydrogenase (17 β -HSD) is located on the Z gene, which could provide a partial explanation for how sexual differentiation occurs regardless of the presence or absence of the gonads (Itoh *et al.*, 2006, London & Clayton, 2010). While there has not been a sex difference described in steroidogenic acute regulatory protein (StAR), CYP11A1, 3 β -hydroxysteroid dehydrogenase (3 β -HSD) (London *et al.*, 2006) and CYP17 (London *et al.*, 2003) mRNA levels, there is some indication that activity of steroidogenic enzymes are sexually dimorphic. At baseline levels, 3 β -HSD activity is higher in female zebra finch telencephalon than males (Soma *et al.*, 2004). 3 β -HSD activity is also rapidly affected in a sex dependent manner. Female biased baseline activity of 3 β -HSD is reversed under acute

stressors (<10min) during which males have higher activity than females (Soma *et al.*, 2004). Estradiol also rapidly changes 3 β -HSD activity to a greater extent in females than males (Pradhan *et al.*, 2008). Males and females could thus have different strategies to engage steroidogenic responses to environmental cues alongside fluctuations of other peripheral or neural steroids. The lack of sex difference in steroidogenic enzyme expression but presence in activity could be explained by post-translational modifications to these enzymes. Despite the post-translational modifications (i.e., phosphorylation) that have been established for the aromatase enzyme (Balthazart *et al.*, 2001a, Balthazart *et al.*, 2001b, Balthazart *et al.*, 2003, Foidart *et al.*, 1995) there is a great deal of interest now in sorting out how other steroidogenic enzymes such as 3 β -HSD are similarly modified.

3.2 Estradiol and audition in the zebra finch

While gross morphological sex differences have not been described in the auditory lobule of zebra finches as have been described in the motor circuit, there is some evidence that steroid actions maybe be sexually dimorphic in the songbird auditory forebrain. In particular, aromatase expression differs in certain regions of the auditory lobule. In the caudomedial nidopallium (NCM), adult males have more aromatase ir-positive fibers as compared to females (Saldanha *et al.*, 2000) and there is more aromatase activity in the male caudal forebrain (Rohmann *et al.*, 2007). Males also have more aromatase pre-synaptic boutons, total synapses, and proportion of synaptic aromatase expression in NCM (Peterson *et al.*, 2005). These sex differences have been observed in adult animals and not juveniles (Saldanha *et al.*, 2000), and it is unclear whether this is organized early in development or whether this difference is sensitive to gonadal status. It is also unclear if this difference in expression and activity translates to a difference in downstream mechanisms within the NCM (see 3.3 and 4.2.2).

In addition to a role for local neuroestrogen fluctuations (see 3.3), gonadal steroid hormones contribute substantially to both neuronal development and auditory perception. In adult females, the ovaries produce large quantities of estradiol as compared to the male testes (Schlinger & Arnold, 1991, Schlinger & Arnold, 1992); it is thought that the major source of estradiol in adult male zebra finches is the CNS itself (Schlinger & Arnold, 1991, Schlinger & Arnold, 1992). Interestingly, adult male and female serum level of estradiol do not differ (Adkins-Regan *et al.*, 1990), indicating that the differences in brain-derived concentrations in males and females may be compensated by ovarian estradiol production, though this has not been explicitly tested. When removing the gonads of adult zebra finch males and females, serum levels of estradiol actually increase in both males and females, but with males having a much higher mean difference than females (Adkins-Regan *et al.*, 1990). One hypothesis to explain these patterns is that there is negative feedback from the gonads on alternative sources of steroids, such as the CNS (Schlinger & Arnold, 1991, Schlinger & Arnold, 1992). Other estrogens, such as estrone, could also be important in the context of sexually dimorphic production and action, but to our knowledge, this has not been directly explored in the zebra finch.

Peripheral sources of steroids have impacts on auditory responsiveness in the auditory cortex. Exogenous estradiol implants enhance auditory responsiveness of cells in the

auditory lobule (Maney *et al.*, 2006) and changes in cellular responsiveness to song also depend on breeding season in seasonal songbirds (Heimovics *et al.*, 2012). Exogenous implants of estradiol also influence auditory responsiveness in subregions of the auditory lobule (Sanford *et al.*, 2010). The relationship between gonadal supplies of estradiol and local brain supplies of estradiol could be a very intriguing aspect of the neuromodulatory role for estradiol in the auditory forebrain (Maney, 2012).

3.3 Neuroestrogens and auditory function

Experiments directly measuring neuroestrogen concentrations in the brain of zebra finches have been bolstered by the validation of *in vivo* microdialysis that allows measurement and manipulation of 17-beta-estradiol in the forebrain (E2; Ramage-Healey *et al.*, 2008). Initial experiments confirmed that local estradiol synthesis within NCM is suppressed by local reverse microdialysis of the aromatase inhibitor fadrozole (FAD) in adult males, and that baseline and fluctuating concentrations of E2 were detectable using commercial ELISAs (confirmed using GC/MS; Ramage-Healey *et al.*, 2008). The NCM has been the target of most of these experiments to date, partially because it is a relatively large brain region to target for microdialysis experiments, and also because it is particularly enriched with the aromatase enzyme (see above). In light of the topic of this review, one intriguing research avenue has become to determine whether sex differences exist in the forebrain production of estrogens *in vivo*, in awake freely-behaving zebra finches.

The abundance of aromatase fibers and presynaptic terminals in the NCM of male zebra finches as compared to females (see references above) has led to the prediction that a sex difference in E2 concentrations could be detectable via *in vivo* microdialysis. The first experimental test of this hypothesis to directly compare microdialysate concentrations in males vs. females showed no detectable differences in baseline concentrations of E2 in NCM (Fig. 1; Ramage-Healey *et al.*, 2012). Dialysates from 12 males and 10 females were measured in the same ELISA run to minimize the influence of plate-to-plate variability that may mask differences. These findings indicated that, within the NCM, E2 levels at baseline (i.e., in the absence of social/visual/auditory input from conspecifics) were similar in males and females (Fig. 1). A second relevant finding in these early studies was that E2 levels differed by greater magnitudes between *brain regions* (i.e. E2 levels were higher within the estrogenic NCM than within other regions of the pallium) in both males (Ramage-Healey *et al.*, 2008) and females (Ramage-Healey *et al.*, 2012). Therefore, the most relevant source of variation in neuro-estradiol levels was not sex but sub-regions within the CNS itself.

Instead, the presence of sex differences in synaptic aromatase in NCM may be associated with rapid fluctuations in neuroestrogens in the NCM that are sex-specific. By and large, the responses of NCM neuroestrogens to auditory playback stimuli have been similar between males and females (Ramage-Healey *et al.*, 2012, Ramage-Healey *et al.*, 2008). In adult males and females, E2 is elevated during the 30 min playback of conspecific song, and is unchanged from baseline in response to similar playback of white noise in both sexes. Therefore, E2 levels are elevated in auditory contexts in both males and females, perhaps reflecting a basic feature of neuroestrogen modulation of auditory processing regardless of sex. However, one finding from the study by Ramage-Healey *et al.* (2012) indicates a degree

of sex-specificity in acute neuroestrogen fluctuations. That is, when females were presented with visual stimuli of male or female conspecifics alone (via LCD screen inside the microdialysis chamber) NCM E2 levels were unchanged from baseline. This was also true when males were presented with visual stimuli of conspecific males. However, males presented with conspecific female visual stimuli exhibited a significant elevation in NCM E2 levels, even in the absence of any auditory playback associated with the video. It is possible that neuroestrogen elevations during visual contact with females, prior to engaging in acoustic interactions, enables a sensory ‘preparedness’ in which NCM neurons are primed for the processing of auditory stimuli, such as female calls, conspecific male vocalizations, or self-generated auditory feedback stimuli. The potential sex-specific role of neuroestrogens as participants in multi-sensory integration in higher-order cortical regions is an intriguing future direction of this line of research.

More recent work has explored the sex-specificity of E2 fluctuations in the NCM of juvenile zebra finches. As mentioned above, juvenile songbirds have been the focus of a great deal of research attention in the areas of the neurobiology of critical periods, behavioral plasticity, and sexual differentiation (Adkins-Regan *et al.*, 1994, Arnold, 1997, Brainard & Doupe, 2000, Gong *et al.*, 1999, Jarvis *et al.*, 1995, Konishi, 2004, Mooney, 2009). The major portion of sexual differentiation in young zebra finches occurs during the incubation and post-hatching periods, which has allowed particular accessibility to manipulations during critical windows of differentiation and song learning. Treatment with E2 in the first two weeks of hatching is a potent manipulation that can masculinize female hatchlings via organizational actions, leading to females that are able to sing in adulthood (Adkins-Regan *et al.*, 1994, Grisham *et al.*, 2008, Gurney & Konishi, 1980, Konishi & Akutagawa, 1988, Nordeen *et al.*, 1986, Thompson *et al.*, 2011).

Despite the indications that neuroestrogens can masculinize the zebra finch song circuit in vitro (Holloway & Clayton, 2001), our recent microdialysis studies in juvenile zebra finches reveal that E2 levels in the NCM are undifferentiated between males and females during the critical masculinization window (Chao *et al.*, 2014). Specifically, while E2 levels were statistically indistinguishable between males and females during the early sensory (25-35 dph) and sensorimotor (35-60 dph) age ranges, Chao *et al.* (2014) observed a significant elevated in baseline E2 levels within NCM during the late juvenile period, prior to sexual maturity (Fig. 2). Therefore, while NCM accounts for the predominant source of nearby estrogen synthesis to the song circuit for potential masculinization, the local levels of E2 within NCM during parts of the critical masculinization period are undifferentiated between males and females (Chao *et al.*, 2014). It therefore remains to be determined whether local estrogen microenvironments within the song pre-motor circuitry (HVC-RA) are differentiated between males and females during the masculinization window. However, it appears that baseline E2 levels within NCM are elevated in males as compared to females just prior to sexual maturity, although the functional implications of this divergence remain unclear. It is possible that neuroestrogens are important for the late-stage auditory feedback that is essential for song production in males as they reach sexual maturity and their song ‘crystalizes’ into its adult form.

The relative paucity of sex differences in fluctuating neuroestrogens in NCM has raised the question of whether downstream mechanisms of acute estrogen actions within NCM (or as they propagate to other brain regions) are also similar between males and females. While this hypothesis has not been directly tested to date, there are indications from electrophysiological experiments that estrogens in NCM exert largely similar modulatory actions on the firing patterns of NCM neurons in male and female adult zebra finches. The first study to examine acute estrogen actions on NCM neurons observed that exogenous E2 treatment enhanced the auditory-evoked firing patterns of NCM neurons in males and females (Tremere *et al.*, 2009). In this study, the authors reported no sex difference for the influence of estradiol, and so results from males and females were combined. Subsequent experimental work reported largely similar findings with adult males (Remage-Healey *et al.*, 2010) and adult females (Remage-Healey *et al.*, 2012), in which retrodialysis of E2 into NCM acutely enhanced the auditory-evoked firing rates of NCM neurons. The responses of NCM neurons to E2 treatment were similar in magnitude in the above studies, but it is important to note that in neither case were males and females directly compared in a statistical model. In general, therefore, the acute neuromodulatory actions of E2 in the NCM appear to be similar between adult males and female zebra finches. It remains to be determined whether juvenile zebra finches exhibit similar estradiol-dependent enhancement of auditory processing in NCM, and whether the molecular and/or receptor mechanism of acute neuroestrogen actions in NCM are similar or divergent in males vs. females. Similarly, the receptor mediated mechanism is unclear at present. Co-expression of estrogen receptors as well as aromatase in forebrain auditory perceptive regions are well conserved in vertebrates such as fish (Forlano *et al.*, 2005) and birds (Metzdorf *et al.*, 1999). Co-expression studies have shown that in NCM that ER β is expressed in the same cells as aromatase (Jeong *et al.*, 2011) where ER α has little to no co-expression with aromatase (Metzdorf *et al.*, 1999, Saldanha & Coomaringam, 2005). Sex differences have not been found for either receptor expression in NCM. Selective agonists for both classical receptors have not been able to reproduce the rapid auditory evoked effects of E2 (Remage-Healey *et al.*, 2013), suggesting that other membrane receptors (ex: GPER1 or ER-X) could control this signaling. GPER1 expression is sexually dimorphic in zebra finch telencephalon around the critical period of song-learning; however, this sex differences disappears by adulthood (Acharya & Veney, 2012).

4. Egr-1 signal transduction mechanisms and sex differences

Another way to probe for molecular mechanisms of rapid estrogen signaling is by examining the signal transduction effects that occur within neurons activated by stimuli and/or estrogens. Immediate-early genes (IEGs) have been used extensively as a tool for exploring neuronal activation patterns, and it has been suggested that they are markers for what is known as a genomic action potential (Clayton, 2000). It is thought that the genomic action potential is a way for neurons to code for lasting, significant events and initiate the process of memory encoding (Clayton, 2000). One such immediate early gene is early growth response-1 or Egr-1 (also known as ZENK in songbirds). Egr-1 is a particularly interesting protein because of its known role in memory through targeting proteins that are essential for synaptic plasticity (Knapska & Kaczmarek, 2004).

Below, we focus on the relationship between estradiol and Egr-1, as a way of mapping a molecular mechanism of signal transduction within the brain and how this mechanism may differ between the sexes. Egr-1 has been the primary immediate-early gene used in song bird research to probe for changes in neuronal activity within the brain. Egr-1 is known to be auditory responsive (Jarvis & Nottebohm, 1997, Mello & Clayton, 1994, Mello *et al.*, 1992), and has been used as an anatomical guide for physiological investigations. Characterizing the cellular and molecular mechanisms that control Egr-1 expression could therefore provide insight into new directions for auditory research.

A working model for estradiol signaling in NCM is that estradiol acts via membrane-bound estrogen receptors to cause changes in the MEK-ERK pathway, which ultimately regulates transcription factors that target immediate early genes such as Egr-1 (Maney & Pinaud, 2011). Egr-1 is an important transcription factor in regulating proteins essential for learning and memory in the hippocampus (Davis *et al.*, 2003, Knapska & Kaczmarek, 2004, Veyrac *et al.*, 2013). Because of its responsiveness in the auditory lobule as well as its implication in memory formation, Egr-1 could be a key protein involved in coding for auditory memories in regions such as NCM and CMM (caudomedial mesopallium). Before we turn to Egr-1 associations with auditory processing, memory, and non-genomic estradiol signaling in songbirds, we will first consider evidence that sex differences occur within this pathway in other model systems.

4.1 Sex differences in the signal transduction of ERK, MAPK, and Egr-1

The link between estradiol and Egr-1 regulation through MAPK-MEK-ERK signaling has been well documented in *in vitro* cell lines. In rat uteri cell culture, higher concentration of estradiol application resulted in elevated Egr-1 expression (Suva *et al.*, 1991). In human carcinogenic cell lines responsive to estrogens, autophosphorylation of Raf-1 induced Egr-1 expression (Pratt *et al.*, 1998) and Egr-1's responsiveness to hormone was blocked by MAPKK inhibitors (Chen *et al.*, 2004). In myocardium rat tissue, Egr-1 mRNA and protein is rapidly induced by estradiol through both ER α and ER β , and this effect is regulated through ERK1/2 (de Jager *et al.*, 2001). In glioma cells that only express ER β , E2 increased Egr-1 expression and regulated by phosphorylation of Raf-1 and Erk1/2, indicating that the Raf/MEK1/Erk-MAPK pathway involvement in signaling to Egr-1 (Kim *et al.*, 2011). While most studies do not report an estrogen response element (ERE) consistently on the Egr-1 promoter (Knapska & Kaczmarek, 2004), it does contain steroid responsive elements (SRE) and cAMP response elements (CRE) that have been previously shown to be activated by extranuclear estrogen mechanisms and associated with non-classical estrogen receptors (Aronica *et al.*, 1994, Dong *et al.*, 1999, Duan *et al.*, 2002). Deletion of the SRE from the Egr-1 promotor eliminates the responsiveness to E2 specifically due to Elk-1 binding (Chen *et al.*, 2004) further implicating the importance of the MAPK pathway in regulation of Egr-1. Regardless of tissue type and species, Egr-1 sensitivity to estradiol through the MAPK ERK pathway seems to be conserved.

Activation of the ERK-MAPK pathway is also sexually dimorphic in different contexts as well as across species. Male drosophila exhibit a more profound regulation of the MAPK pathway in response to neuropharmacological manipulations as compared to females

(Sharma *et al.*, 2009). Male and female piglets have different cerebrovasodilation effects resulting from brain injury when administered inhibitors for the ERK-MAPK pathway (Armstead *et al.*, 2011). In male rat hippocampus, there is an increase of phosphorylation of both ERK and CREB after contextual fear conditioning that corresponds with a sex difference in the retention of the fear response (Gresack *et al.*, 2009, Kudo *et al.*, 2004). Sexually dimorphic phosphorylation patterns of this pathway also seem to be responsive to changes in gonadal hormones. Barabas *et al.* (2006) report both sex- and region-specific differences in hypothalamic regions of the mouse brain in phosphorylation of MAPK after gonadectomy and estrogen treatment.

One study by Abraham and Herbison (2005) found sex and regional differences in the phosphorylation of CREB to estradiol treatment in the mouse brain. CREB is a transcription factor that targets and regulates Egr-1 transcription through the CRE promotor site (Knapska & Kaczmarek, 2004). While there was not a sex difference in the expression of CREB, females had an increase in pCREB following E2 treatment in more brain regions than males. Boulware *et al.* (2005) also found similar effects in female hippocampal tissue culture where induction of pCREB after estradiol was specifically regulated by the MAPK pathway, but they did not see this effect in the male tissue. The same group also showed that this sex difference is due to aromatization of testosterone into estradiol early in development and masculinized females resembled males in pCREB induction from estradiol (Meitzen *et al.*, 2012; also discussed in 2.2). Szego *et al.* (2006) found female pCREB was sensitive to estradiol treatment but Grove-Strawser *et al.* (2010) did not find these effects in males, suggesting that a sex difference emerges early on for the response of pCREB to estradiol (Meitzen *et al.*, 2012, reviewed by Laredo *et al.*, 2014).

Egr-1 expression can also be sexually dimorphic depending on brain region, and Egr-1 expression is modulated between the sexes based on context (this is further discussed in 4.2.1 for song birds), and these differences can have direct effects on behavior. An example of this is from Stack *et al.* (2010) in which blocking Egr-1 expression in the medial prefrontal cortex brought male anxiety levels up to female levels in mice. Together, these studies provide strong evidence that the signaling pathway between estradiol and Egr-1 activation is highly sensitive sex at multiple levels. This work has largely explored this connection for the purposes of furthering basic understanding of memory, anxiety, cancer, and the biology of signal transduction, but it also provides a new source of questions to better understand how these pathways may or may not be sexually dimorphic in the context of auditory representations in the songbird brain.

4.2 Egr-1 and signal transduction in the songbird

The link between estradiol signaling and Egr-1 regulation is a promising mechanism for auditory responsiveness in the telencephalon of the songbird brain. As described, the songbird auditory forebrain circuit is not only Egr-1 responsive to hearing song but it is also associated with local estradiol synthesis and IEG modulation – suggesting an opportunity to explore this link in the context of naturalistic sensory experiences.

4.2.1 Egr-1 is song-inducible—The sex differences in neuronal activation of Egr-1 expression in the songbird auditory lobule are diverse and somewhat conflicting. Importantly, these differences seem to depend on the context for the auditory exposure (summarized in Table 1). One example is the representation of tutor song in the auditory lobule as measured by Egr-1 expression and the question of whether NCM and CMM (caudomedial mesopallium) might code different aspects of song based on sex and rearing experience. Song tutoring has distinct purposes for males and females. During the juvenile period, males learn songs from their fathers and produce adult song that is similar to father's song as adults (Brainard & Doupe, 2000, Mooney, 2009, Williams, 2004). While female zebra finches do not sing, exposure to father's song also seems to be important for auditory perception. Females raised without tutor song lack a preference for father's song and lose their preference for higher quality song (Lauay *et al.*, 2004, Riebel, 2000). Thus, while exposure to song early in development is critical for both males and females, Egr-1 studies have been some of the first indications that song is represented differently in adult auditory brain regions.

As we have noted, context is crucial for understanding how song is represented by Egr-1 between the sexes. Females have been shown to have increased Egr-1 IEG induction in CMM to conspecific male courtship song, although no sex difference is observed in the NCM (Avey *et al.*, 2005). Females also have more Egr-1 expression to long calls (Gobes *et al.*, 2009). However, other studies show that regional-differences or lack of differences in response to song presentation based on type of stimuli and rearing environment for these two regions. Females who have a preference for father's song also show a corresponding increase in Egr-1 expression in the CMM but not NCM (Terpstra *et al.*, 2006). This difference in IEG expression to father's or tutors song is not seen in males in the CMM, however there is a correlation in strength of Egr-1 expression in NCM to strength of song learning (Terpstra *et al.*, 2004). While in tutored males and females there is no sex difference in the auditory lobule to responsiveness to songs in zebra finches, untutored females show more Egr-1 induction in both the dorsal NCM and CMM to all auditory stimuli (social song, untutored song) as compared to untutored males (Tomaszycki *et al.*, 2006). In other song birds, such as the canary, CMM seems to encode other types of song perception in females. In particular, Egr-1 is upregulated in CMM when a female hears "sexy" syllables of male song (Leitner *et al.*, 2005). Since females prefer mate's song that more resembles father's song, CMM could be the source of father's song memory in female songbirds that also synthesizes this mate preference. For females, it matters whether or not songs are familiar or unfamiliar and females tend to prefer mate song to other conspecific songs (Woolley & Doupe, 2008). It is also important whether songs are directed (a male singing facing the female with visual displays) or undirected (a male singing with no particular direction to the female or in a female's absence). In females, the NCM seems to be coding for novelty, since unfamiliar directed song has the highest Egr-1 expression as compared to mate directed and mate undirected. However, CMM instead has equally high expression of Egr-1 expression to both unfamiliar and mate directed song as compared to undirected song (Woolley & Doupe, 2008). Sex differences in region specificity and the regulation of Egr-1 expression could be attributed to many factors such as attention and storage/retrieval of song memories. It is also important to note that many of the stimuli

presented here are not identical or even presented under equitable conditions, so it is difficult to conclude exactly how CMM and NCM respond to auditory stimuli presentations in males vs. females. This could be one of the many reasons that sex differences in the zebra finch auditory lobule are unclear, considering the variability at which they are reported for Egr-1 studies. Understanding the nature and prevalence of sex differences in cell signaling pathways is important in the songbird auditory forebrain because of some recent work at the level of extracellular physiology. Yoder *et al.* (2014) report a sex difference for the auditory-evoked firing rates of NCM neurons. While both females and males show tutor song representations in NCM, females had a diminished response magnitude to novel songs than males, although the functional significance of this difference is not yet clear. It has been suggested that NCM stores tutor-song memory in males, which has been supported by both Egr-1 and electrophysiology studies (Bolhuis *et al.*, 2000, Phan *et al.*, 2006, Terpstra *et al.*, 2004, Yoder *et al.*, 2014). The studies discussed above suggest that NCM may play a similar role in female song memory along with CMM, but further exploration is needed to determine similarities and differences of male and female tutor song memory.

The above-mentioned song-presentation differences could be due to organizational effects early in development or activational effects that depend on breeding status or social context. During the sensory period of song learning, females at post-hatch day 30 increase c-FOS expression in response to song in the auditory lobule, where males increase Egr-1 expression (Bailey & Wade, 2003) and this difference disappears at day 45 (Bailey & Wade, 2005) where both males and females increase Egr-1 expression equally in response to song. This suggests that during the sensory period males and females could be using different mechanisms to code for song-learning. Considering the importance estradiol plays in shaping sex differences of the motor circuit of the song system, it is possible that estradiol is also a necessary organizational steroid during the critical sensory and sensorimotor periods for auditory processing as well. While the former studies did not take into account neuroestrogens, examining the role that estradiol has on song-inducible Egr-1 expression in the adult could lend important insights into the mechanisms occurring in early development.

4.2.2 The role of estradiol on song-inducible Egr-1 expression—Estradiol has been shown to influence Egr-1 expression in the auditory lobule in response to song playback. Female white-throated sparrows breed seasonally, and in the winter months, their ovaries regress and estradiol serum levels reach low-baseline levels. This makes them a great model for studying systemic estradiol effects on the brain. Maney *et al.* (2006) implanted these seasonal breeders with E2 and blank control capsules to measure how the presence of systemic estrogens affects the Egr-1 response to conspecific song. They found overall that E2 birds had a higher Egr-1 expression to conspecific song but no other tones, and that E2 birds had more Egr-1-positive cells in response to song than blank birds in auditory regions. Interestingly, the birds that heard tone-only had fewer Egr-1 positive cells with E2 treatment compared to the blank capsules in NCM and CMM. This indicates that the sensory discrimination of song compared to other noises is modulated by estradiol's actions coupled to the genomic Egr-1 response. In a follow up study, the same group examined E2's effects on the social behavior network and found that E2 implants increased Egr-1 response and selectivity to song as compared to blank controls throughout the social

behavior network. They also found this effect in the hippocampus (Maney *et al.*, 2008), which is congruent with mammalian literature on Egr-1-responsive cells and their regulation by estradiol.

Sanford *et al.* (2010) mapped out the topography of the estradiol-modulated genomic response of Egr-1 expression in the female white-throated sparrow. Using systemic E2-implants like the above study, they identified seven distinct subregions in NCM in this species that are unequally responsive and sensitive to song and estradiol treatment. They report that the rostral-medial domains appear to be E2 selective for song, and that in the rostral NCM overall, Egr-1 is more responsive to E2 regardless of song treatment.

However, Egr-1 expression and E2 do not always exhibit a synergistic regulatory relationship in all areas of the brain or across song-bird species. The ventromedial hypothalamus has a decreased Egr-1 activation in estradiol-treated female zebra finches (Svec & Wade, 2009). The same group also found that estradiol decreased Egr-1 for tutored song compared to untutored song and silence in the NCM and CMM. This is somewhat at odds with the sensitivity of these regions to E2 in the female white-throated sparrow, although it is important to note that zebra finches are opportunistic breeders as compared to seasonally-breeding white-throated sparrows. Local administration of E2 into discrete brain areas is also necessary and sufficient for modulating NCM responsiveness and sensitivity to conspecific songs and tones for the Egr-1 genomic response (Tremere *et al.*, 2009) demonstrating that this regulation of genomic response may also be locally controlled.

As mentioned before in other models, the MEK-ERK pathway has also been associated with IEG regulation as well as auditory function in the zebra finch. Cheng and Clayton (2004) demonstrated the necessity of the MEK-ERK pathway in Egr-1 regulation specifically in the zebra finch model. Using adult, male zebra finches, there was a rapid increase in phosphorylated ERK activation after song exposure and MEK inhibitor UO126 decreased song-induced Egr-1 expression. London and Clayton (2008) also demonstrated that ERK phosphorylation is essential for early tutor memory formation in male juvenile zebra finches.

Tremere *et al.* (2012) mapped out the MAPK pathway in the NCM of zebra finches after exploring the link between estradiol and Egr-1. The group found that not only do auditory signals increase pERK, but this phosphorylation of ERK is dependent on local estradiol synthesis through ER β associating with MEKK1. This work is consistent with the studies in mammalian in vivo and cell culture. While this study included both sexes overall, no sex comparisons were reported so it still remains unclear how male and female zebra finches may differ in this molecular pathway. Whereas this study addressed intracellular changes from local manipulations in NCM, studies in other songbirds have provided an alternative understanding to the more global hormonal effects via intracellular, estradiol-dependent signaling. Heimovics *et al.* (2012) tested the estradiol dependent phosphorylation of ERK and CREB in male song-sparrows and found that this activity differs based on season. Overall, E2 decreased pCREB in the NCM in the breeding season only. This suggests that alternative mechanisms may change how the MEK-ERK pathway regulates Egr-1 expression not only between sexes, but according to breeding/seasonal context as well. Recent evidence (Maney *et al.*, 2006, Maney *et al.*, 2008) has indicated that systemic levels

of estradiol are playing an important role in regulating neuronal Egr-1 expression, but that the local vs. systemic relationship of estradiol needs to be further explored in the context of song-inducible gene expression. While Tremere *et al.* (2009)(local E2 administration) do not report explicit sex comparisons, the work from Maney's group in white-throated female sparrows indicates that at least in females, there needs to be a systemic access to estradiol for the Egr-1 response to conspecific song. A direct comparison between males and females, with and without a gonadal supply of steroids is now needed. It is possible that the relationship between Egr-1 and estradiol may depend on de novo synthesis of estradiol in the brain, or it may depend primarily on peripheral access to hormones from the gonads, or an interaction between, gonads, sex and brain steroidogenesis (Maney, 2012; See Fig. 3).

While inferences have been made about male and female responsiveness to estradiol in the auditory lobule based on single sexed studies and/or studies with males and females, this question cannot be truly answered without direct sex comparisons in the research design. We have recently explored the connection between estradiol and Egr-1 by directly testing how the regulation of song-inducible Egr-1 expression differs between males and females. We have found that inhibition of aromatase through an acute, oral administration of fadrozole decreases song-induced Egr-1 expression depending on sex and subregions of the auditory lobule (Krentzel & Ramage-Healey, 2014), which coincides with the exogenous E2 work from Maney *et al.* (2006) and Sanford *et al.* (2010). However, we did not find sex or aromatase inhibition effects on phosphorylated CREB expression within the same animals (Krentzel & Ramage-Healey, 2014). As far as we are aware, this is the first study to directly compare how endogenous E2 synthesis effects Egr-1 expression in both male and female zebra finches in several subregions of the auditory lobule. Considering that we are observing sex differences at a subregional level of the auditory lobule, this is likely an indication that differential estradiol responsiveness occurs at a local level dependent on aromatase expression, although this possibility has yet to be explored. There are also several unaddressed questions relating to systemic access to gonadal steroids and the functional role this has on aromatase expression in the auditory lobule as well as song-inducible immediate early gene expression in the zebra finch.

5. Conclusions

The role of sex in regulating differential sensory processing and responses to neuroestrogens is likely not limited to songbirds. Asking specific questions about auditory representations in relation to estradiol neuromodulation in the zebra finch could also have translational implications. Language development in humans tends to be sex biased, where girls outperform boys in verbal skills from early childhood through adolescence; however, this gender bias heavily depends on the measure of verbal fluency as well as measurements of writing and reading literacy (Halpern *et al.*, 2007). There are indications of a small but potentially important female bias of verbal episodic memory, which has been implied as a potential mechanism for this sex difference (Herlitz & Rehnman, 2008). Human sex differences in cognitive skills such as language are highly controversial. Biological mechanisms that have been considered are left language lateralization bias for males (Shaywitz *et al.*, 1995) and greater inter-hemispheric communication in females (Bitan *et al.*, 2010). Gonadal hormones have also been suggested to have a role in language sex

biases, and sequence variants of the human aromatase gene CYP19A1 have been correlated with language deficits (Anthoni *et al.*, 2012). Steroidogenesis has also been described throughout the human brain (as reviewed by Stoffel-Wagner, 2003). In particular, the temporal cortex, which is responsible for many of the sensory experiences of audition and language, is rich in steroidogenic enzyme expression, including aromatase (Yague *et al.*, 2006). Much like in the telencephalon of the song bird brain (Saldanha *et al.*, 2000), aromatase is found in the terminal fibers of neurons in the temporal cortex of the human brain (Yague *et al.*, 2006) indicating that estradiol could be important for regulation of auditory processing at the level of discrete synapses. Peripheral hormones also seem to be important regulators of language development in humans. Levels of estradiol unbound to steroid hormone binding globin (SHBG) are positively correlated to complexity of melody in crying of human infants, a precursory indicator to later language development (Wermke *et al.*, 2014). While there has not been a sex difference described for neurosteroid production enzymes in the human brain, there are differences in peripheral levels of estradiol early in infancy (Wermke *et al.*, 2014) which may have impacts on early language development through several different molecular mechanisms. Fluctuations of hormonal state later in adult life also have impacts on auditory representations, specifically in maintaining normal hearing in young and middle aged women (Charitidi *et al.*, 2009). Overall, there is a need at the level of basic neuroscience to understand how sexually differentiated mechanisms may impact language perception and learning, and the zebra finch provides an advantageous model system of exploring these questions as compared to other model organisms.

Here we have presented a framework to approach questions of estradiol signaling and its neuromodulatory impacts on auditory representations in the brain with an emphasis focusing on sex. Sex differences in estradiol signaling, from gonadal steroid secretions to local and rapid synthesis in the brain have been observed in several model organisms. The downstream, intracellular mechanisms, such as the MAPK-ERK pathway, recruited by this hormonal and/or neuromodulatory signaling also may be shaped by sexual differentiation. Because of the sensitivity that this system has demonstrated to differences in sex and the more recent resurgent interest in balancing the sexes in study design, we think that turning our attention to questions directly comparing males and females will provide a more complete understanding of estradiol signaling and its effects on sensory acquisition and processing.

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Highlights

- Sex differences in rapid estradiol signaling span model organisms.
- Neuroestrogens influence auditory representations in both males and females.
- The MAPK intracellular pathway is a potential compensatory mechanism for the sexes.

Figure 1. Baseline estradiol content does not differ between adult males and females in NCM
Estradiol was collected from adult male and female zebra finches through microdialysis in NCM and run on a single ELISA plate. E2 content for males: $M=13.95$ pg/ml, $SEM=1.68$ pg/ml ($n=12$); and females: $M=12.29$ pg/mL, $SEM= 3.14$ pg/ml ($n=10$). $p=0.65$ for unpaired t-test. The data for this figure were originally presented in text form in Remage-Healey *et al.* (2012).

Figure 2. Estradiol increases with age in male juvenile zebra finches only

Estradiol was collected from juvenile male (A) and female (B) zebra finches through microdialysis and measured using ELISA. Males have a significant linear relationship between days post hatch (which is a mean age over multi-day collections) and estradiol content as measured within NCM ($F_{(1,23)}=30.718$, $R^2=0.57$, $p<.0001$). Females did not have a significant relationship between estradiol content and age ($F_{(1,23)}=1.349$, $R^2=0.10$, $p=.268$). Phases of song-development are depicted across age: sensory stage from 25-35 dph; sensorimotor stage from 30-60 dph; and subadult from 60-80 dph. Data in both panels were adapted from Chao *et al.* (2014).

Figure 3. A proposed model for estradiol compensation between the sexes on auditory representations in estrogen-sensitive neurons

A conceptual model depicting compensation of sex differences based on reported findings in the NCM of male and female zebra finches. The left side of the chart is the summary of male zebra finch studies (blue) and the right side of the chart is the summary of female zebra finch studies (red), with the center serving as a summary of where both sexes are similar (purple). The top of the chart illustrates the relationship between gonadal hormones and neuroestrogens based on aromatase expression and activity in the NCM. The center of the chart illustrates how song changes estradiol levels. The bottom of the chart summarizes four levels of downstream auditory events, specifically membrane events, phosphorylated ERK, phosphorylated CREB, and Egr-1 expression. These boxes summarize studies that have measured these outcomes in males (blue text), females (red text), and both (purple text). Finally, from Egr-1, we propose potential functional outcomes of this neuroestrogen signaling pathway in the zebra finch based on the synaptic plasticity and learning and memory literature for mammalian models. Numbers within the image are the following citations: 1 (Saldanha *et al.*, 2000); 2 (Rohmann *et al.*, 2007); 3 (Remage-Healey *et al.*, 2012); 4 (Remage-Healey *et al.*, 2008); 5 (Krentzel and Remage-Healey, *unpublished*); 6 (Yoder *et al.*, 2014); 7 (Remage-Healey *et al.*, 2010); 8 (Tremere *et al.*, 2009); 9 (Cheng & Clayton, 2004); 10 (Tremere *et al.*, 2012); 11 (Heimovics *et al.*, 2012); 12 (Krentzel & Remage-Healey, 2014); 13 (Maney *et al.*, 2006). Asterisks (*) indicate that this citation was from another songbird species.

Table 1

Sex differences of Egr-1 expression in zebra finch

Authors	Stimuli/context	Sex difference direction of Egr-1	Brain region	Species/age
Avey et al. 2005	Auditory/video playback	F>M	CMM NCM	Zebra finch, adults
Gobes et al. 2009	Long calls	F>M	CMM, NCM, HP	Zebra finch, adults
Terpstra et al. 2004; 2006	Father's Song vs. novel song	M diff only F diff only	NCM CMM	Zebra finch, adults
Tomasyzcki et al. 2006	Untutored with multiple song stimuli Tutored with multiple song stimuli	F>M F=M	NCMd, CMM	Zebra finch, adults
Bailey and Wade 2003; 2005	Conspecific song vs. silence	F<M** F=M	NCM, CMM, HP	Zebra finch d30 1 Zebra finch d45

* These are comparisons based on two separate studies and not direct comparisons of males and females.

** No change in female Egr-1 expression to conspecific song but there was an observed change in c-Fos.