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**SEX DIFFERENCES IN ESTRADIOL SIGNALING IN THE ZEBRA FINCH
(TAENIOPYGIA GUTTATA) AUDITORY CORTEX**

A Dissertation Presented

by

AMANDA A. KRENTZEL

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

September 2017

Neuroscience and Behavior Program

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AMANDA A. KRENTZEL

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ABSTRACT

SEX DIFFERENCES IN RAPID ESTRADIOL SIGNALING IN THE ZEBRA FINCH (TAENIOPYGIA GUTTATA) AUDITORY CORTEX

SEPTEMBER 2017

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Directed by: Professor Luke Ramage-Healey

Although several sex differences have been described in brain structure, function, and development, sex as a biological factor is underrepresented in neuroscience studies. In the mammalian brain, there are sex differences in the mechanism of rapid estradiol actions on neuronal physiology. In the songbird, the brain is a major source of estradiol production, and estradiol rapidly modulates auditory responsiveness through dynamic changes and an unknown receptor mechanism. I set out to determine if there are sex differences in rapid estradiol modulation of auditory cortical activity, as has been shown in other systems. I tested this hypothesis through three aims: 1) to determine whether the identity of interneurons in the auditory regions of the brain differs between the sexes, 2) test whether acute, endogenous estradiol production is necessary for auditory responsiveness in both sexes and 3) test whether the membrane estrogen receptor GPER1 is necessary and sufficient to shape auditory-evoked activity in both sexes. I found that male and female estrogen-producing and estrogen-sensitive cells did not differ in coexpression with interneuron subtype markers in auditory cortical regions. I also determined that more regions of the male auditory cortex depend on acute, endogenous estrogen production for auditory-induced gene expression than that of females, indicating

that males are more sensitive to acute-synthesis of estrogens than females. Finally, I found that narrow-spiking (NS) neurons in the caudomedial nidopallium are more associated with auditory responses than broad-spiking (BS) neurons in males whereas in females these cell types are similar. GPER1 is necessary for the full auditory responsiveness and coding but only in NS neurons of males, indicating an alternative receptor mechanism in females. In this dissertation, I describe a mechanism by which rapid estrogen modulates auditory responsiveness in males, but females have differences in the reliance on brain derived estradiol as well as receptors that mediate estradiol's actions. This dissertation provides a framework to study sex differences using a mechanistic approach, and highlights the importance of sex as a biological variable in physiological studies even in brain regions with anatomical similarities.

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CHAPTER I

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 I 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. I 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 I review evidence that non-genomic mechanisms of neuroestrogen actions are sexually differentiated, and I 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 bio- medical research (Clayton and 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, I 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 potentially sensitive to the masculinizing effects of estrogens during development, brain estrogen levels (within the auditory forebrain) are not detectably different between males 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. I 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, I 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.

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 and 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 and Arnold, 2011). Previous reviews have considered how organizational effects of testosterone and estradiol direct sexual differentiation during critical periods in mammals (Forger and de Vries, 2010; McCarthy, 2010) as well as birds and lizards (Balthazart et al., 1996; Ball and Wade, 2013). Here, I 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, I 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 neurobehavioral 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, I review recent work on sex differences in molecular mechanisms for the rapid actions of estradiol signaling and the control of auditory representations in the songbird brain.

Sex differences in acute effects of estrogens on 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., 2005; 2007; Heimovics et al., 2012) and behavioral changes have been described in as little as 15 min to an hour (Cross and Roselli, 1999; Taziaux et al., 2004; Cornil et al., 2006; Fernandez et al., 2008; Trainor et al., 2008; Phan et al., 2012). For the purposes of this essay, I refer to acute events as changes in cellular physiology, signal transduction, or genetic expression that occur within 60 min, 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 and 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; Toran-Allerand et al., 2002; Revankar et al., 2005), and there is ample evidence to support that membrane receptors can mediate acute effects (Filardo et al., 2000; Revankar et al., 2005; Srivastava and Evans, 2013). Here, I 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.

Electrophysiology in rodents

Estradiol can initiate cellular responses via membrane-associated actions in a variety of brain regions (Meitzen and Mermelstein, 2011; Roepke et al., 2011;

Srivastava et al., 2011; Luine and Frankfurt, 2013), and sex differences have been reported since the very beginning of this literature. The initial findings in the hypothalamus were shown to fluctuate firing rate 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 and 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 attention on potential sex differences in the acute effects of steroids like estrogens has become ever more apparent.

Intracellular estradiol-dependent effect

Some rapid intracellular signaling events initiated by estradiol actions at the cellular membrane are also sex specific in the 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 testosterone 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 (Rhen and Crews, 2000; Riebel et al., 2002; Adkins-Regan and Leung, 2006; McCarthy et al., 2012), 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 (Fan et al., 2010; Boulware et al., 2013; Fortress et al., 2013; Luine and Frankfurt, 2013). Despite the importance of this literature, to date, most of this work has been conducted in females and to my 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 and 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.

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; Wohlgenuth 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 (Brainard and Doupe, 2000; Jarvis, 2004; Bolhuis and Gahr, 2006; Mooney, 2009; Hahnloser and Kotowicz, 2010). Steroid hormones have been shown to be essential for development and masculinization of the song circuit (Holloway and Clayton, 2001) as well as playing an important neuromodulatory role in auditory perception and discrimination in adults (Maney and Pinaud, 2011; Pinaud and Tremere, 2012; Pawlisch and Ramage-Healey, 2015). 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 and 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.

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, amphibians, reptiles 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 (Nordeen et al., 1986; Grisham et al., 2002), in particular estradiol made in the brain independent of gonadal steroids.

One proximate explanation for sex differences 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 and 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 have sex differences. 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 (<10 min) 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 (Foidart et al., 1995; Balthazart et al., 2001b, a) there is a great deal of interest now in sorting out how other steroidogenic enzymes such as 3 β -HSD are similarly modified.

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 may have sex differences 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 Sections 4.3 and 5.2.2).

In addition to a role for local neuroestrogen fluctuations (see Section 4.3), 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 and Arnold, 1991, 1992); it is thought that the major source of estradiol in adult male zebra finches is the CNS itself (Schlinger and Arnold, 1991, 1992). Interestingly, adult male and female serum levels of estradiol do not differ (Adkins-Regan et al., 1990), indicating that the differences in brain-derived concentrations in males and females maybe 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 and Arnold, 1991, 1992). Other estrogens, such as estrone, could also be important in the context of sex differences in production and action, but to my 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).

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.

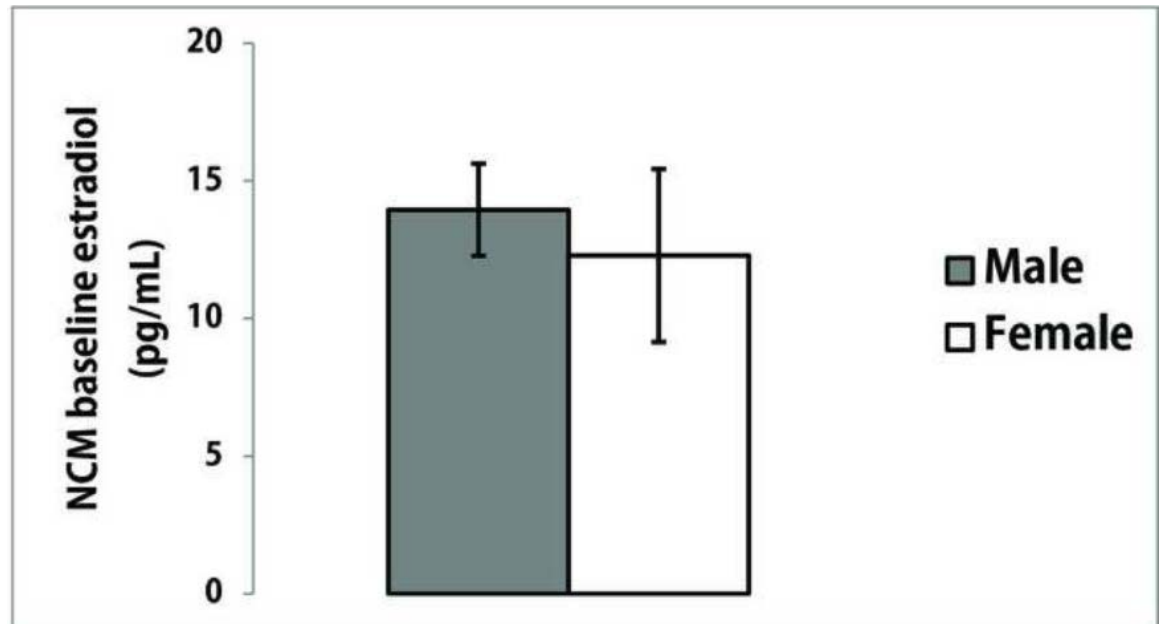


Figure 1: Baseline estradiol content does not differ between adult males and females in NCM.

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).

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 (Remage-Healey et al., 2008, 2012). In adult males and females, E2 is elevated during the 30 min play-back 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 Remage-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

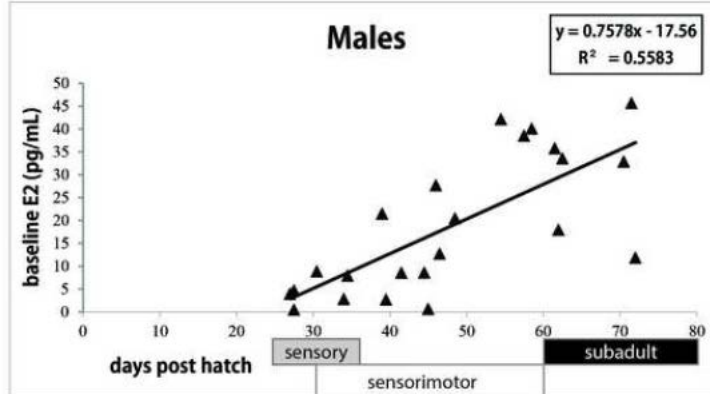
LCD screen inside the microdialysis chamber) NCM E2 levels were unchanged from baseline. This was also true when males were presented with visual stimuli of conspecific males. However, males presented with conspecific female visual stimuli exhibited a significant elevation in NCM E2 levels, even in the 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; Jarvis et al., 1995; Arnold, 1997; Gong et al., 1999; Brainard and Doupe, 2000; 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 (Gurney and Konishi, 1980; Nordeen et al., 1986;

Konishi and Akutagawa, 1988; Adkins-Regan et al., 1994; Grisham et al., 2008; Thompson et al., 2011).

Despite the indications that neuroestrogens can masculinize the zebra finch song circuit *in vitro* (Holloway and Clayton, 2001), 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., 2015). 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. (2015) observed a significant elevation 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.

A



B

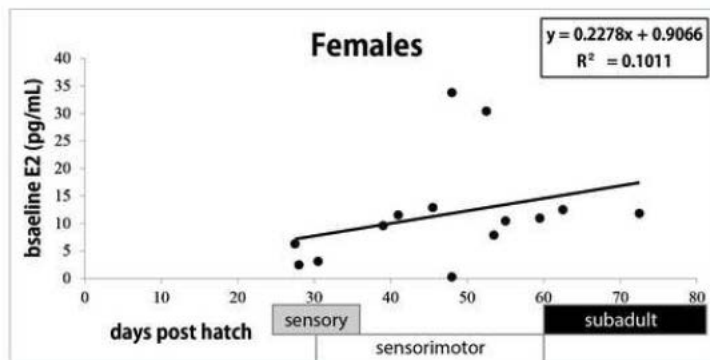


Figure 2: Estradiol increases with age in male juvenile zebra finches.

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 to 35 dph; sensorimotor stage from 30 to 60 dph; and subadult from 60 to 80 dph. Data in both panels were adapted from Chao et al. (2015).

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 (Ramage-Healey et al., 2010) and adult females (Ramage-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 and 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 and Veney, 2012).

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 and Kaczmarek, 2004). Below, I 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 (Mello et al., 1992;

Mello and Clayton, 1994; Jarvis and Nottebohm, 1997), 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 and 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 and 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 I turn to Egr-1 associations with auditory processing, memory, and non-genomic estradiol signaling in songbirds, I will first consider evidence that sex differences occur within this pathway in other model systems.

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 and 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 promoter 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 also has sex differences in different contexts as well as across species. Male drosophila exhibits 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 (Kudo et al., 2004; Gresack et al., 2009). Sex differences in 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 promoter site (Knapska and 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 Section 3.2). 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 Section 5.2.1 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 have sex differences in the context of auditory representations in the songbird brain.

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 – suggesting an opportunity to explore this link in the context of naturalistic sensory experiences.

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 and Doupe, 2000; Williams, 2004; Mooney, 2009). 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 I 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 tutor's 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 con-specific songs (Woolley and 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 and 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. (2015) 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; Terpstra et al., 2004; Phan et al., 2006; Yoder et al., 2015). 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, whereas males increase Egr-1 expression (Bailey and Wade, 2003) and this difference disappears at day 45 (Bailey and 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.

Table 1. Sex differences of Egr-1 expression in zebra finch

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 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
Tomaszczki 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 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.

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 not to 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 and 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 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; 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 sex studies and/or studies with males and females, this question cannot be truly answered without direct sex comparisons in the research design. I 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. I 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 and Ramage-Healey, 2014), which coincides with the exogenous E2 work from Maney et al. (2006) and Sanford et al.

(2010). However, I did not find sex or aromatase inhibition effects on phosphorylated CREB expression within the same animals (Krentzel and Ramage-Healey, 2014). As far as I am aware, this is the first study to directly compare how endogenous E2 synthesis affects Egr-1 expression in both male and female zebra finches in several subregions of the auditory lobule. Considering that I am 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.

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 implicated as a potential mechanism for this sex difference (Herlitz and 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 I 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, I think that turning 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.

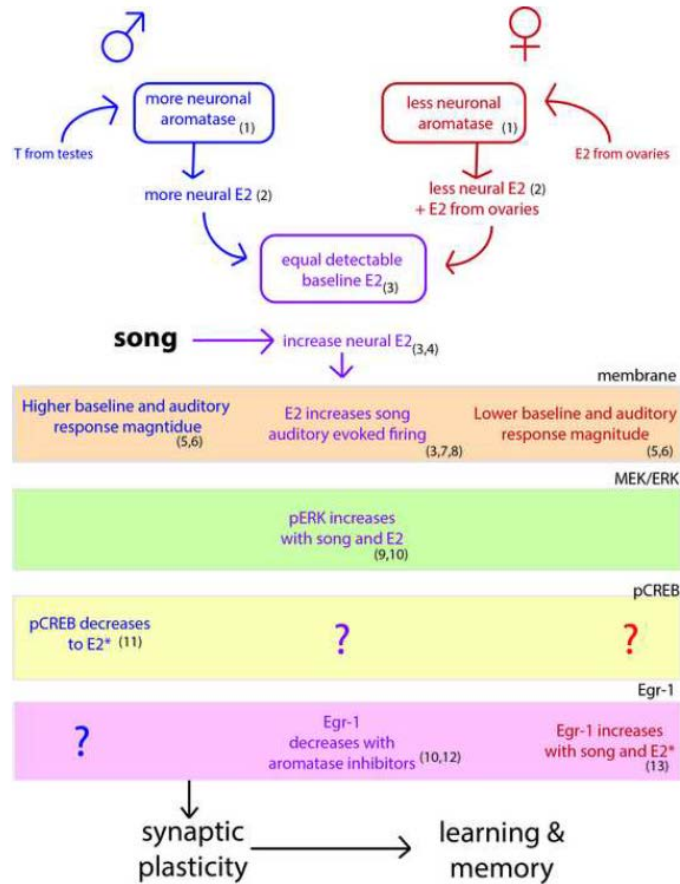


Figure 3: A proposed model for estradiol compensation between the sexes on auditory representations in estrogen-sensitive neurons.

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, I 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 and Clayton, 2004); 10 (Tremere et al., 2012); 11 (Heimovics et al., 2012); 12 (Krentzel and Remage-Healey, 2014); 13 (Maney et al., 2006). Asterisks (*) indicate that this citation was from another songbird species.

Research hypothesis and specific aims

In this chapter, I summarized the mechanisms by which estradiol can rapidly alter neuronal physiology. I also demonstrate that while there are some sex differences described in these mechanisms, especially in mammals, there has been a lack of investigation on how rapid estradiol signaling might be different in male and female songbirds. In this dissertation, I turn my attention to secondary auditory cortical structures of the zebra finch brain, investigating whether different components of rapid estradiol signaling (i.e., acute brain synthesis, neuronal cell types, and steroid receptor mediation) differ between the sexes. I hypothesize that rapid estrogen signaling in the zebra finch forebrain has sex differences in mechanisms for regulating audition. To test this hypothesis, I have addressed three specific aims:

- 1) Characterize interneuron cell-types within male and female auditory regions and their relationship to estrogen-producing cells as well as estrogen-sensitive cells.
- 2) Determine whether acute, endogenous estradiol production affects auditory responsiveness of immediate early genes between males and females.
- 3) Investigate a role for membrane estrogen receptors in the auditory response properties of male and female forebrain neurons.

These approaches address the hypothesis of sex differences in rapid estradiol signaling by testing for differentiation from different levels of the mechanism (ie. cell types, steroid production, and receptor mediation). Aim 1 uses the neuroanatomical approach to characterize cell types involved in rapid estradiol signaling as GABAergic interneurons. Aim 2 tests whether active production of estradiol as birds are hearing

song affect intracellular markers for audition involved in key pathways of membrane estradiol signaling. Finally, Aim 3 determines whether one estrogen membrane receptor, GPER1, is necessary and sufficient to auditory representations within the caudomedial nidopallium. All three of these aims consider sex as a biological variable to determine how estradiol acts through non-canonical pathways to shape auditory processing.

CHAPTER II

AROMATASE AND GPER1 CELLS HAVE SIMILAR EXPRESSION AND COEXPRESSION WITH INTERNEURON SUBTYPES ACROSS THE SEXES

Abstract

Songbirds have evolved the specialized capacity to learn complex vocalizations are a model for human speech and auditory processing. While production pathways have been well characterized, auditory forebrain structures are less understood and contain heterogeneity in cell types. Estrogens modulate auditory-evoked neuronal firing in regions such as NCM which contain cell types that express aromatase, the estrogen synthase, and membrane estrogen receptors such as GPER1, which are proposed to mediate rapid changes. GABAergic neurons are known to be auditory as well as sexually dimorphic in several brain regions, so I set out to characterize estrogen-producing (aromatase+) and estrogen-receiving (GPER1+) neurons by colabeling with GABAergic markers in several auditory structures. I performed a double-label immunofluorescence staining for aromatase and GPER1 with interneuron markers calbindin, parvalbumin, and GABAergic marker GAD67. I observed regional differences in expression of most markers, and found largely no sex differences, with a few exceptions. I also did not observe sex differences in coexpression of aromatase or GPER1 with any GABAergic marker. Aromatase did not coexpress with calbindin, though there was modest coexpression with parvalbumin. GPER1 did not coexpress with either interneuron subtype but had some coexpression with GAD67. Notably, GPER1 was expressed in regions of the brain in which somatic aromatase was absent, suggesting a role for rapid-estrogen signaling at a network level. Although males and

females were similar across these measures, I noted aromatase cells tended to cluster with each other, and we found that males had higher proportions of clustering with larger cluster sizes than in females. I determined that while males and females are similar in expression density of these markers, organization may be different and could have functional consequences. Future experiments examining the relationship between terminal aromatase expression and GPER1 expression may elucidate these networks and connectivity patterns.

Introduction

Songbirds learn complex vocalizations, which is rare in mammals. This has made them an ideal animal model to study how the brain learns to produce and respond to complex vocalizations akin to human language (Jarvis, 2006; Petkov and Jarvis, 2012). While the neuroanatomy and cell identities of the song production system have been meticulously characterized (Braun et al., 1991; Hara et al., 2012; Zengin-Toktas and Woolley, 2017), the auditory regions involved in complex, higher order processing, are less well understood. Certain auditory regions such as the caudal medial nidopallium (NCM) have been implicated as a locus of tutor memory (Bolhuis and Gahr, 2006; London and Clayton, 2008; Bell et al., 2015; Bolhuis and Moorman, 2015; Yanagihara and Yazaki-Sugiyama, 2016), responsive to complex song stimuli (Ramage-Healey et al., 2010; Ikeda et al., 2015), and have been compared to secondary auditory cortex and Wernicke's Area of the human brain (Gobes and Bolhuis, 2007). NCM is also rich in expression of aromatase (Saldanha et al., 2000), the enzyme that synthesizes estradiol from testosterone. This expression is similar to that found in human and monkey temporal cortex (Yague et al., 2006; Yague et al., 2008), a region

that contains analogous structure to NCM. Estradiol has a pervasive role in auditory function. It increases auditory-evoked activity in the NCM (Ramage-Healey et al., 2010; Ramage-Healey et al., 2012), and acute production of estradiol content increases when birds hear song (Ramage-Healey et al., 2008; Ramage-Healey et al., 2010). Despite the implications of estradiol as a neuromodulator in auditory functioning, the cellular identities of estrogen-producing and estrogen-responsive cells are unknown for any auditory system.

The auditory forebrain of the zebra finch is largely described as similar between males and females, whereas the song-production motor pathways exhibit robust sexual dimorphisms in terms of size (Nottebohm and Arnold, 1976; Hamaide et al., 2017) and neurochemical cellular identities (Grisham and Arnold, 1994; Cornez et al., 2015). These sex differences are, in part, organized by central actions of estradiol early in development (Holloway and Clayton, 2001; Wade and Arnold, 2004). Interestingly, the auditory forebrain structures contain most of the neuronal aromatase expression in the brain (Shen et al., 1995; Saldanha et al., 2000), and there is not a consensus on whether estradiol's function is different between sexes. Aromatase is the rate-limiting step for estradiol production, and in the songbird forebrain, aromatase is expressed in somas, axons, and terminals (Saldanha et al., 2000), like the temporal cortex of humans (Yague et al., 2006). This cellular localization within terminals presumably allows for the fast, dynamic production of estradiol to participate in acute, rapid signaling. Aromatase is also expressed in the cell bodies of neurons, which can allow for paracrine signaling to cells nearby. Both males and females in adulthood express similar quantities of aromatase cell number as measured by somatic expression in NCM (Saldanha et al.,

2000); however, fiber density and aromatase activity in synaptosomes are each higher in males (Saldanha et al., 2000; Peterson et al., 2005). Despite this, estradiol production as measured by in vivo microdialysis in NCM shows that both sexes have similar baseline production of estradiol content (when in silence) (Ramage-Healey et al., 2012) and when hearing song, both sexes increase estradiol content rapidly (Ramage-Healey et al., 2008; Ramage-Healey et al., 2012). There is evidence that aromatase is activity dependent in quail (Balthazart et al., 2004; Balthazart et al., 2006; Cornil et al., 2013) and zebra finch brain (Freking et al., 1998; Ramage-Healey et al., 2011), and in the zebra finch, there is regional specificity of the regulatory control of aromatase activity (Comito et al., 2015) suggesting that aromatase may be expressed and regulated in different cell types depending on region. Whether there are sex differences is unknown.

Synaptic aromatase expression and rapid effects observed in physiology suggest that estradiol is acting through a membrane estrogen receptor to exert its effects on audition. Both males and females increase auditory-evoked activity rapidly in NCM when estradiol is infused (Ramage-Healey et al., 2010; Ramage-Healey et al., 2012) suggesting a membrane estrogen receptor as a mechanism. In mammals and birds, ER α and ER β can be inserted into cell membranes, and through associating with metabotropic glutamate receptors, can influence intracellular signaling for fast modulation (Boulware et al., 2005; Meitzen and Mermelstein, 2011; Meitzen et al., 2013; Seredynski et al., 2015). Both receptor subtypes are expressed in the songbird brain (Jacobs et al., 1996; Metzdorf et al., 1999; Fusani et al., 2000; Jeong et al., 2011; Horton et al., 2014), yet a previous study from our lab has shown that agonists specific for ER α and ER β are not sufficient to induce auditory responsiveness as seen with

estradiol (Ramage-Healey et al., 2013). GPER1 is a g-protein estrogen receptor expressed in cell membranes in the brain and body (Srivastava and Evans, 2013). It has previously been characterized in the zebra finch brain, and is known to be expressed in NCM similarly between males and females in adulthood (Acharya and Veney, 2012). However, this is the only other study to date to visualize this receptor type in zebra finches.

Auditory forebrain cortical structures are largely heterogenous. GABAergic cells are abundant in NCM and express immediate early genes when birds hear song (Pinaud et al., 2004), suggesting that these cell types are important in auditory representations and encoding. Interneurons can be subdivided into a complex array of cell-types. In mammals, interneuron subtypes have been shown to mediate important auditory functions such as auditory tuning, corollary discharge, and odd-ball tasks (Moore and Wehr, 2013; Schneider et al., 2014; Chen et al., 2015). Previous papers have shown the expression of both calbindin and parvalbumin in the zebra finch forebrain. Calbindin is expressed more in males in the NCM than in females (Pinaud et al., 2006), raising the possibility that calbindin can account for sex differences in auditory physiology. Parvalbumin has not been compared between the sexes in auditory cortical regions but in motor pathways males have higher expression and connectivity (Grisham and Arnold, 1994; Cornez et al., 2015). In the human temporal cortex, aromatase cells coexpress with both parvalbumin and calbindin (Yague et al., 2006), demonstrating a compelling rationale for species comparison with zebra finches. The identities of GPER1 neurons have never been characterized in songbirds.

In this study, I characterized estrogen-producing (aromatase) and estrogen-responsive (GPER1) cell types of the auditory forebrain, particularly focusing on whether there are regional and sex differences in coexpression with calbindin and parvalbumin. Because I did not find coexpression of GPER1 with either interneuron subtype, I also performed a double-label with GAD67, the enzyme that synthesizes GABA and is expressed in somas. Overall, I did not detect sex differences in auditory regions with respect to aromatase and GPER1 expression, as well as coexpression with the GABAergic subtypes, with a few exceptions. However, I did observe that there are sex differences in clustering of aromatase cells in ventral NCM, a novel observation in the nidopallium.

Methods

Animals

Subjects were kept in single sexed cages housed in a sex-mixed aviary ideal for breeding conditions with food and water available *ad libitum*. I separated adult zebra finches into two studies: study 1 (male n=6, female n=6) and study 2 (male n=7, female n=7). Each study is segregated by objective (Study 1 characterizing estrogen-producing cells and Study 2 characterizing estrogen-sensitive cells); however, most of the methods are the same unless explicitly noted. After collecting animals, I used some of the serial sections from the other study to increase the subject number for comparisons. I isolated all animals into sound attenuation chambers overnight before exposing them to a song playback paradigm ideal for immediate-early gene activation (Mello et al. 1994). I did this with intention of future auditory characterizations; however, these results are not germane to the current study.

After song exposure, birds were killed by an overdose of isoflurane and then perfused transcardially with 0.1M phosphate buffer made in 0.9% saline (PBS) followed by 4% paraformaldehyde. After brains were extracted, they were post fixed in 4% paraformaldehyde for 2 hours and then transferred to 30% sucrose/PBS solution in 4 °C for at least 48 hours. Brains were kept in this solution until sectioning.

Immunofluorescence

Brains were sectioned serially (3) in the sagittal plane at 35 µm using a cryostat (Leica, Germany). Sections were stored at -20°C in cryoprotectant until tissue processing. When tissue was used for immunostaining, sections were transferred from cryoprotectant to 0.1M PB overnight at 4 °C. All sections were washed in 0.1M PB three times and then blocked using a 10% normal goat serum solution made in 0.3% PBT (phosphate saline buffer with Triton X) for 2 hours at room temperature. Sections were then incubated in primary antibodies as described in Table 2. Incubation lasted 1 hour at room temperature followed by 48 hours at 4 °C. Sections were then washed with 0.1% PBT and incubated in secondary antibodies that were either anti-rabbit, anti-mouse, and/or anti-chicken [Alexa 488-or Alex 594-conjugated] raised in goat (Thermo Fisher Scientific Inc., MA, USA; 1/200 except for GPER1 stained tissue where the secondary was a 1/2000 dilution). Sections were then washed again in 0.1% PBT and mounted onto gelatin-coated slides with ProLong Diamond Antifade Mounting Medium (Thermo Fisher). Specificity for aromatase, parvalbumin, and calbindin antibodies used are described in Ikeda et al. 2017. Anti-GPER1 has previously been confirmed with a western blot in zebra finch at this concentration (Acharya and Veney, 2012). The

GAD67 antibody has not been included in a published article. Personal communication with other researchers in the field about lack of success using other GAD antibodies allowed me to rule out other options. I selected this antibody because of reports from the manufacturer of its success in other avian species. I confirmed through a double-label pilot with GAD67 and calbindin that this antibody only marks a subset of GABAergic neurons (ex: 100% coexpression in cerebellum but minimal coexpression in telencephalon; data not shown).

Table 2. Primary antibodies and concentrations

Antibody	Protein description	Host species	Company and catalog number	Dilution	Type
Anti-aromatase	enzyme that synthesizes estradiol	rabbit	Gift from Dr. Saldanha	1:2000	polyclonal
Anti-GPER1	Membrane bound G-protein coupled estrogen receptor	rabbit	MBL Intl LS-A4268	1:2000	polyclonal
Anti-calbindin	Calcium binding protein	mouse	Sigma Aldrich C9848	1:2000	monoclonal
Anti-parvalbumin	Calcium binding protein, a marker for fast-spiking interneurons	mouse	Millipore MAB1572	1:10,000	monoclonal
Anti-GAD67	Enzyme that synthesizes GABA expressed in somas	chicken	Abcam ab75712	1:100	polyclonal

Confocal imaging

Slides were imaged using a confocal microscope (NA1, Nikon, Tokyo, Japan) with NIS-Elements imaging software (Ar). Sections with regions of interest were

identified at 10x and a 3x3 stitch image would be taken to confirm landmarks to correctly identify regions under higher magnification. For lateral sections that contained HVC (proper name), HVC shelf and arcopallium, both HVC and/or arcopallium needed to be present in the same section (1.7-2.5mm lateral to the midline). Medial sections which contained regions of interests such as caudomedial nidopallium (NCM), caudomedial mesopallium (CMM), Field L, and hippocampus (HP) (0.2-1mm lateral to the midline) were defined by thickness of the hippocampus and complete lack of nucleus taenia (Tn) and the arcopallium. Once regions were identified, images would be taken under the 60x oil objective in zstacks of 9-15 μm thick at 1 μm per step. Laser strength and gain were adjusted for each section, but kept consistent across regions on the same section unless there was a dramatic difference where the objective needed to be refocused. I was unable to successfully identify HVC and the shelf in GPER1/GAD67 tissues. Aromatase is exclusively expressed in the shelf and not HVC (Saldanha et al. 2000, Ikeda et al. 2017), so I was more successful in the identification of these regions for this marker. GPER1 appeared to be uniformly dispersed, so it was difficult to locate HVC and the shelf, and these regions were not considered for further analysis for GPER1. Hippocampus (HP) was collected as a non-auditory region that expresses aromatase and GPER1 (Saldanha et al., 2000; Acharya and Veney, 2012). CMM was used as a negative control for aromatase expression that is also an auditory region.

Image analysis

All images were blinded for cell counting using FIJI software (National Institutes of Health, USA). All cell counts were performed with the maximal z-projection image, while scanning through the z-slices for confirmation. After cell counts, all counts were normalized to number of DAPI cells for that image and depicted as a percentage of DAPI. For coexpression, both aromatase and GPER1 are depicted as percentage of the interneuron subtype label of interest. Cell counts had a CV within raters <15%. I noticed that aromatase cells tended to form clusters with each other, so I also analyzed clusters of aromatase cells. Clusters were defined as a group of two or more cells forming somato-somatic contacts. The number of cells within each cluster were counted manually by a using the 60x z-stack images from NCMv, because qualitatively, this region appeared to have the most clusters. Aromatase cells in clusters were determined to be neurons by a NeuN double stain (Ikeda et al. 2017). I also ran a simulation using MATLAB to determine whether clustering of aromatase somas is different from what can be expected by random association. I sampled from a normal distribution using the mean and SEM from the aromatase cell count for the cluster analysis (91.7 ± 7.29). I created random plots of spheres varying by cell number and size (also sampling from a normal distribution: average diameter of 50 somas measured was $9.16 \pm 0.29 \mu\text{m}$). The plot fit the dimensions of the confocal images, including the volume ($15 \mu\text{m}$). I ran 1000 simulations and measured the distance between all the spheres per simulation. Points were defined as touching or “a cluster” when their distance was shorter than the sum of each sphere’s radius. After 1000 simulations, a percentage of spheres found in cluster sizes of 1, 2, 3... etc. were quantified using the

union-find algorithm. This simulation quantification mirrors that of the manual aromatase cell clustering counts performed on the images.

Statistics

Two-way between subject ANOVAS were run for all analyses with post-hoc Bonferonni analyses for significant region effects. For most analyses, I did not have statistical power to detect region*sex interactions. Since the focus of this study was on individual sex comparisons, and there were compelling mean differences, I ran individual t-tests for each region to compare the sexes and determine if there were meaningful effects that would be masked in a larger model. I report significant differences as $p < .05$. For the clustering analysis, since I ran 1000 simulations, I used the percentages generated as the population mean for random clustering. I ran one-sample t tests for each sex against the population for each cluster size. I also compared males and females together in a mixed factor two-way ANOVA for cluster sizes to determine sex differences, followed by Tukey's pairwise comparisons for post-hoc analyses for male vs. female comparisons and Bonferroni post-hocs for cluster size comparisons.

Results

I performed region and sex comparisons for all measurements. Somatic aromatase expression did not differ by sex overall ($F(1,62)=1.72$, $p=0.19$, $d=0.30$, Power 25%). I also did not detect any sex differences in most individual brain regions through individual t-tests ($p > .05$: dNCM, vNCM, aHVCshelf, HP, highest Power=16% Figure 4A) with the exception of posterior HVCshelf where females expressed more aromatase than males ($t(7)=2.89$, $p=.023$, $d=2.04$, Power=70% Figure 4 right). I

observed differences in aromatase expression by region with the HP having the most aromatase and CMM having no somatic aromatase expression ($F(7,62) = 6.06$, $p < 0.0001$; post-hoc t-tests: HP vs any region, $p < 0.05$; CMM vs any region, $p < 0.01$; Ikeda et al. 2017). Dorsal and ventral NCM, anterior and posterior HVCshelf, and NCL did not differ in expression ($p > .05$). I compared GPER1 expression for region and sex. I did not detect sex differences in GPER1 cell number for any regions (overall: $F(1,45)=0.65$, $p=0.42$, $d=0.21$, Power=12%, individual regions sex comparisons $p > .05$, Figure 4B). GPER1 expression also did not differ by the regions I analyzed ($F(5,45)=0.91$, $p=0.48$, Power=30%, Figure 4 right).

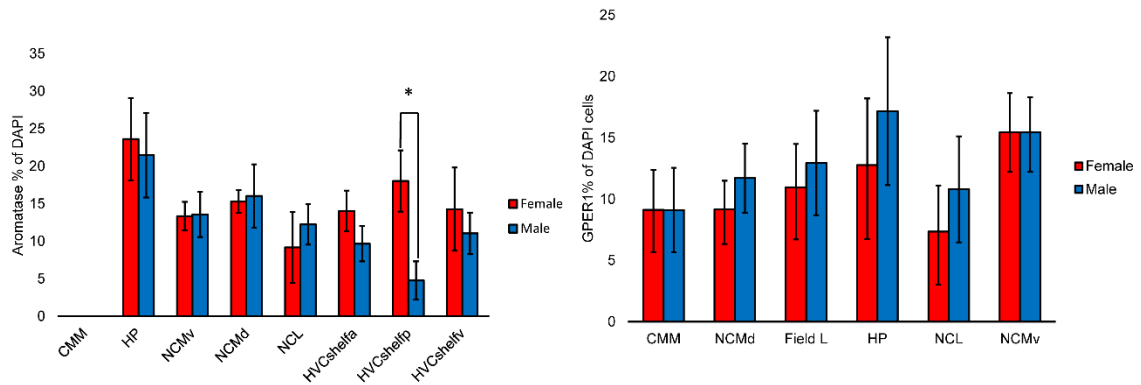


Figure 4: Aromatase and GPER1 expression is similar in males and females except posterior HVC shelf.

Left – percentage of aromatase cells within each quantified brain region. Right – percentage of GPER1 cells within each quantified brain region. Females are red bars and males are blue bars. $p < .05$ * Left - CMM, F N=6, M N=3; NCMv; HP, F N=6, M N=5; NCMv, F N=6, M N=6; NCMd, F N=6, M N=5; NCL F N=3, M N=5; HVCshelfa, F N=3, M N=6; HVCshelfp, F N=3, M N=6; HVCshelfv, F N=4, M N=5. Right –CMM, F N=4, M N=4; DNCM F N=6, M N=7; FIELD L, F N=4, M N=4; HP F N=4, M N=5; NCL, F N=4, M N=4; VNCM, F N=4, M N=4.

I did not detect a difference in parvalbumin expression by sex ($F(1,57)=0.86$, $p=0.36$, Power=15%, all regions t-test: $p > .05$; Figure 5A), but there was a difference by region ($F(7,57)=2.56$, $p=.023$; Bonferroni post-hoc: HVCshelfa vs. NCL $t(57)=3.48$,

$p=0.027$; Figure 5A). Calbindin also differed by region ($F(7,64)=7.31$, $p<.001$; Bonferroni post-hocs: CMM vs. HVCshelp $t(64)=3.79$, $p=.0095$, NCMv vs. all other regions except HVCshelp: $p>.05$; Figure 5B) and I did not detect a difference by sex ($F(1,64)=0.065$, $p=0.80$, Power=5.7%, all regions: $p>.05$; Figure 2C) with the exception of a trending difference in NCMv where females expressed more calbindin than males ($t(14)=1.93$, $p=0.074$, Figure 5C) contradictory to previous sex differences described in NCM (Pinaud et al., 2006), and a trending difference in CMM where males had more expression than females ($t(9)=-2.34$, $p=0.052$; Figure 5C).

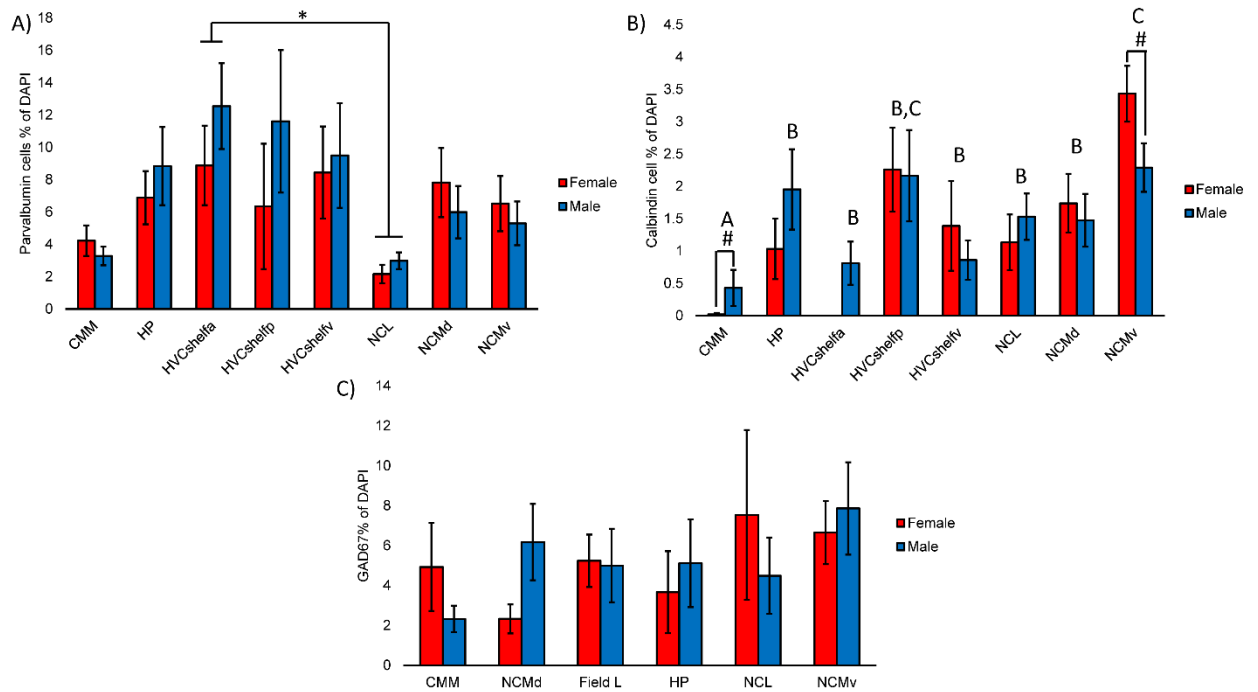


Figure 5: Interneurons are different by region but not sex.

A) Percentage of parvalbumin cells quantified for each region. B) Percentage of calbindin cells quantified for each region. C) Percentage of GAD67 cells quantified for each region. Females are red bars and males are blue bars. $p=.074^{\#}$. Letters above regions indicate Bonferroni post-hoc comparisons. A) Parvalbumin: CMM, F N=6, M N=4; HP, F N=5, M=4; HVCshelfa, F N=3, M N=5; HVCshelfp, F N=3, M N=5; HVCshelfv, F N=3, M N=5; NCL, F N=4, M N=4; NCMd, F N=6, M N=5; NCMv, F N=6, M N=5. B) Calbindin: CMM, F N=5, M N=4; HP, F N=6, M=4; HVCshelfa, F N=3, M N=4; HVCshelfp, F N=3, M N=4; HVCshelfv, F N=3, M N=4; NCL, F N=4, M N=4; NCMd, F N=9, M N=7; NCMv, F N=9, M N=7. C) GAD67: CMM, F N=4, M N=4; NCMd, F N=6, M N=7; Field L, F N=4, M N=4; HP, F N=4, M N=5; NCL, F N=4, M N=4; NCMv, F N=6, M N=7.

I also wanted to examine coexpression patterns and determine if there were sex differences dependent on region and sex. First, from previous pilot tissue that we sampled from Study 1, I did not observe any coexpression of GPER1 with either calbindin or parvalbumin in any region (Figure 6). I also did not observe coexpression of aromatase with calbindin for either sex (Figure 7). There was however a moderate

coexpression pattern with parvalbumin, and this coexpression did not differ by sex ($F(1,47)=0.75$, $p=0.39$, Figure 8A left). However, there was a significant difference in coexpression of aromatase and parvalbumin cells as a percentage of parvalbumin by region ($F(7,44)=8.25$, $p<0.001$; Bonferroni posthocs: NCMv vs. all other regions $p<.05$, Ikeda et al. 2017; Figure 8B left).

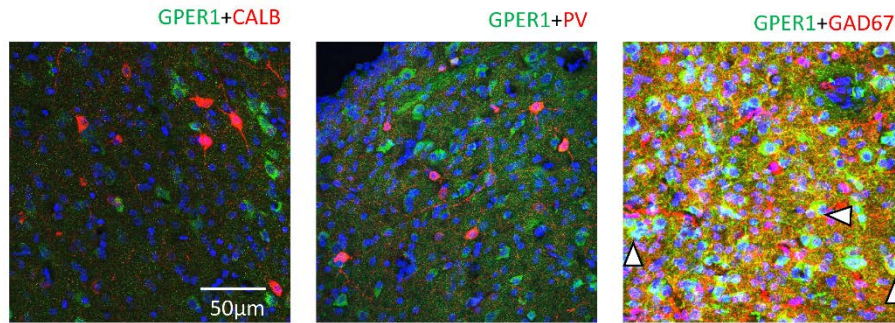


Figure 6: GPER1 expresses with GAD67 but not CALB or PV.

Each image was taken from different regions to represent general lack of expression (CALB and PV) or coexpression (GAD67). All images are maximal projection images from 15 μm zstacks. White arrows indicate cells containing both GPER1 and GAD67.

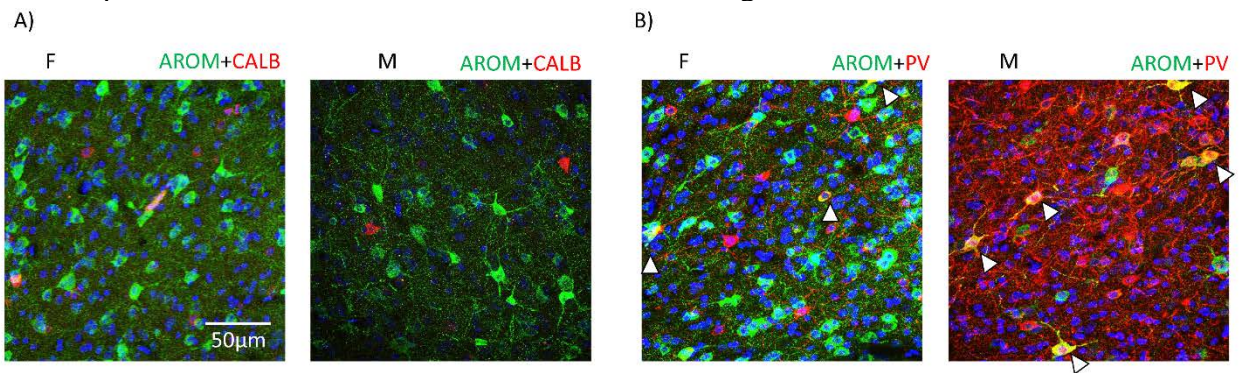


Figure 7: Aromatase coexpresses with PV but not CALB.

Each image was taken from different regions to represent general lack of expression (A;CALB) or coexpression (B;PV). All images are maximal projection images from 15 μm zstacks. White arrows indicate cells containing both aromatase and parvalbumin.

Since I could not detect any coexpression with either interneuron subtype and GPER1, I used an antibody against GAD67, the enzyme that synthesizes GABA and is preferentially expressed in somas (Erlander et al., 1991; Schwab et al., 2013). This antibody does not mark all GABAergic neurons; however, I did see coexpression with GPER1 (observations not quantified from pilot tissue), indicating I was marking cells that were not calbindin or parvalbumin, therefore a different population (see description in Methods). I performed colabeling study with this marker so that I could address the hypothesis of whether GPER1 was sexually dimorphic in its expression of this subtype of GABAergic cells. First, I did not observe sex differences in GAD67 expression (overall: $F(1,45)=0.092$, $p=0.76$, Power=6.0% all regions: $p>.05$, Figure 5C). I also did not detect regional differences in GAD67 expression ($F(5,45)=0.97$, $p=0.45$, Power=31.2% Figure 5C). There were no sex differences in coexpression of GPER1 with GAD67 with the exception of CMM which had a trend towards males having higher expression ($t(6)=-2.41$, $p=.053$ Figure 8A right). I was also not able to detect a regional difference in GPER1+GAD67 coexpression as a percentage of DAPI ($F(5,45)=1.20$, $p=0.32$, Figure 8A right). I detected a regional difference in GPER1+GAD67 expressing cells as a % of all GAD67 cells ($F(5,45)=1.09$, $p=0.38$, Figure 8B right).

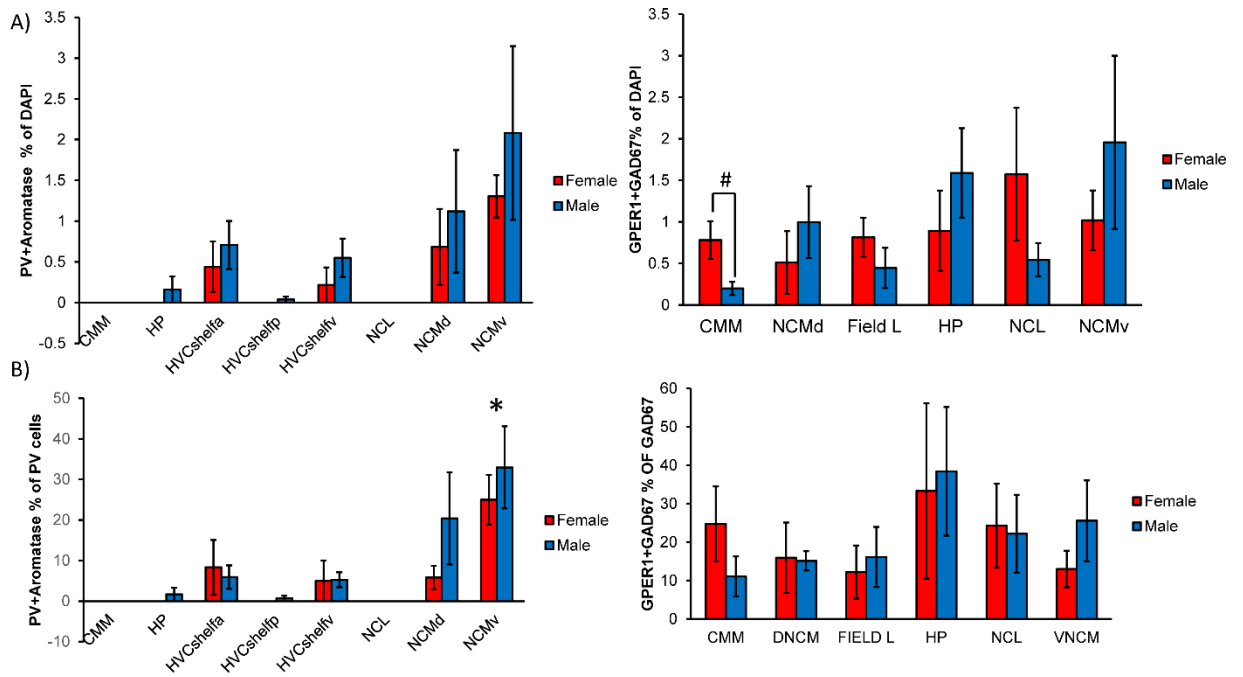


Figure 8: Males and females are similar in coexpression of aromatase and GPER1 with GABAergic markers.

A) Left: Percentage of parvalbumin + aromatase as % of DAPI cells quantified for each region. Right: Percentage of GPER1+GAD67 as % of DAPI cells quantified for each region. Females are red bars and males are blue bars. B) Left: Percentage of parvalbumin + aromatase cells as % of parvalbumin. Right: Percentage of GPER1+GAD67 as % of DAPI cells quantified for each region. A) AROM+PV: CMM, F N=6, M N=3; HP, F N=5, M=4; HVCshelfa, F N=3, M N=5; HVCshelfp, F N=3, M N=5; HVCshelfv, F N=3, M N=5; NCL, F N=3, M N=3; NCMd, F N=6, M N=5; NCMv, F N=6, M N=5. B) GPER1+GAD67: CMM, F N=4, M N=4; NCMd, F N=6, M N=7; Field L, F N=4, M N=4; HP, F N=4, M N=5; NCL, F N=4, M N=4; NCMv, F N=6, M N=7.

Finally, I observed that aromatase cell formed somato-somato clusters particularly in the NCM. I decided to determine how large aromatase cluster sizes were and how often aromatase cells were found clustered. More descriptions of regional differences, neuronal identity, and example images of clusters are described in Ikeda et al. (2017). Here, I observed a sex by cluster size interaction in the occurrence of clusters in ventral NCM ($F(8,72)=3.10$, $p=.0046$). More aromatase cells in females (47.4%) are not clustered as opposed to in males (26.4%; Bonferroni pairwise comparison $t(72)=5.27$, $p<.001$, Figure 9)B, Most aromatase cells are clustered with at least another aromatase cell (females 52.6%, males 73.6%). I wanted to determine the extent to which these clustered distributions would be observed by chance and I ran simulations to determine how often similar cell-sized spheres would cluster together in silico. I found that both males ($t(4)=-10.89$, $p<.001$) and females ($t(5)=-3.04$, $p=.029$) have a lower proportion of cells not clustered than random (males=26.4%, females=47.4%, random=72.8%; Figure 9), as well as a higher proportion of clustered cells (males: $t(4)=11.05$, $p<.001$, females: $t(5)=3.13$, $p=.026$, random 26.53%). As illustrated in Figure 9A, most males (4/5) had more frequent occurrences of cell clusters as large as 7 cells in a cluster, which was rare in the simulation and did not occur often in females. I detect a significant main effect of cluster size ($F(8,72)=31.39$, $p<0.00001$). The decay of percent of aromatase cells found in increasing cluster sizes differed between the sexes. For males, aromatase cells found solo (1), with another aromatase cell (2), and in a cluster of 3 or 4 did not significantly differ from each other (pairwise Bonferroni comparisons: 1 vs.2 $t(72)=0.44$, $p>.05$, 1 vs.3 $t(72)=0.59$, $p>.05$, 1 vs.4 $t(72)=3.72$, $p=0.06$). However, cluster sizes of 5 and above were significantly different than non-

clustered aromatase cells ($1 \text{ vs } \geq 5$, $p < .05$), but not significantly different from each other (Figure 9B). For females, non-clustered aromatase cells were significantly different than all cluster sizes (pairwise Bonferroni comparisons: $1 \text{ vs } 2$ $t(72) = 3.81$, $p < .0001$, etc., Figure 9B) and continued to decay. One reason males may have more clustering than females is if they have a greater cell density within this region. However, I compared DAPI measurements from ventral NCM in both studies and found no differences between either sex in NCMv ($t(21) = -0.71$, $p = 0.48$; data not shown).

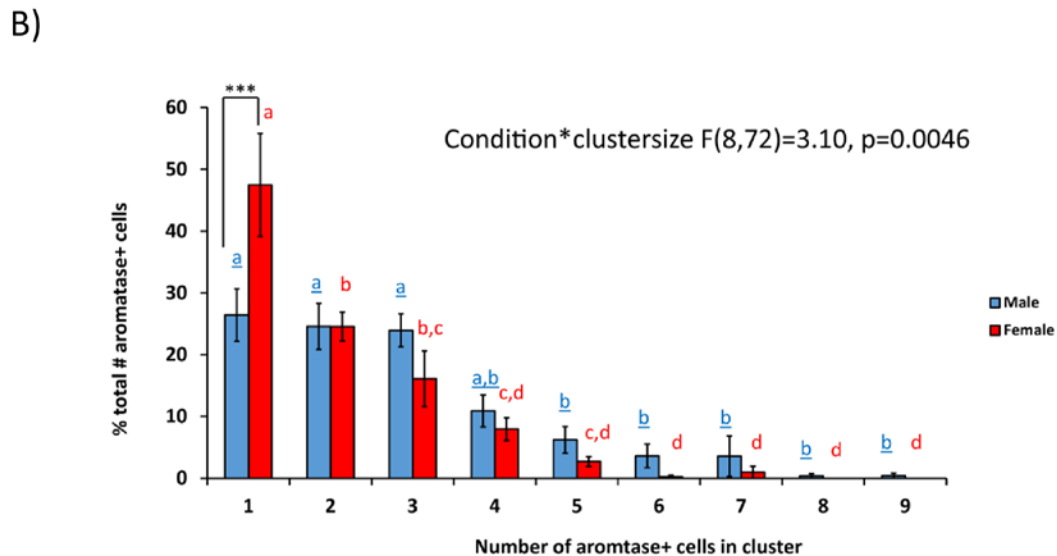
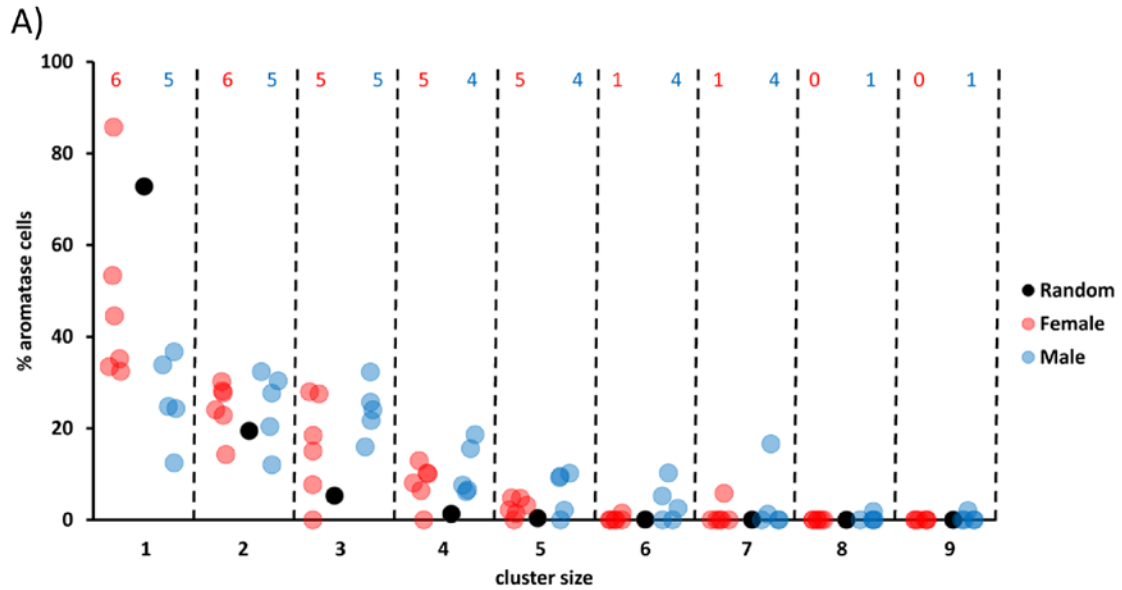


Figure 9: Males and females have different percentages of aromatase cells found in clusters.

A) Percentage of total aromatase+ cells per subject NCMv found in increasing cluster sizes. Aromatase cells that are 1 cell are not considered a cluster. Females are red dots and males are blue dots. Black dots are representation of the population mean for 1000 simulations of random. Numbers above each column indicate the number of females (red) and males (blue) that exhibit cases of clusters of that size out of 6 females and 5 males. B) Bar graphs depicting the mean and standard error of the mean for percentage of total aromatase+ cells in males (blue bars) and females (red bars). Asterisks (***) $p < .001$ for M vs. F. Letters indicate Bonferroni posthoc comparisons within sex for each cluster size ($p < .05$).

Discussion

This is the first study to systematically characterize interneuron cell types of estrogen-producing and estrogen-responsive cells of several subregions the auditory lobule, and also to compare males and females. In general, males and females were similar in expression of somatic aromatase, GPER1, parvalbumin, calbindin, and the GAD67+ subtype. Despite overall similarities between the sexes, some sex differences emerged. Aromatase expression in posterior HVCshelf was higher in females. GPER1+GAD67 positive neurons were trending for higher expression in CMM of females, and calbindin+ neurons were also trending higher in female NCMv. There were also sex differences in cell clustering of aromatase+ cells in NCMv. Specifically, males had fewer non-clustered aromatase cells than females and more incidences of larger cluster sizes. Finally, I found that aromatase+ cells across brain regions coexpress with parvalbumin but not calbindin. GPER+1 cells never coexpressed with calbindin or parvalbumin, but there was modest coexpression with GAD67. These results indicate that estrogen-producing cells and estrogen-receiving cells are each made up of a population of GABAergic neurons.

This study has replicated prior work reported in the literature concerning lack of sex differences in aromatase and GPER1 expression. I did not find sex differences in somatic aromatase expression in NCM, which was first reported by Saldanha et al. (2000). I also found no expression of somatic aromatase in CMM, which has long been noted by the field, confirming the consistency and reliability of the protocol (Shen et al., 1995; Saldanha et al., 2000). I also replicated lack of sex differences in GPER1 expression in adulthood (Acharya and Veney, 2012). One inconsistency between my

study and other is that Pinaud et al. (2006) reported males had more calbindin expression across NCM, where I find a similarity in dorsal NCM and a trending increase in expression in females in ventral NCM. Pinaud et al. (2006) quantified global calbindin expression across the caudal nidopallium; however, I sampled from smaller regions of the caudal nidopallium, dividing the region into lateral (NCL) and dorsal and ventral medial sections (NCMd and NCMv). Subregions of the nidopallium have differences in responsiveness to songs and estradiol exposure (Maney et al., 2006; Sanford et al., 2010). Analyzing cell types and cell densities in subregions may indicate local differences in microcircuitry.

One important observation that I made in this study is that GPER1 is expressed in many more auditory regions than aromatase, specifically in CMM and Field L as well as HVC (not quantified in this study; Acharya and Veney, 2012), in which aromatase expression is notably absent. Although my study does not include a colabeling of aromatase and GPER1 in the same sections, future work may determine the densities of aromatase and GPER1 cells in auditory subregions, as well as possible coexpression. A resultant hypothesis is that aromatase cells, which are mostly dense in secondary auditory regions such as NCM and HVCshelf, may be projecting to areas that express GPER1, the receptor for estradiol. NCM and HVC have neuromodulatory connections exhibited by changes in HVC selectivity to bird's own songs when E2 is infused into NCM (Remage-Healey and Joshi, 2012). The shelf also sends projections into HVC (Vates et al., 1996), which may be another source of estradiol influencing HVC physiology. NCM and CMM are secondary auditory regions that also have reciprocal connections (Vates et al. 1996). Although there is not somatic expression of aromatase

in CMM, there may be terminal expression that could influence rapid estradiol signaling onto GPER1 expressing neurons in this region. There are some indications that “ghost cells” I observed in Study 1 in regions like the arcopallium may reflect terminal aromatase expression (Ikeda et al. 2017). Localization of aromatase synapses will need to be further explored using tracer and EM studies to determine the network and synaptic connections of these regions.

Previous reports indicated sex differences in auditory activity of subregions of the auditory lobule (Caras et al., 2015; Giret et al., 2015; Yoder et al., 2015) and interneuron expression in the motor song circuit (Grisham and Arnold, 1994; Cornez et al., 2015). I showed that despite regional heterogeneity in cell types, there are large similarities in expression of interneurons and estrogenic cells types across the auditory lobule, and between the sexes. This is the first study to do a broad sampling of several forebrain regions to compare males and females and examine several neuronal cell types. Future work with larger samples sizes will be necessary to rule out any undetectable sex differences. I only compared somatic expression of all the markers used in this study, using cell count as a dependent measure. There is considerable evidence for sex differences in aromatase fiber expression and aromatase activity (Saldanha et al., 2000; Peterson et al., 2005), suggesting sex differences in estradiol production and synaptic function may be contributing more to differences in physiology than cell types.

Although I did not observe sex differences in coexpression, this does not rule out that there may be sex differences in cell communication between interneurons and estrogen-producing and estrogen-responsive cell types. For example, if calbindin is

more globally expressed in NCM in males rather than females as reported in Pinaud et al. (2006), then there may be more connections with either aromatase-positive or GPER1-positive cells that could facilitate physiological differences in how songs are represented within the NCM. One finding that we reported in Ikeda et al. 2017 from Study 1 is that aromatase cells are found in somato-somatic clusters with other aromatase cells. I performed additional sex comparisons and found that males and females differ in the proportion of aromatase cells clustering together, suggesting that organization of estrogen-producing cell types in the region I analyzed, NCMv, are sexually dimorphic and may have functional consequences. The role of somato-somato clustering of these cell types is unclear. One proposal is that gap junctions may be involved to synchronize communication between cells which has previously been described in a neighboring region HVC (Gahr and Garcia-Segura, 1996); however, there is a lack of physical evidence currently for NCM – whether it be electrophysiology recordings or dye transfers – to conclude this may be functional purpose for clustering. Alternatively, vocal learners such as songbirds and parrots have large cell densities packed into the telencephalon, which surpasses the mammalian cortex outside of primates (Olkowicz et al., 2016). Since birds do not have a neocortex that sits on the outer layer of the telencephalon with sulci and gyri to increase surface area as in mammals, another strategy must be used to pack in more cells. High cell density and packing may explain why clustering occurs; however, it is unclear how this would relate to sex differences in cluster as males and females have similar cell densities in this region.

Most of the aromatase and GPER1 cells did not coexpress with any of the cell type markers, demonstrating that there are large populations of these cells that are still uncharacterized. There are other notable markers for interneurons that are important for auditory physiology. Other interneuron subtypes such as somatostatin and VIP are important cell types for auditory processes in mammalian cortex (Pi et al., 2013; Chen et al., 2015; Phillips and Hasenstaub, 2016). These, as well as glutamatergic cell types, can further inform the heterogeneity of the auditory lobule and may give insight into how rapid estradiol signaling is being regulated by specific cell types. In the human temporal cortex, which contains auditory association regions analogous to NCM and CMM, most aromatase expressing cells are excitatory pyramidal neurons (Yague et al., 2006). Studies measuring mRNA have shown that inhibitory neurons consist of about half of all auditory neurons (Pinaud et al., 2004) and aromatase cells are 40:60 inhibitory to excitatory (Jeong et al., 2011). These results have not been validated with protein measurements, and the markers do not represent all GABAergic neurons markers.

A portion of this study was inspired by descriptions of aromatase interneurons within the human temporal cortex (Yague et al., 2006). From a species comparison standpoint, I find similar coexpression of aromatase with parvalbumin between zebra finches and humans; however, the lack of coexpression of calbindin with aromatase in zebra finches differs from the coexpression in the human brain. This finding might provide some species-specific mechanisms for regulation of neuroestradiol on auditory activity. Although there have not been gross anatomical descriptions of GPER1 in the human brain, GPER1 is expressed in the primate prefrontal cortex on dendrites

(Crimins et al., 2017). Given the robust role of rapid estradiol signaling in auditory processing of the zebra finch, which uses complex, learned vocalizations analogous to human speech, membrane estrogen receptors such as GPER1 and translocated ER α and ER β should be further explored in context of cortical organization in the human brain as well as how these receptors mediate sensory and perception of language.

In this study, I have shown that males and females are largely similar in the GABAergic cell identities of estrogen-producing and estrogen-responding cells of the auditory forebrain, but that the songbird auditory forebrain is regionally distinct by the organization of these cell types. Although a large subpopulation of aromatase-positive and GPER1-positive cells are still uncharacterized, this work provides anatomical and cell density descriptions for future projects to elucidate that relationship of the neuroestradiol circuit within the brain.

CHAPTER III

ACUTE ENDOGENOUS ESTRADIOL PRODUCTION IS NECESSARY FOR SONG-INDUCED IMMEDIATE-EARLY GENE EXPRESSION WITH REGIONAL AND SEX SPECIFIC DIFFERENCES

Abstract

Estrogens are synthesized in the brain and can act through non-canonical mechanisms to modulate neuronal physiology. An example is auditory responsiveness in songbirds, where peripheral hormones or exogenous hormone manipulations alter auditory-evoked neuronal activation; however, the significance of endogenous neuronal estrogen production is less understood. Both males and females can synthesize estradiol (E2) rapidly in the caudal nidopallium (NCM). It is unclear whether the sexes need acutely synthesized estradiol for neuronal auditory activity. Here, we examine whether endogenous estradiol synthesis is necessary for auditory induced immediate early gene expression of Egr-1 in both sexes in several cortical regions known to differentially expression aromatase. I also determined if expression of phosphorylated CREB, a transcription factor that targets Egr-1, is regulated in parallel, and whether it is auditory inducible. I administered a peripheral dose of the aromatase inhibitor fadrozole, and measured auditory induced Egr-1 across several auditory brain areas. I found that males administered fadrozole decreased Egr-1 expression in more brain regions than females. Regions such as CMM which lack aromatase expression were unaffected, highlighting the region specificity of pharmacological aromatase inhibition within areas that express aromatase. I also found that females have more aromatase expression than do males in posterior HVCshelf, which has been previously unreported. In contrast to Egr1, I did

not observe any changes in pCREB expression with song presentation or aromatase inhibition. These findings are consistent with the hypothesis that endogenous estradiol is acutely synthesized during auditory playback and leads to a downstream transcriptional response in several subregions of the male brain. Females appear to be less effected from this acute inhibition in most regions. I also draw into question the role that pCREB plays in the proposed regulation of Egr-1 by estradiol. Finally, I propose that HVCshelf may be a region of sexual dimorphism that has been previously overlooked, specifically the role for brain-derived estradiol in auditory responsiveness.

Significance

In this chapter, I demonstrate the necessity of endogenous estradiol synthesis to facilitate auditory responsiveness in several secondary auditory cortical regions. I also show a region-specific sex difference where this dependency is stronger in males than it is in females. Together, these findings suggest that compensatory estradiol synthesis in females may protect auditory responsiveness when acute neural estradiol synthesis is blocked. These findings highlight the significance of examining sex differences in physiological properties that can compensate for and/or augment sex differences in neuroanatomy and behavior.

Introduction

Within neuroendocrinology, there has been a focus on transcriptional mechanisms of steroid hormone signaling through nuclear receptors that lead to long-lasting changes to the brain and behavior. However, recent attention has been given to non-canonical mechanisms that act through membrane receptors and intracellular signaling cascades to change neuronal firing states. What is sometimes overlooked is

that these non-canonical mechanisms can also enact long-term changes, specifically through activating kinase cascades which target transcriptional mechanisms to change protein expression (Micevych et al., 2015).

Neuromodulatory actions of estradiol have been described in many brain regions, such as in the hypothalamus where it controls reproductive behaviors (Balthazart et al., 2009; Micevych et al., 2015) and in hippocampal-dependent memory and neuronal activity in rodents (Woolley, 2007; Fernandez et al., 2008; Frick, 2013). Estradiol infused into the caudal medial nidopallium (NCM) enhances auditory evoked activity in both sexes (Remage-Healey et al., 2010b; Remage-Healey et al., 2012), and estradiol content rapidly increases when birds hear conspecific song (Remage-Healey et al., 2008). Together, this work indicated a neuromodulatory role for estradiol in songbird audition, and that endogenous estradiol synthesis is likely shaping auditory events.

One marker for auditory activation and memory is the immediate early gene *Egr-1* (also known as ZENK in the songbird literature; Mello et al., 1992). There has been indirect evidence that estradiol actions on *Egr-1* auditory responsiveness is through non-canonical mechanisms. Long-term implants of estradiol can shift song-induced immediate early gene (IEG) expression in zebra finch auditory forebrain (Maney et al., 2006) in a region dependent manner (Sanford et al., 2010). Injections of E2 directly into NCM induce *Egr-1* message in the absence of song (Tremere et al., 2009). To date, there has not been a study to directly test whether endogenous estradiol synthesis is necessary for sensory-induced expression of *Egr-1* or other IEGs. There is suggestion that chronic fadrozole administration dampens memory formation (Yoder et al., 2012),

which may indicate an Egr-1 mechanism of action because of the role this IEG plays in memory formation in other systems (Knapska and Kaczmarek, 2004; Moorman et al., 2011). Evidence suggests Egr-1 is regulated by estradiol via non-canonical mechanisms. The MEK-ERK pathway is necessary for song-induced expression in birds (Cheng and Clayton, 2004), as well as estradiol induced expression in mammalian in vitro tissues (Suva et al., 1991; Pratt et al., 1998; de Jager et al., 2001; Chen et al., 2004). Exogenous estradiol also rapidly regulates phosphorylation patterns of proteins such as ERK and CREB in the songbird forebrain in response to song (Heimovics et al., 2012). Phosphorylated CREB can bind to the promotor of Egr-1 through CRE sites (Knapska and Kaczmarek, 2004); however, its role in regulation of auditory-induced Egr-1 is unknown in songbirds.

Gross anatomical comparisons have shown that males and females are similar in auditory subregions of the forebrain, although neuroestrogens may have sex differences. Somatic aromatase expression in NCM are the same between the sexes; however, males have more aromatase fibers and terminal aromatase activity in this region (Saldanha et al., 2000; Peterson et al., 2005) suggesting sex differences in the role for rapid estradiol synthesis. Peripheral estradiol is also similar between males and females (Adkins-Regan et al., 1990; Prior et al., 2014) despite the peripheral source from the ovaries in females (Schlinger and Arnold, 1992), indicating that alternative sources, such as brain aromatase in males, may be involved in compensation. Peripheral steroid hormones can be dynamic in other behavioral processing such as stress and aggression (Shors et al., 2009; Heimovics et al., 2016), but in songbirds, peripheral estradiol levels remain stable in acute timeframes when birds hear song, despite concurrent robust changes in

estradiol levels within the brain (Ramage-Healey et al., 2008). Fecal estrogen content indicates there is changes to peripheral estradiol in different contexts of song playback (Tchernichovski et al., 1998), but this long-term (several days) detection method does not capture acute changes . This indicates that in auditory activity, likely dynamic, neuronal synthesis of estrogens are playing the major role in acute neuromodulation of auditory activity rather than peripheral sources.

Downstream targets may reveal sex differences in auditory cellular signaling as well (Krentzel and Ramage-Healey, 2015; Chapter 4). In mammalian hippocampus, rapid E2-induced phosphorylation of CREB only occurs in females but not males (Abraham and Herbison, 2005; Boulware et al., 2005; Meitzen et al., 2012). By examining expression of both Egr-1 and pCREB, the current study can therefore determine if there are sex differences in the response to endogenous estradiol synthesis within the intracellular pathway. I can also determine if changes to Egr-1 and pCREB expression change together as a result of aromatase inhibition, and if this is similar between males and females.

I set out to test four hypotheses in this study. First, I tested whether acute estradiol synthesis is necessary for auditory-induced Egr-1 expression in subregions of the zebra finch telencephalon. Second, I tested whether phosphorylation of CREB is similarly regulated by acute estradiol synthesis (in parallel with regulation of Egr-1). Third, I tested whether phosphorylation of CREB is inducible by song-exposure. Finally, I examined the extent to which any of these mechanisms depend on sex as a biological factor, predicting that females would be less dependent on acute estradiol synthesis due to compensation from peripheral sources.

Methods

Animals

Adult (>120days) male (n=20) and female (n=21) zebra finches were raised in a breeding aviary (light cycle) with food and water available ad libitum. After fledging, birds were moved to single sex cages to mature into adulthood. For all experiments, birds were removed from aviary ~12-24 hours before manipulations.

Stimuli playback

Study 1: Males (n=8) and females (n=8) were isolated from the colony into sound-attenuation chambers. One hour before playback an oral dose (30uL) of either saline or the aromatase inhibitor fadrozole (1mg/mL Novartis; n=4/sex by treatment group) was administered. This dosing has been successfully used in prior studies to reduce aromatase activity and impair estrogen-dependent behaviors (Wade et al., 1994; Saldanha et al., 2004; Rensel et al., 2013; Rensel et al., 2015). I waited one hour before playback because this is the timeframe for oral fadrozole to begin to have measurable changes (Kochak et al., 1990). This is also when the playback speaker was placed in the chamber. Stimuli playback consisted of novel triplicate song played for 30 minutes (three conspecific songs played back to back (ISI: 5s) for ~15 seconds and then 45 seconds of silence. Repeated across 30 minutes; ~70dB), which has been validated and replicated as the ideal exposure for Egr-1 expression (Mello et al., 1992). This period was followed by 30 minutes of silence in the dark to avoid male singing.

Study 2: Based on the results of Study 1, I wanted to determine if the effects on Egr-1 expression of the fadrozole treatment were specific to the synthesis of auditory-induced Egr-1 expression, rather than a toxic, degradation of Egr-1 expression. Rather

than exposing birds to song playback during the timeframe of decreased aromatase activity and estradiol production (~1 hour after administration in Study 1; Kochak et al., 1990), I followed the same design as study 1 with males and females n=3 per treatment and sex; total=12), with the exception that I administered the song playback paradigm immediately after fadrozole administration. Song playback occurred before fadrozole has reported effects on aromatase activity (see prior citations), and fadrozole would only be active after the protein expression of Egr-1 has occurred.

Study 3: Males (n=9) and females (n=10) were collected by two separate experimenters in two separate studies so sex differences were not directly compared. I was interested in whether phosphorylation of CREB has different patterns based on song presentations. For each study, there were two stimuli groups: no song, 85 seconds of song or 15 minutes of song. The stimuli duration choices were selected based on previous publications. 85 seconds was chosen because it is the ideal length for pERK, an upstream kinase known to be responsive to song in males (Cheng and Clayton, 2004). 15 minutes was selected because prior work has used this playback design for other phosphorylation studies (Heimovics et al., 2012). Song groups consisted of triplicate song playback protocol for the duration of time of that group. Since I had a no song control, to avoid any song being heard by males through their own singing, all subjects were kept in the dark ~2 hours before the experiment began and throughout playback. The playback speaker was placed ~ 5 minutes before the start of the playback session. For the 15-minute group, triplicate song was played back for the entirety of the session and then birds were immediately killed through rapid decapitation. For the 85 second group, birds sat in silence for 13 minutes and 35 seconds and the song paradigm

was played at the end. For the silence group, birds sat in silence for the entirety of the 15 minutes (Figure 10).

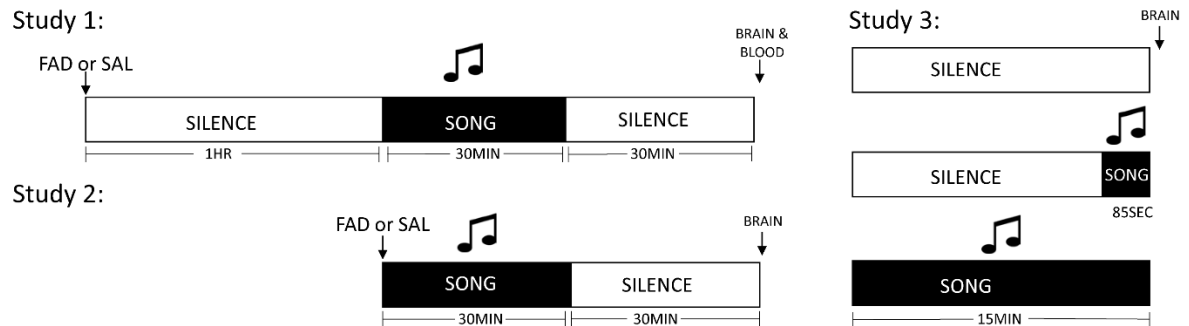


Figure 10: Timelines for song presentation and drug administration of each study. Timelines are all organized based on day of experimentation to illustrate both drug administration and song condition. Study 1 and 2: Fadrozole (FAD) and saline (SAL) administration is indicated by black arrows. Fadrozole administered orally requires about an hour to become active to shut down aromatase activity (Kochak et al., 1990). Black boxes indicate time periods when triplicate song is played back to the subjects. White boxes indicate periods of silence. All timescales are indicated below the boxes.

Hormone assay

Whole blood was collected from animals at time of sacrifice for Study 1. I performed a solid phase extraction on the whole blood and then used EIA (Caymann) to measure estradiol. Four out of sixteen sample were compromised during extraction, and I was unable to get any detectable levels so these samples were removed from the analysis. I did not detect any differences in peripheral estradiol levels regardless of drug treatment ($F(1,8)=0.56$, $p=.475$) or sex ($F(1,8)=.099$, $p=.72$; data not shown), indicating that the 60-min treatment with fadrozole did not significantly shift peripheral estradiol levels. This finding is consistent with prior work (Prior et al., 2014), however due to the loss of samples, these measurements are underpowered. (Power is 10%, $d=1.06$).

Immunostaining

After all stimuli presentations, birds were immediately killed by rapid decapitation and their brains were extracted and dropped into a 5% acrolein solution made in 0.1M phosphate buffer (PB). Brains sat in fixation overnight at room temperature. The following morning, brains were transferred to a 20% sucrose in 0.1M phosphate buffer/0.9% saline (PBS) solution at 4 °C for at least two days. Brains were sections using a cryostat (Leica, Germany) at 45µm in serial sections and stored in cryoprotectant at -20°C. For each study, all subjects were processed at once in the same free-floating immunostaining run. Sections were washed using 0.1M PB and then treated with 0.5% sodium borohydride (NaBH₄) in PBS. After 3-5minute washes in 0.1M PB and 3-15minute washes in 0.1M PB, sections were incubated in 10% normal goat serum (S-1000, Vector Labs) made in 0.3% Triton-x/0.1M PBS (PBT) for 1 hour. Sections were then incubated with either anti-Egr-1 rabbit polyclonal antibody (1:10,000, sc-189, Santa Cruz, antibody used for all Egr-1 staining in zebra finch) or anti-pCREB rabbit polyclonal antibody (1:5,000, Ser133, sc-101663, Santa Cruz; same epitope as antibody used in sparrow brain Heimovics et al., 2012) made in 0.3% PBT for 1 hour at room temperature followed by ~48 hours at 4 °C. Following washes in 0.1% PBT, sections were incubated in the secondary antibody biotinylated goat anti-rabbit (BA-1000, Vector Labs) made in 0.3% PBT at a 1:200 dilution for 1 hour. Sections were washed and then incubated in Vector A:B (1:500 dilution; Vectastain Elite ABC Kit PK 6100, Vector Labs) solution for 90 minutes followed by washes. For development, I used the Vector SG hydrogen peroxide and chromatin (Vector SG Peroxidase (HRP Substrate Kit, SK-4700, Vector Labs) kit, dropping one drop of each

into the wells and the sections developed for 10 minutes. After mounting, sections were dehydrated using a 100%-95%-75% ethanol washes followed by HemeD. Sections were coverslipped using permount. I validated the pCREB antibody by first reserving some sections before the experiment, following this same process but incubating the primary in the respective blocking peptide (Ser133, Cell Signaling Technology). I observed total absorption of the antibody and observed no non-specific binding (data not shown).

For aromatase comparisons, I followed a similar protocol. Animals (males n=6 and females n=6) were transcardially perfused using 4% paraformaldehyde (PFA) in PBS. After perfusion and a post-fixation of 2 hours with 4% PFA, brains were switched to a 30% sucrose/saline solution. Brains were sectioned at 35 μ m. Changes to the immunostaining protocol reflect differences in fixation and using fluorescent secondary antibodies. After sectioning and storage in cyroprotectant, sections were washed with 0.1M PB 3x for 15 minutes each. A specific zebra finch anti-aromatase rabbit antibody (supplied by Colin Saldanha; Saldanha et al., 2000) was used at 1:2000 and incubated for 1 hour at room temperature and then ~48 hours at 4 °C. After washing in 0.1% PBT 3x for 15 minutes, I incubated the sections in secondaries raised in goat for rabbit antibodies conjugated to Alexa 488 and 594. Because of using fluorescence, sections were mounted with Pro Diamond Anti-Fade with DAPI (Thermofisher).

Imaging

All regions of interest were predetermined because of known aromatase expression patterns. I selected the caudal medial nidopallium (NCM) because of its aromatase fiber and activity sex difference (Saldanha et al., 2000; Peterson et al., 2005). I divided the region into dorsal and ventral subregions. I defined NCM sections by the

thickness of the hippocampus and the absence of nucleus taenia (Tn) which contain medial sections ~ 0.2-1.0mm from the midline. I selected HVCshelf as another region of interest because it is known to express aromatase and is auditory responsive. I divided the shelf into anterior and posterior and used the absence of Egr-1 in HVC to determine where shelf begins. Sections were selected from ~1.7-2.5mm from the midline. For females, HVC location was difficult due to small size. In some subjects HVC was not always reliably visible, so I selected sections that appeared similar based on other anatomy markers such as thinness of the hippocampus and presence of the arcopallium and took images of dorsal caudolateral nidopallium. Because pCREB does not have a noticeable distribution difference between HVC and HVCshelf as does Egr-1, for Study 1, since Egr-1 and pCREB were stained on serial sections of the same animal, the Egr-1 immunoreactivity guided the region of interest for HVCshelf for the pCREB immunoreactivity in adjacent sections. For Egr-1, I also quantified cells in CMM as a auditory region that does not have somatic aromatase expression (Saldanha et al., 2000, Chapter 2), therefore, I hypothesized I would not see changes in Egr-1 expression with fadrozole. For Study 2 and 3, I collected images only from the dorsal and ventral NCM.

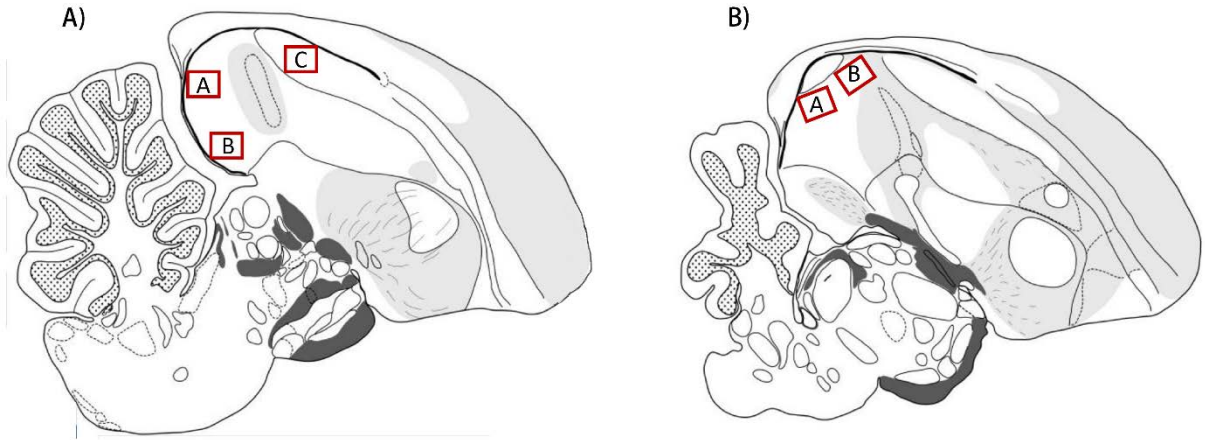


Figure 11: Auditory regions of the zebra finch forebrain.

A) Schematic of a sagittal section of the zebra finch brain at ~1mm. This is representative of sections I targeted for imaging. Boxes represent subregions as follows: A] dorsal NCM, B] ventral NCM, C] CMM. B) Schematic of a sagittal section at ~1.7 mm. Boxes represent representative regions for HVC shelf: A posterior and B anterior. Images adapted from ZEBra zebrafinchatlas.org.

Images were taken at 20x using brightfield microscopy (Zeiss Axio Lab A.1) and the Zeiss software ZEN 2012 blue edition. I took 3-4 images per region per animal. Images were quantified by an experimenter blinded to treatment condition. The experimenter counted cells in the entire image. Since both Egr-1 and pCREB are nuclear stains, discrete nuclei were counted manually using Image J. Inter-counter variability was 10.5% for Egr-1 and 7.6% for pCREB.

For aromatase fluorescence, detailed methods are described in Chapter 2 as well as the full extent of the study and all the regions analyzed. Briefly, I used a confocal microscope (NA1, Nikon, Tokyo, Japan) with NIS-Elements imaging software (Ar) was used to take pictures at 60x with z-slices that ranged from 9-15 μ m. These images came from regions like the regions outlined in the above studies. I could only unequivocally identify HVCshelf reliably in 5 males and 3 females. Aromatase counts are represented as a percentage of DAPI.

Analysis

I averaged sections from repeated regions of each subject for one representative count of that region. As is standard in the Egr-1 literature, I represent the cell counts within an area of mm^2 . All statistics were performed using Origin 2017. I ran two way mixed effects ANOVAS for region and sex and region and treatment initially. For sex by treatment by region comparisons, due to the low subject number per group and lack of power to detect complex interaction of treatment, region, and sex, I used non-parametric statistics and ran Mann-Whitney U's fixing the factors region and sex and compared treatment groups. I did the same for the pCREB analysis. For Study 3, since males and females were collected in two different studies, I did not run them together in a statistical model and instead performed a one-way within-subject ANOVA for each sex and region.

Results

I tested the hypotheses that estradiol synthesis is necessary for auditory-evoked IEG induction in an acute timescale. First, I found a decrease in auditory-induced Egr-1 expression by fadrozole pre-treatment ($F(1,14)=7.44$, $p=0.016$) and this change in expression depended on brain region (region: $F(4,56)=34.17$, $p<.0001$; region*treatment: $F(4,560)=5.08$, $p=.0015$). I did not detect a main effect of sex ($F(1,14)=2.73$, $p=0.12$). I lacked power to detect a treatment by sex interaction (23%) and sex by treatment by region (12%). The *a priori* hypothesis was that auditory activation would be more reliant on estrogen synthesis in males than females, so I fixed the factors sex and region and ran Mann-Whitney U tests for saline vs. fadrozole for each region and each sex to determine. For males, Egr-1 expression was significantly

lower in fadrozole treated animals in dNCM (U=16, p=0.029; Figure 12&13), anterior HVCshelf (U=16, p=0.029; Figure 13), and posterior HVCshelf (U=16, p=0.029; Figure 13). Ventral NCM (U=10, p=0.69; Figure 13) and CMM were unaffected (U=12, p=0.34; Figure 13). For females, Egr-1 expression was only significantly lower for fadrozole-treated animals in anterior HVC shelf (U=16, p=0.029; Figure 13) and all other regions were unaffected (dNCM U=12, p=0.34 [Figure 13], posterior HVCshelf U=10, p=0.69, vNCM U=10, p=0.69, and CMM U=11, p=0.49; Figure 13). Therefore, the data show that more regions in males depend on acute estradiol synthesis for Egr-1 auditory induction than female brain regions. This decrease in induction also is region-specific in both sexes.

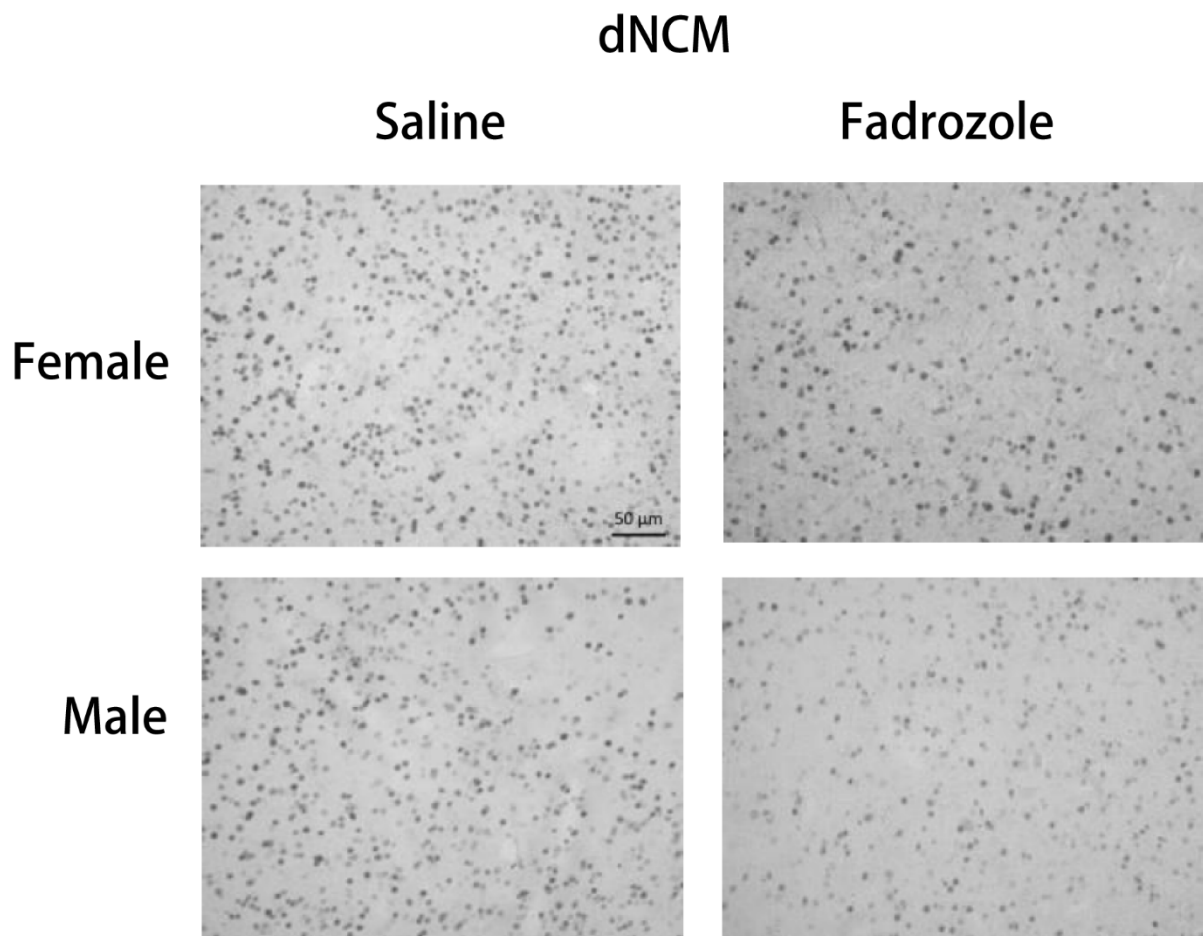


Figure 12: Song-induced Egr-1 expression decreases with fadrozole administration.

Representative images from dNCM for each sex and treatment group. Images were taken at 20x magnification. Left column is saline treated, right column is fadrozole treated, top row are females, and bottom row are males. Scale bar is 50 μ m.

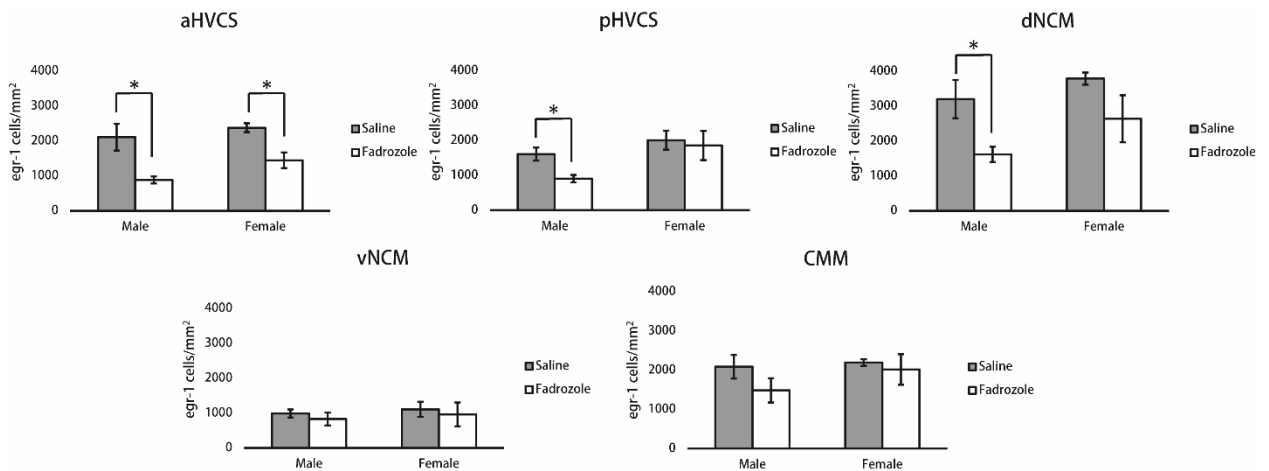


Figure 13: Summary of aromatase inhibition of song-induced Egr-1 expression. Means and standard errors (error bars) for Egr-1 expression per mm² of tissue. Gray bars are saline treated animals and white bars are fadrozole treated animals. n=4/sex*treatment. p<.05*.

I next examined expression of pCREB in serial sections from the same experiment. There was a significant difference in expression across regions (F(3,42)=7.84, p=0.00029; significant Bonferroni posthoc: dNCM vs. pHVCshelf: t(42)=5.15, p=0.0040, vNCM vs. pHVCshelf: t(42)=6.39, p=0.00028); however there was not a difference overall within sex (F(1,14)=0.25, p=0.62) or a change with fadrozole administration (F(1,14)=0.25, p=0.63). I did not observe any differences across all four brain regions I quantified for either sex (Males: aHVCshelf U=12, p=0.34, pHVCshelf U=7, p=0.89, vNCM U=7, p=0.86, dNCM U=8; Figure 14, p=1.00; Females: aHVCshelf U=7, p=0.56, pHVCshelf U=6, p=0.66, vNCM U=10.5, p=0.54, dNCM U=9, p=0.89; Figure 14 & 15 for all), indicating that phosphorylation of CREB is not regulated in the same way as Egr-1 by sex or acute estradiol synthesis. However, I did not see sufficient evidence in the literature that 1) pCREB is song inducible or 2)

that the timecourse necessary for Egr-1 induction would maintain changes to phosphorylation patterns. Therefore I tested in a new experiment whether shorter song-exposure could prompt induction of pCREB. I saw that pCREB expression did not change in either dorsal or ventral NCM for silence, 85 secs, and 15 min song exposures (Males: dNCM $F(2,4)=0.46$, $p=0.66$, vNCM $F(2,4)=0.14$, $p=0.88$; Females: dNCM $F(2,4)=0.035$, $p=0.97$, vNCM $F(2,4)=0.23$, $p=0.80$; Figure 16). Males and females were examined in two separate studies so no direct sex comparisons were possible.

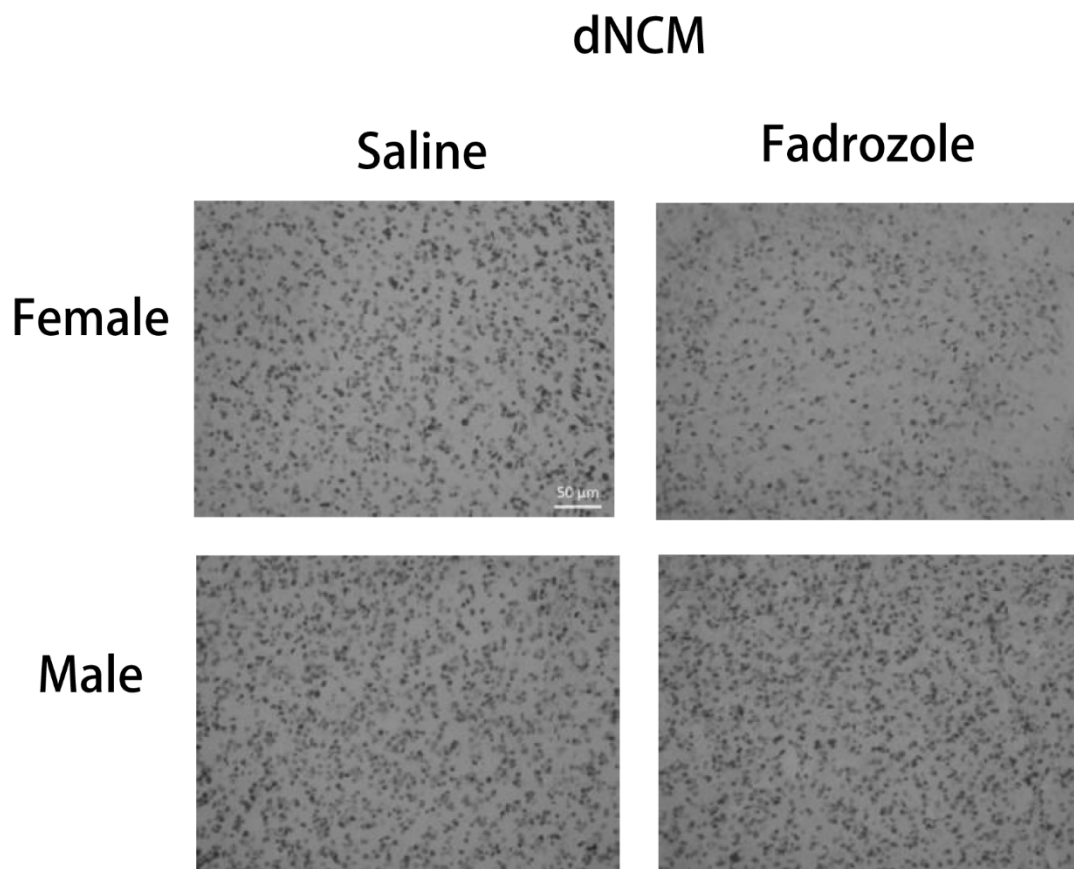


Figure 14: Expression of pCREB is unaffected by aromatase inhibition. Representative images from dNCM for each sex and treatment group. Images were taken at 20x magnification. Left column is saline treated, right column is fadrozole treated, top row are females, and bottom row are males. Scale bar is 50 μm.

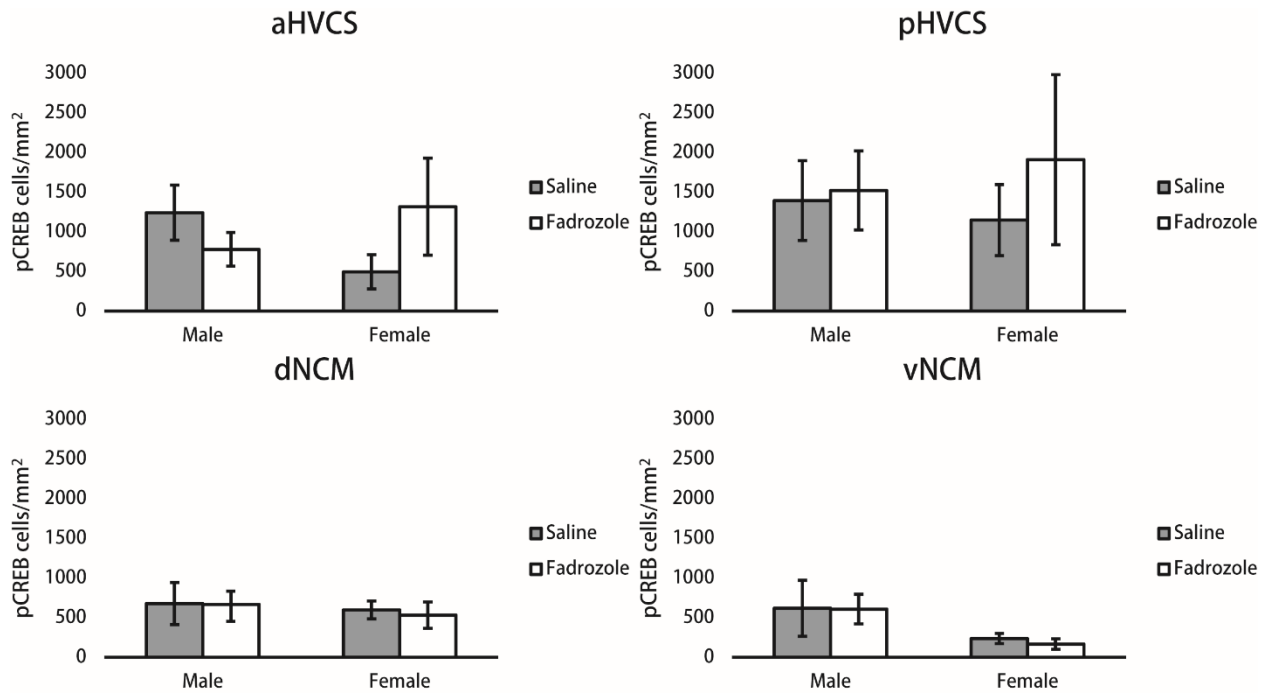


Figure 15: Summary of pCREB expression in the zebra finch forebrain. Means and standard errors (error bars) for pCREB expression per mm² of tissue. Gray bars are saline treated animals and white bars are fadrozole treated animals. N=4/sex*treatment.

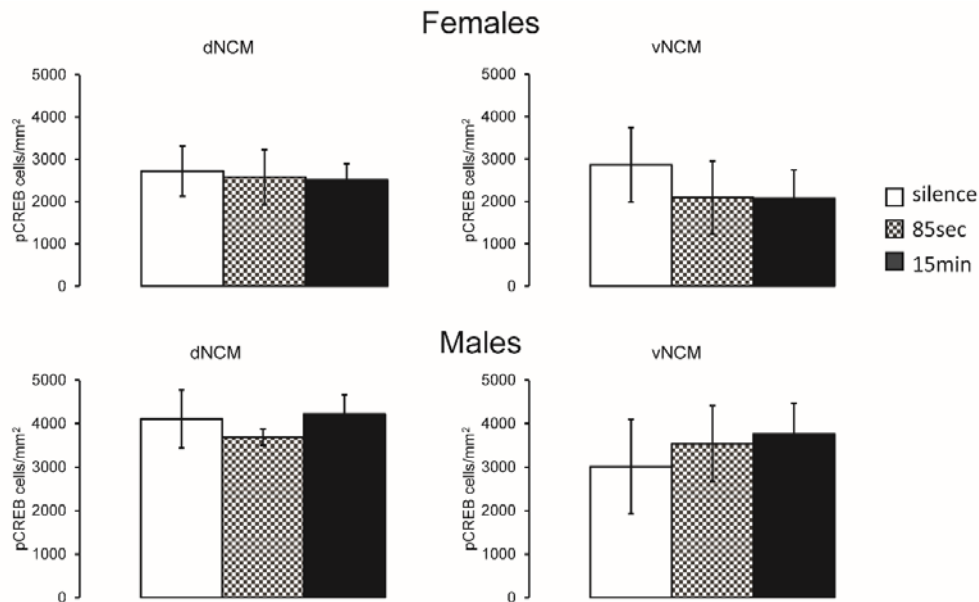


Figure 16: pCREB expression does not fluctuate with song playback. Summary of pCREB expression for males and females from Study 3. Means and standard errors (error bars) for pCREB expression per mm² of tissue for dorsal and ventral NCM. White bars are animals exposed to no song, checkered bars for 85 seconds, and black bars for 15 minutes. Females: silence (n=3), 85 sec (n=3), 15 min (n=4). Males: silence (n=3), 85 sec (n=3), 15 min (n=3).

The regulation of Egr-1 induction by fadrozole could be explained by estrogen-dependent regulation of the active synthesis of the IEG or alternatively a nonspecific ‘toxic’ protein degradation by the drug fadrozole. To distinguish between these possibilities, I administered fadrozole and saline orally immediately prior to song playback in a new set of animals. In principle, this treatment timecourse is not pharmacokinetically sufficient to suppress brain aromatase activity during the subsequent 30-min song exposure, yet song-induced Egr-1 protein during the following 30 min induction period is very likely to be exposed to the drug. I did not observe a difference between saline and fadrozole birds (dNCM: $F(1,8)=0.021$, $p=0.89$; vNCM: $F(1,8)=0.24$, $p=0.64$; Figure 17), indicating that the fadrozole is not sufficient to reverse or degrade Egr-1 induction.

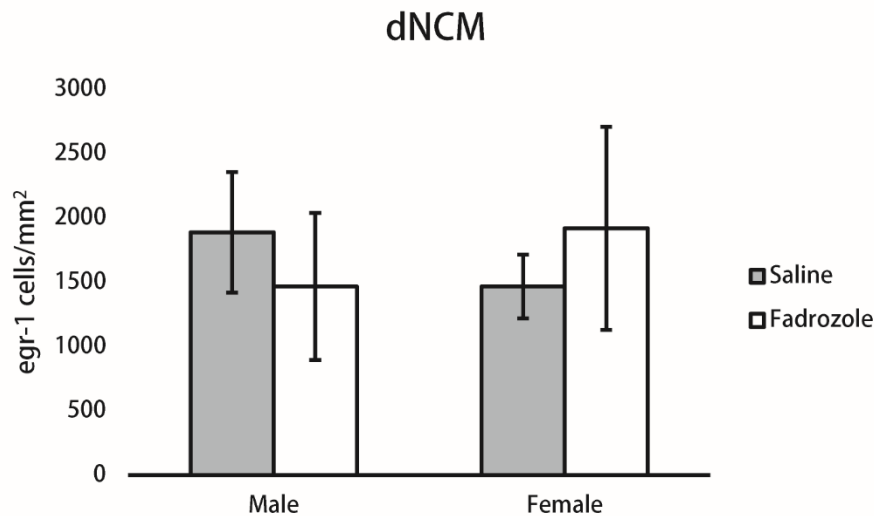


Figure 17: Aromatase inhibition after song exposure does not impact Egr-1 expression.

Summary of means and standard errors (error bars) for egr-1 expression per mm² of tissue in dorsal NCM (dNCM). Gray bars are saline treated animals and white bars are fadrozole treated animals. $N=3/\text{sex}*\text{treatment}$. $p>.05$.

Chapter 2 contains all of the subregions of the aromatase somatic aromatase expression in the the auditory telencephalon. I describe here the findings from that study that are relevant to the regions I quantified for Egr-1, to test the hypothesis that sex differences in regional aromatase expression are contributing to the differences in Egr-1 expression with fadrozole administration. Here, I did not find any sex differences in expression in any region analyzed with the exception of the pHVCshelf where females have more aromatase expression ($F(1,8)=8.33$, $p=.023$; Figure 18A). By contrast, in aHVCshelf, there was not a sex difference ($F(1,8)=1.27$, $p=.30$; Figure 18A), or in dNCM ($F(1,10)=.033$, $p=.86$; Figure 18B) or vNCM ($F(1,11)=.005$, $p=.95$; Figure 18B). CMM did not have any somatic aromatase expression. Therefore, the same region in which we observed sex-specific, rapid suppressive effects of aromatase inhibition on Egr-1 induction (pHVCshelf, Fig. 4) I also observed a sex difference in expression of aromatase protein itself.

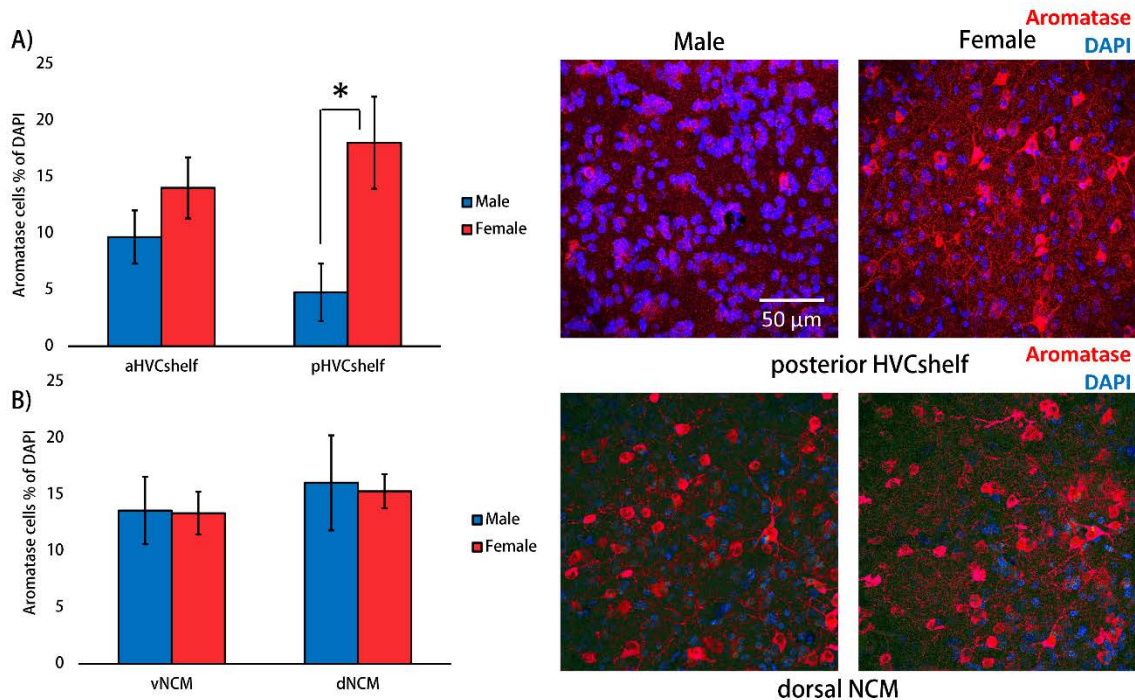


Figure 18: Females have more aromatase expression in pHVCshelf than males. A) Left: Summary of aromatase expression as a percentage of total cells counted by DAPI for aHVCshelf and pHVCshelf. Right: Representative images of cell counts near the mean for posterior HVCshelf. B) Left: Summary of aromatase expression as a percentage of total cells counted by DAPI for ventral and dorsal NCM (vNCM, dNCM). Right: Representative images of cell counts near the mean for dorsal NCM. Males are blue bars and females are red bars means and standard errors. Each image is a z-projection stack image of 15 μm thick. Images have been altered for brightness and contrast only for presentation, not analysis. Dorsal NCM images were stained with Alexa 488 and quantified as such but shifted to red for consistency in presentation only. $p < .05^*$. Scale bar is 50 μm . HVCshelf males $n=5$, females $n=3$. NCM males $n=6$, females $n=6$.

Discussion

I found that acute estradiol synthesis is necessary for complete auditory responsiveness of cells within aromatase rich auditory regions of the zebra finch forebrain. This is the first paper to show a direct link between endogenous estradiol synthesis and auditory-evoked gene expression in the brain. This is also the first study to address this question in both sexes. While prior papers have shown that exogenous

E2 application can change auditory-induced Egr-1 expression (Maney et al., 2006; Tremere et al., 2009; Sanford et al., 2010) demonstrating sufficiency, to my knowledge this is the first published study to test the necessity of endogenous estradiol synthesis on auditory-induced Egr-1 expression. This is also the first paper to establish that males have more regions of the auditory forebrain that depend on endogenous estradiol synthesis than females. Some sex differences have been described relating to brain derived estrogens and how they may impact audition (Saldanha et al., 2000; Peterson et al., 2005; Rohmann et al., 2007; Chao et al., 2015), but this is the first to report an auditory consequence of sex differences likely due to differences in brain-derived estradiol production.

I found that depending on brain region, males and females had differing degrees of suppressed auditory-induced Egr-1 expression when pre-treated with an aromatase inhibitor. In dNCM, aHVCshelf, and pHVCshelf, males have less Egr-1 expression when administered fadrozole as opposed to saline. However, this effect only exists in aHVCshelf for females, indicating that in dNCM and pHVCshelf, there is a sex difference in dependency of estrogen synthesis on auditory activation. I hypothesized that this effect was due to a difference in aromatase expression in these regions, and I confirmed this in pHVCshelf by observing a larger somatic aromatase expression in female pHVCshelf as compared to that in males. However, for dNCM, I found that males and females have similar somatic aromatase expression (Chapter 2) which replicates prior literature (Saldanha et al., 2000).

Two non-competing hypotheses may explain why males have a stronger decrease of auditory-induced Egr-1 expression when aromatase is acutely inhibited in

more brain regions. The first hypothesis is that auditory-induced Egr-1 expression is dependent on neural estradiol and differences between males and females are related to how this estradiol is synthesized and metabolized locally within brain regions. The second hypothesis is that due to ovarian estradiol in females, peripheral estradiol levels are maintaining neural responses to songs in females.

For the first hypothesis, I have several pieces of evidence that the effects I observed are due to differences in neuronal sources of estradiol. Because of the region-specific differences, I observed a decrease in Egr-1 expression in fadrozole treated groups, these effects may be due to brain-derived aromatase activity. For example, regions such as CMM that contain estrogen receptors (see Chapter 2) but no somatic aromatase (Saldanha et al., 2000, Chapter 2) did not exhibit altered Egr-1 induction in response to fadrozole administration. One surprising region that I did not see an effect was ventral NCM which is known to have robust aromatase expression (Chapter 2); however, I did not see an effect of aromatase inhibition in either sex on Egr-1 auditory evoked expression. Ventral NCM had the lowest Egr-1 expression of the regions analyzed, so it is possible I have a floor effect on its overall Egr-1 expression. Given that I observed a decrease in auditory induced Egr-1 expression in males administered fadrozole, this is consistent with the idea that males are more reliant on acute, neuronal synthesis of estradiol than females in dNCM. I found a similar effect in pHVCshelf, however, I detected an actual sex difference in somatic aromatase expression where females have more aromatase than males in the posterior shelf but not in dNCM. A sex difference has been described in terminal activity (Peterson et al., 2005; Rohmann et al., 2007) and aromatase fiber expression (Saldanha et al., 2000). This may seem

contradictory, however, there is likely a functional difference in somatic vs. terminal expression of aromatase. Somatic aromatase may be relevant for paracrine signaling of estradiol, where estradiol is being released locally within the brain but not targeted to specific synapses. Terminal expression is likely involved in neuromodulator/neurotransmitter like functions (Remage-Healey et al., 2010a). Fiber expression and terminal activity has not been described in detail in the shelf, so it is unclear if there is a similar relationship in this region that may explain this effect. More somatic aromatase may be protecting auditory responsiveness in females by an accumulation of estradiol still being present within the acute timescale.

For the second hypothesis, there is sufficient evidence that there is compensation in males and females for overall estradiol levels. Males and females do not differ in measurable peripheral levels (Adkins-Regan et al., 1990; Prior et al., 2014), despite there being a significant amount of estradiol synthesized from female ovaries (Schlinger and Arnold, 1991, 1992). There is also evidence that for both sexes there are sources of estradiol that are non-gonadal, specifically when zebra finches are gonadectomized there is an increase in peripheral estradiol (Adkins-Regan et al., 1990). A major source of this E2 is likely the brain as there is detectable and robust production of neuroestradiol than can be measured in the periphery (Schlinger and Arnold, 1992). In NCM which is dense with aromatase expression, both sexes express somatic aromatase; however, males have more aromatase expression on fibers (Saldanha et al., 2000) and activity of the enzyme in terminals (Peterson et al., 2005), indicating that rapid estrogen signaling may be more abundant in males. However, there has not been a detectable difference in estradiol content between the sexes in adulthood when sampling via

microdialysis in gonadally intact males and females (Remage-Healey et al., 2012). This suggests that despite the alternative sources from either the brain or periphery, males and females achieve similar estradiol content that is measurable in discrete regions.

In this study, there is reasonable evidence to suggest that acute oral fadrozole is not significantly decreasing peripheral sources of estradiol for either sex. I found that fadrozole did not alter peripheral estradiol levels in either sex within this 60-90 min timeframe. Prior evidence suggests that acute exposure to aromatase inhibitors is not sufficient to decrease peripheral estradiol in either zebra finch sex (Prior et al., 2014) although in sheep it decreases peripheral estradiol by 2-8 hours (Benoit et al., 1992). Likely, peripheral steroids are not rapidly changing to song exposure. Peripheral estradiol fluctuations in response to song exposure in females have been measured in the feces, and it takes several days to detect changes (Tchernichovski et al., 1998). When measuring plasma testosterone and estradiol after song playback, male zebra finches do not have changes in the periphery despite changes in steroid content in the brain (Remage-Healey et al., 2008). These results suggest that in zebra finches, in the timeframe of this study, song is not sufficient to dynamically change peripheral E2, though this would need to be more systematically explored. Given this evidence, it is possible that gonadal estradiol in females has not been yet metabolized and decreased significantly by fadrozole. This provides another mechanism by which the aromatase inhibitor suppression of auditory-induced Egr-1 is blunted in females as opposed to males.

Future work will need to determine the role that brain-derived estradiol and gonadal-derived estradiol plays in auditory physiology, particularly in females.

Experiments directly testing the necessity of ovaries in auditory responsiveness are needed to confirm or refute the sufficiency of brain-derived estradiol to maintain auditory-induced gene expression. Conversely, targeting specific brain regions, such as dNCM or pHVCshelf for aromatase inhibition can determine whether auditory responsiveness is maintained as seen in this study.

Sex differences in estrogen dependent Egr-1 and aromatase expression in the shelf may indicate that the shelf is a sexually dimorphic nucleus. There is little known about the shelf of HVC other than it is auditory and send projections into HVC and RA cup (Vates et al., 1996), which are sexually dimorphic motor nuclei that is critical for production of birdsong in males. Since the sex difference is dependent on the anterior-posterior axis of the shelf, where anterior shelf has a similar response between the sexes and the posterior shelf is differential, this may indicate a true sex difference in shelf neurochemical functioning or a sex difference in size and shape which affects what region was sampled. HVC is smaller in females than in males (Nottebohm and Arnold, 1976; Hamaide et al., 2017), and shelf is defined by the parameters of this nucleus. Although I was careful in the identification of shelf, there is a lack of thorough anatomical description of this region, particularly in females. This difference might represent portions of the dorsal caudolateral nidopallium (NCL).

I also reported that pCREB is not song-inducible, nor is it regulated in parallel with Egr-1 expression changes in either sex. I proposed that pCREB was a likely candidate for a transcription factor that targets Egr-1 and is sensitive to E2 signaling of the MEK-ERK pathway. However, unlike the phosphorylation of ERK (Cheng and Clayton, 2004), I did not see changes in pCREB expression after short song exposure in

dorsal or ventral NCM. Phosphorylation of CREB also has sex specific induction of estradiol in the mammalian hippocampus, which made it another enticing candidate for testing sex differences of downstream targets of E2. However, I did not observe any sex or treatment specific effects in the study. This indicates that pCREB is likely not regulated in the same pathway as pERK and Egr-1 in audition, though there are still open questions about its sensitivity to acute estradiol application. There are other transcription factors that can target the Egr-1 promotor such as Elk-1 which also is a target of the MEK-ERK pathway (Chen et al., 2004; Knapska and Kaczmarek, 2004) .

Although I did not detect changes in pCREB due to auditory or endogenous estradiol manipulations, this does not rule out that sex differences in Egr-1 expression may be due to sex differences in cell signaling mechanisms. There is a lack of literature on receptor types that mediate estrogen signaling in the zebra finch forebrain and whether these are similar between the sexes. Phosphorylation of ERK and its role in audition has exclusively been characterized in male but not female zebra finches (Cheng and Clayton, 2004), as well as the rapid effect of estradiol on pCREB, pTH, and pERK in male song sparrows (Heimovics et al., 2012). Although pCREB seemed a potential candidate due to the mammalian literature (Abraham and Herbison, 2005), this study indicates that the role of pCREB in sexually different signaling is highly unlikely, however this does not mean males and females are utilizing the same intracellular mechanisms. Many ubiquitous kinase cascades such as MEK-ERK are sensitive to perturbations in a sex specific manner (Gresack et al., 2009; Sharma et al., 2009; Armstead et al., 2011). Future studies examining downstream pathways of E2

regulation of audition will need to seriously consider sex as a biological variable to determine how males and females are utilizing E2.

Although Egr-1 expression is often used as a neuronal activation marker for auditory regions in the songbird, Egr-1 is a transcription factor that has been implicated in learning and memory (Davis et al., 2003; Knapska and Kaczmarek, 2004; Veyrac et al., 2013). Egr-1 targets promotor regions of genes involved in synapse formation and maintenance (Knapska and Kaczmarek, 2004; Moorman et al., 2011). Egr-1 knockout mice have memory deficits (Han et al., 2014). Within songbirds, Egr-1 has been described as a marker for the “genomic action potential” (Clayton, 2000), which is that process that neurons use to encode salient stimuli and translate the signal from an action potential to changes that occur in gene expression having long term consequences on protein expression and neuronal architecture. Secondary auditory regions such as NCM have been implicated as a region that stores “tutor memory” or a template for male song (Bolhuis and Gahr, 2006; Bolhuis and Moorman, 2015; Yanagihara and Yazaki-Sugiyama, 2016). Egr-1 may be a mechanism by which these memories are stored. If this is the case, the sensitivity of Egr-1 to neural estradiol suggests that estradiol may be an important signal in auditory memories and song development.

This study demonstrates that acute estradiol synthesis is necessary for auditory responsiveness in multiple cortical regions of the zebra finch. This is particularly evident in males, supporting the hypothesis that males are more reliant on active estrogen synthesis than females for auditory responsiveness. Future work is necessary to unpack the regional specific regulation of aromatase activity and E2 sensitive signaling

pathways within auditory regions of the forebrains comparing the sexes to further understand the role estradiol has as a neuromodulator in auditory processing.

CHAPTER IV
**A MEMBRANE G-PROTEIN COUPLED ESTROGEN RECEPTOR IS
NECESSARY BUT NOT SUFFICIENT FOR SEX-DIFFERENCES IN
SONGBIRD AUDITORY CODING**

Abstract

Estradiol can act as a neuromodulator in brain regions important for cognition and sensory processing. Estradiol can also shape sex differences, but rarely have these two concepts been considered simultaneously. In both male and female songbirds, estradiol rapidly increases within the auditory forebrain during song exposure and enhances local auditory processing. I tested whether GPER1, a membrane bound estrogen receptor, is necessary and sufficient for neuroestrogen regulation of forebrain auditory processing in both male and female zebra finches (*Taeniopygia guttata*). At baseline, I observed a robust sex difference in single-neuron responses to songs. Specifically, in males only, narrow-spiking neurons (NS) carried more auditory information than broad-spiking neurons (BS). Following acute inactivation of GPER1, auditory responsiveness and coding were suppressed in male NS, yet unchanged in female NS, and also unchanged in BS of both sexes. By contrast, GPER1 activation did not mimic previously-established actions of estradiol in either sex. Lastly, the expression of GPER1 and its coexpression with the inhibitory neuron marker GAD67 were similarly abundant in males and females. To my knowledge, this is the first description in any organism of: 1) a role for GPER1 in regulating sensory processing, and 2) a sex difference in auditory processing of complex vocalizations in a cell-type specific manner. These results reveal sex-specificity in rapid estrogen signaling,

consistent with neuromodulation that accounts for and/or compensates for brain sex differences, in a cell-type specific manner, in brain regions that are anatomically similar in males and females.

Significance statement

In this study, I report a cell-type specific mechanism of auditory processing that differs between the sexes, and that is also mediated by a membrane estrogen receptor in males. Sex differences in the physiological parameters of neurons in regions that are anatomically similar could have important implications for understanding neural networks and behavior, and this work in particular indicates sex differences *in vivo* in the modulatory actions of neuroestrogens. Broadly, my study exemplifies that sensory regions can be physiologically differentiated for sex-specific encoding of complex stimuli, including learned vocalizations.

Introduction

Sex differences in neuroanatomy occur throughout the brain in vertebrates. For regions that do not differ structurally between males and females, anatomical similarity could belie underlying sex differences in physiology and modulation. For example, song production is male-specific in many songbirds and associated with robust sex differences in the song motor pathway (Nottebohm and Arnold, 1976; Hamaide et al., 2017), yet the songbird auditory network is considered largely similar in males and females (Krentzel and Ramage-Healey, 2015; Brenowitz and Ramage-Healey, 2016). Specifically, the songbird caudomedial nidopallium (NCM; analogous to secondary auditory cortex) is not sexually dimorphic, yet some NCM firing states appear divergent between males and females (Dagostin et al., 2012; Yoder et al., 2015). It is currently

unclear whether and how these differences manifest in the auditory response properties of single NCM neurons. In both sexes, NCM is selective for complex song stimuli (Terpstra et al., 2006; Tomaszycski et al., 2006; Ramage-Healey et al., 2010; Ikeda et al., 2015), and NCM is hypothesized to be a locus for auditory memory and discrimination (Bolhuis and Gahr, 2006; Bell et al., 2015; Bolhuis and Moorman, 2015; Yanagihara and Yazaki-Sugiyama, 2016). More broadly, examining sex differences in songbird auditory physiology can provide insights into sex differences in hearing, language development, and neuroendocrine mechanisms in other species, including humans (Charitidi et al., 2009; Wermke et al., 2014; Quast et al., 2016).

Hormones and genes organize brains into “male-like” and “female-like” states (Wade and Arnold, 2004; McCarthy, 2010; Maekawa et al., 2014). Sexually dimorphic neural circuits can support differentiated motor behaviors and traits (Breedlove and Arnold, 1983), but they can also compensate to maintain similarity between sexes when hormonal, genetic, and/or morphologic factors differ (De Vries, 2004). A key hormonal mechanism associated with sex differences in vertebrates is the neural production of estradiol, which can shape long-term gene expression in the brain in addition to acute effects (Krentzel and Ramage-Healey, 2015; Micevych et al., 2015).

In humans and songbirds estrogens are locally produced within the auditory cortex, including at synaptic terminals (Naftolin et al., 1996; Saldanha et al., 2000; Yague et al., 2006). The abundance of aromatase-positive neurons in NCM is similar in male and female zebra finches; notably males have more elevated aromatase expression and enzymatic activity within presynaptic terminals (Saldanha et al., 2000; Peterson et al., 2005). Still, in both sexes, local NCM estradiol is acutely elevated when hearing

conspecific song (Ramage-Healey et al., 2008; 2012), and exogenous estradiol increases auditory responsiveness (Ramage-Healey et al., 2010; 2012). Thus, local increases in brain estradiol can rapidly modulate audition – similarly – in both sexes.

Despite similarities in neuroestrogen production, the receptor mechanism that mediates rapid neuroestrogen signaling is unknown, and may be sex-specific. Classical nuclear receptor agonists do not mimic rapid estradiol actions in NCM (Ramage-Healey et al., 2013). Alternatively, the membrane-bound g-protein coupled estrogen receptor 1 (GPER1) can mediate rapid estrogen signaling in other models (Srivastava and Evans, 2013) and is expressed in mammalian, avian, and teleost brain (Acharya and Veney, 2012; Almey et al., 2016; Crimins et al., 2016; Mangiamele et al., 2017). In mammals, rapid estrogen signaling is mediated by GPER1 in both the hippocampus (Briz et al., 2015; Kumar et al., 2015; Waters et al., 2015; Kim et al., 2016), and striatum (Almey et al., 2016), and the acute actions of estrogens on hippocampal synaptic transmission are sex-specific, and mediated in part by GPER1 (Oberlander and Woolley, 2016).

For these reasons, I tested two primary hypotheses, that: (1) the response properties of NCM differ between males and females, and (2) auditory processing and coding are regulated by GPER1 at the level of single neurons in NCM. I report sex differences in auditory processing and information coding at the level of single neurons that are cell-type specific. I further show that GPER1 is necessary to maintain this sex difference, but that activation of GPER1 alone does not mimic the actions of estradiol.

Methods

Animals

Adult (>120 dph) male and female zebra finches were housed in single sex cages in flight aviaries with food and water available ad libidum (14 day/10 night). All animals were gonadally intact. Protocols for animal care and use were approved by the Institutional Animal Care and Use Committee at the University of Massachusetts. Males (n=27) and females (n=27) were collected across four electrophysiological studies that had the same within subject design (aCSF, drug, aCSF). To examine potential sex differences in firing at pre-drug conditions, results from the first aCSF trial were pooled across all four studies (N = 27 males and 27 females each). The three drug treatment studies with antagonist G36 (100 μ M; males n=5, females n=5) and agonist G1 (low dose 100 nM: males n=10, females n=8; high dose 100 μ M: males n=5, females n=6) include all data from a pre, drug, and post drug trials. These doses were selected based on the specificity of binding to the GPER1 receptor (Dennis et al., 2011). Additional animals (males n=7, females n=6) were added from trial 1 aCSF only recordings for a larger comparison across studies. A separate set of males (n=7) and females (n=6) were collected from the same aviaries for the immunofluorescence study.

Surgery

I used protocols adapted from previously published methods (Remage-Healey et al., 2010; 2012; Remage-Healey and Joshi, 2012; Ikeda et al., 2015; Pawlisch and Remage-Healey, 2015). Animals underwent stereotaxic surgery to affix head posts and draw markings on the skull for NCM coordinates. Animals were removed from the larger aviary just before surgery, and were isolated from food for ~20 minutes to

prevent aspiration during anesthesia. Based on weight, 35-45 μ L of equithesin was injected into the pectoralis muscle. Approximately twenty minutes following injection, birds were affixed to a stereotax at a 50° head angle.

A local lidocaine injection (10-20 μ L, 2% in ethanol; Sigma-Aldrich) was subcutaneously administered under the scalp and the skull was exposed. The bifurcation of the midsagittal sinus ('zero point') was identified by cutting a small window into the upper leaflet of skull. From here, hatched marks were made in the upper leaflet of skull over the NCM coordinates (rostral 0.8mm; lateral 0.8mm) from the zero point on both left and right hemispheres. A small hole was also made in the upper skull leaflet above the cerebellum and a silver wire was inserted for grounding during physiology recordings and secured using cyanoacrylate. The scalp was resealed using cyanoacrylate and dental cement secured the head post. Birds recovered from anesthesia within 4 hours and were placed in an isolation chamber housed with at least one other bird for 1-2 days until recording.

Electrophysiology & Retrodialysis

I performed anaesthetized *in vivo*, single electrode electrophysiology on the subjects with a retrodialysis probe implanted in NCM. Previous studies testing neuromodulation of auditory neurons in songbirds have used this method in order to achieve long-term (2-3 hrs), stable recording sites alongside acute vehicle, drug, and washout administration with retrodialysis (Ramage-Healey et al., 2010; 2012; Ikeda et al., 2015; Pawlisch and Ramage-Healey, 2015). Prior to recordings each subject was administered 20% urethane over the course of 2-2.5 hours in at least three doses at 30 μ L each. Total volume of anesthesia ranged from 90-120 μ L depending on the size of the

bird. Birds were then secured by the head post to a custom stereotax (Herb Adams Engineering) at a fixed 50° angle. Each subject was kept on a heating pad to maintain body temperature (DC Neurocraft). All experiments took place inside a sound attenuation booth (Industrial Acoustics) on an air table (TMC).

The lower skull leaflet and dura were dissected away from the marked exposure. The left NCM was always exposed first and most subjects had recordings from the left hemisphere (50/54 recordings). If recordings were unsuccessful in the left hemisphere, then the right hemisphere was exposed (4/54 recordings). Right hemisphere recordings were not systematically different from left hemisphere recordings for auditory responsiveness and were therefore pooled in the analyses. A pre-filled microdialysis probe (CMA 7 with 1 mm membrane; Microdialysis Probe, CMA Microdialysis) was inserted caudal to the hatched markings and descended ~1.5mm ventral into NCM. The flow rate of aCSF was 2 μ L/min. After 30 minutes of delay from implantation, a single carbon fiber electrode (Carbostar-1, Kation Scientific) was descended immediately caudal (~200-500 μ m) to the probe to search for auditory sites. Sites were selected between ~0.8-1.5mm ventral from the surface. An auditory site was selected by online confirmation that peri-stimulus time histograms (PSTHs) displayed a positive auditory response to auditory playback stimuli. All recordings were amplified, bandpass filtered (300 –5000 Hz; A-M Systems), and digitized at 20 kHz (Micro 1401, Spike 2 software; Cambridge Electronic Design). Prior to recordings, song files for auditory playback were normalized to ~70dB.

Once the site was selected, trial 1 of the experiments began. Trial 1 consisted of 30 minutes of retrodialysis of aCSF followed by 15 minutes of continuous retrodialysis of aCSF with concurrent playback of song stimuli. Stimuli were three separate male conspecific songs selected from recordings of birds from the colony at least 3 years prior (i.e., outside of age ranges for the subjects), and a white noise sound file, totaling four sound files. Each sound file was played back for 20 repetitions pseudorandomly at an inter-stimulus interval of 10 ± 2 sec, totaling ~15 minutes of stimuli exposure. After the end of Trial 1, the aCSF was switched for the drug trial (100 μ M of G36 (Azano Scientific); 100 μ M or 100 nM of G1 (Azano Scientific; Stock made in DMSO then diluted to concentrations in aCSF for a final DMSO concentration of maximum 0.5%). Trial 2 followed the same time course and playback. Trial 3 consisted of a washout with aCSF for the same time course. Following Trial 3 washout, recording sites were lesioned (10 μ A for 10 s) for confirmation of electrode placement.

Histology

Birds were decapitated immediately following the lesion. Brains were extracted and stored in 20% sucrose/10% formalin solution in 4 °C. After fixation, brains were frozen in OCT and stored at -80 °C until sectioning. I sectioned at 45 μ m using a cryostat (Leica CM 3050S) and mounted onto Fisher superfrost slides. I performed a nissl stain using 0.25% thionin and slides were then dehydrated through a series of ethanol washes followed by Heme D and coverslipped using permount (Fisher Scientific). Sections were visualized under a bright field microscope (Zeiss Axio Lab.A1) to confirm probe site implantation into NCM. Lesion sites were more difficult to confirm in all cases due to the lack of gliosis. However, all recording sites were

confirmed on-line to exhibit highly phasic, bursting response properties that are characteristic of NCM neurons, and all probes were histologically confirmed to be within NCM.

Auditory responsiveness

Recordings were processed in Spike2 (version 7.04) software. First pass analysis consisted of measuring auditory responsiveness in multiunit recordings. Thresholds were set at least 2-fold above the noise band for rastering multiunit activity. Single units were isolated by sorting spikes based on waveform shape in Spike2 with the same thresholding (n=116). A PCA analysis confirmed isolated units and I obtained 2-3 units from each subject (Fig. 19). All single units included in the analysis had an interspike interval >1ms. Single units were not selected based on amplitude or whether they were consistently responsive to auditory stimuli. Rather, I performed an unbiased categorization solely on the PCA to isolate the most distinct waveforms (Fig. 19A, right). I ran paired t-tests for each unit between spontaneous and stimulus-evoked firing frequencies across all stimulus presentations to determine whether units were statistically responsive ($p < 0.05$) to auditory stimuli for any of the treatment trials. One unit in the G1 low dose study did not meet this criterion and was removed from the analyses.

For rasterized multiunit and single unit spike trains, several parameters were measured. Peristimulus histograms were aligned with each sound stimulus, measuring 2 seconds preceding the stimulus for spontaneous activity and the 2 seconds after stimulus onset for stimulus-evoked activity. Firing frequency (Hz) was measured for both

multiunit and single unit firing during spontaneous and stimulus-evoked periods.

Auditory responsiveness is represented by the zscore:

$$zscore = \frac{Mean(S) - Mean(B)}{\sqrt{Var(S) + Var(B) - 2(Cov(S, B))}}$$

where Mean(S) is the mean number of spikes during the stimulus presentation, Mean(B) is the mean number of spikes during spontaneous firing period, Var(S) is the variance of the stimulus-evoked activity period, Var(B) is the variance of the spontaneous activity and Cov(S,B) is their covariance. I was principally interested in neuromodulation of song-evoked responses rather than differential representation of individual song subtypes. The descriptive means did not demonstrate systematic differences among the three conspecific song types, so I averaged firing frequency and zscores per unit across the three conspecific songs presented for each unit. White noise response had a lower firing frequency and zscore so it was not included in the average.

Response latency for each unit was computed as described by Ono et al. (2016). Briefly, for each stimulus (20 trials each) 5 ms-binned peristimulus time histograms were generated and smoothed with a 5-point boxcar filter. The mean and standard deviation of the spontaneous firing rate of 100 ms preceding the stimulus onset were computed. The latency to fire was defined as the midpoint of the first bin from 0 to 400 ms after stimulus onset when the firing rate exceeded the mean + 3 times the standard deviation of the spontaneous period. If the response did not exceed the threshold within the first 400 ms of stimulus presentation, latency was not calculated for that stimulus; this contingency only occurred in 3 units. For conspecific songs, latency was measured relative to the onset of the first non-introductory note. For each cell, the latencies to fire for each stimulus were averaged (Fig. 19C left).

Auditory coding

I used a custom pattern classifier coded in Python based on a similar classifier originally described by Caras et al. (2015) to determine how auditory-evoked events predict stimuli discrimination in the population of neurons. For each single unit, the classifier pseudorandomly selected one response to each stimulus to serve as templates. The remaining recordings (19 trials per stimulus = 76) were compared to the 4 templates and categorized based on two types of similarity measures: count and timing. This procedure was repeated 1000 times and a mean accuracy score was generated for each stimulus. Spike count accuracy was calculated by using as the number of spikes within the 2 seconds of song presentation. The stimulus type of the template that yielded the most similar number of spikes to the trial in each comparison was considered the predicted stimulus. Ties were resolved pseudorandomly. Spike timing accuracy was determined in a similar manner, but the binary signals were convolved with Gaussian filters before comparison. I systematically varied the standard deviation (σ) of the filter for each cell to determine the optimal integration window, (values used: 1, 2, 4, 8, 16, 32, 64, 128 and 256 ms). The filter that yielded the highest accuracy was selected for each cell (Figure 19A; all cells had a sigma of 16 ms, with the exception of 4 cells that had a sigma of either 1 or 2 ms). Templates and trials were compared using the R_{corr} method (Schreiber et al. 2003, Caras et al. 2015):

$$R_{corr} = \frac{\vec{s}_{trial} \cdot \vec{s}_{template}}{|\vec{s}_{trial}| \times |\vec{s}_{template}|}$$

Where \vec{s} represents the vectors of the trial and the template responses after filtering, which are dot-multiplied then divided by the product of their lengths. This calculation returns a value between 0 and 1, which represent a range from total dissimilarity to total similarity, respectively. The stimulus type of the template that provided the highest $R_{\text{corr}}(\text{trial}, \text{template})$ value was considered the predicted stimulus for the trial in analysis. A confusion matrix was generated for each unit that illustrates the percentage of accuracy for each predicted and observed song type. All timing and count accuracy data presented here are an average of the correctly assigned percentages across all sound types (CON1, CON2, CON3, WN) to represent an overall accuracy score. Excluding responses to WN from the overall accuracy score did not result in changes to the effects reported below, so all classifier data presented below include responses to WN. After generating the confusion matrices, the accuracies of each unit were statistically tested by employing a trial shuffling approach (modified from Caras et al. 2015). Briefly, responses were shuffled and randomly assigned to stimuli. The classifier ran 1000 times using the random responses as input (timing classifiers were run 1000 times for each σ value mentioned above). Finally, the distribution of original accuracies was compared with the distribution of the random accuracies. Because samples sizes were large (1000 x 1000), I opted to use the Cohen's d as an indicator of effect size, $d = \frac{\bar{X}_{\text{original}} - \bar{X}_{\text{shuffled}}}{\sigma_{\text{pooled}}}$, where \bar{X} denotes the means of the distributions and σ_{pooled} is the pooled standard deviation. Accuracies were considered significantly greater than random when Cohen's d was greater than 0.2, which is indicative of a small significant positive effect size (Cohen, 1988). Similar to Caras et al. (2015) single units were categorized as either timing, count, bicoding or neither, based on the effect size

threshold (i.e., if a unit was significant for both timing and count it was categorized as a ‘bicoding’ cell, if a unit was significant for neither timing nor count it was categorized as a ‘neither’ cell).

Spike width

I measured spike quarter width duration for each single unit (averaged across all firing incidents of the first trial) which is the duration of the waveform at 25% of the highest absolute peak value. Quarter width duration of the first largest peak (SQW; both positive and negative deflection) was the most reliable measurement for the diversity of waveform shapes (Figure 19A), and has been validated in zebra finch HVC to assign ‘broad’ units as projection neurons and ‘narrow’ units as interneurons (Rauske et al., 2003; Mooney and Prather, 2005; Day et al., 2013). The resultant distribution of SQW was bimodal with a dip at approximately 0.50 ms (Figure 19B). However, recent work, especially in NCM, has also used spike peak to peak duration to classify cell types, so I therefore performed peak-to-peak measurements as well. Although a consensus about NCM categorizations is still emerging, two characteristics have been generally consistent with broad vs. narrow categories in recent studies: broad cells consistently have a lower firing frequency and a higher latency to fire after stimulus onset (Schneider and Woolley, 2013; Ono et al., 2016; Mouterde et al., 2017). When I examined the cells using the peak to peak classification with a cutoff between broad and narrow at 0.4ms duration, I observed that “broad” peak-to-peak units could be classified either with a canonical action potential shape (two clear high and low peaks) or with a unipolar peak. Unipolar “broad” cells had longer durations than previous cutoffs (Ono et al., 2016), and the peak-to-peak classification scheme did not concomitantly

segregate based on latency or firing rates. By contrast, dividing the population of recorded cells using SQW with a 0.5 ms cut off clearly also distinguished waveform latency ($F(1,109)=6.59$, $p=0.012$, Figure 19C left) and spontaneous firing frequency ($F(1,112)=5.95$, $p=0.016$; data not shown) where broad neurons had a higher latency to fire and higher spontaneous firing frequency. Stimulus-evoked firing frequency was not different between cell types ($F(1,112)=0.079$, $p=0.78$, Figure 19C right). Because of the consistency of categorizing based on shape and the consistent physiological parameters of latency and firing frequency between both types of classification (peak to peak vs. quarter width), I proceeded with SQW for broad vs. narrow unit classification. Prior work has presented these classifications as putative projection neurons (broad) and putative interneurons (narrow). The latency data support this hypothesis, but the firing frequency results do not. Moreover, the direction of the spontaneous firing difference is not consistent with previous observations (broad > narrow) and stimulus-evoked firing was similar between broad and narrow neurons. I therefore restrict the interpretation of broad vs. narrow units as distinct neuronal subtypes but do not make inferences about their putative identities as excitatory vs. inhibitory neurons.

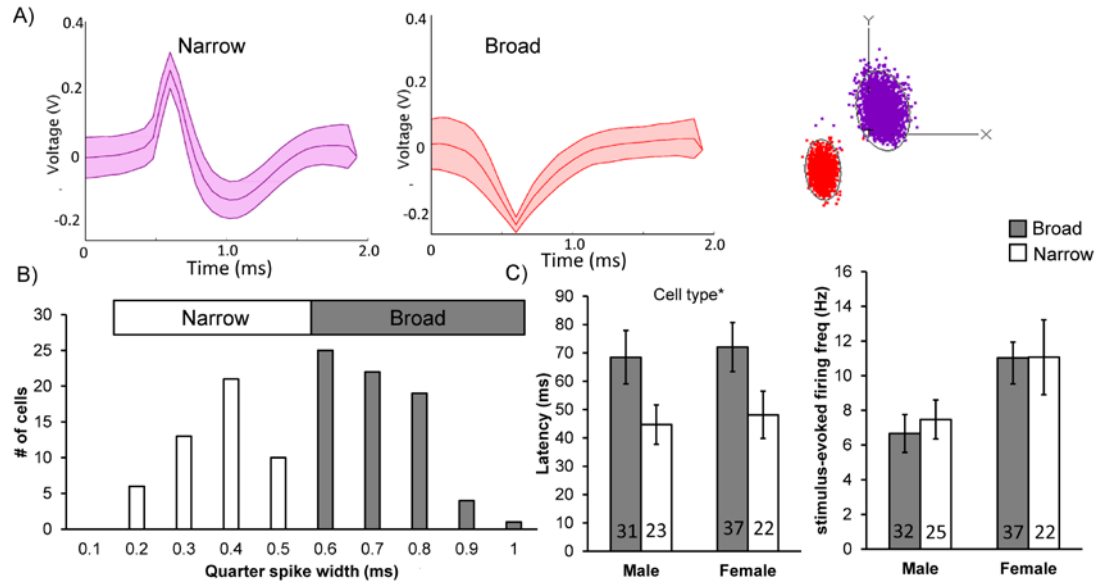


Figure 19: Characteristics of broad and narrow cells.

A) Left. Waveform averages for two example units from the same recording site. The purple unit is a narrow cell and the red unit is a broad cell. The center line indicates the mean waveform, while the shaded area represents the standard deviation for all incidences of that shape in the recording. Right. An example of a PCA for the red and purple cells of that recording. B) Histogram of quarter spike width durations (ms). Dip in histogram at 0.5 ms was used as indicator of cutoff between narrow and broad unit classification. C) Latency (ms) to fire after first syllable (left) and stimulus-evoked firing frequency are depicted as means and SEM. Gray bars are for broad units and white are narrow units. Samples sizes for each group are depicted for each bar. $p < 0.05^*$

Immunofluorescence

I isolated males (n=7) and females (n=7) from single sexed aviaries for ~12-24 hours before sacrifice. I exposed birds to song before sacrifice for another study not relevant to these analyses. I perfused animals with ice-cold saline, then 4% paraformaldehyde, followed by an overnight fix in PFA, and sinking in 30% sucrose in 0.1M phosphate buffer. I sectioned the brains at 35 μ m into cyroprotectant solution. I was interested in double-labeling for GPER1 and markers for inhibitory neurons. Pilot studies indicated there was no co-expression of GPER1 in any brain regions with specific interneuron markers (parvalbumin [PV, 1:10,000, Millipore, AB_2174013] and calbindin [CB; 1:2000, Sigma, AB_476894]; data not shown). We therefore used a polyclonal antibody against GAD67, the enzyme that synthesizes GABA and which is expressed in cell bodies of rat and humans (Erlander et al., 1991; Schwab et al., 2013) and the two analogs GAD65 and GAD67 are found across vertebrates (Bosma et al., 1999). I determined from the manufacturer and control experiments that this antibody only marks a subpopulation of GABAergic cells (i.e., coexpressed with CB neurons in the cerebellum but not the telencephalon), so this marker is not representative of all GABAergic cells, but rather a subtype. Sections were transferred from cyroprotectant and went through a series of 0.1M phosphate buffer washes. Sections were blocked with 10% normal goat serum for 2 hours and then incubated in anti-GPER1 raised in rabbit (MBL International, AB_591551) at 1:2000 dilution which has previously been validated in zebra finches (Acharya and Veney, 2012) and anti-GAD67 raised in chicken (Abcam, RRID: AB_1310248) at 1:100 for 48 hours at 4 °C. Sections were then washed in a 0.1% triton-x phosphate buffer (PBT), followed by an incubation in 1:200

goat anti-rabbit Alexa 488 secondary (Life Technologies, RRID: AB_2576217) and 1:200 goat anti-chicken Alexa 594 (Life Technologies, AB_142803). After a final wash in 0.1% PBT, sections were mounted on slides with Prolong Diamond with DAPI mounting media (Invitrogen). Images were taken of NCM using a Nikon A1SP confocal microscope at 10x and 60x. Figure 21 depicts 15 μm zstacks as a projection image at 60x to illustrate antibody staining of GPER1 and relative expression level within the regions that were recorded from using electrophysiology. All GPER1+ and GAD67+ cell counts in 60x z-stack images of the dorsal and ventral NCM were scored by an experimenter blinded to treatment conditions, and all quantified counts are normalized to DAPI.

Data Analysis

All statistical analyses were performed using IBM SPSS Statistics 22 and Origin 2017. For sex comparisons across all studies during trial 1 aCSF, between-subject ANOVAs were performed and sex-by-cell type comparisons and student's t test for sex comparisons of multiunit dependent measures. For neuromodulation studies (G36 and G1), a three-way mixed factors ANOVA was performed initially for each study. I had power to detect this three-way interaction for auditory responsiveness in the G36 study, so I performed two-way ANOVAS for trial by cell-type fixing the factor sex for all analyses to determine cell-type specific effects within each sex. I was underpowered in other incidences to detect the three-way interaction; however, for other dependent measures in the G36 study (timing accuracy, count accuracy, firing rate) I performed the same two-way ANOVAS and detected significant two-way interactions. In the two-way ANOVAS, when a significant interaction for cell-type and interaction was

detected, I performed one-way ANOVAS fixing the factor cell type to test whether each cell type was significantly altered by drug administration. If a significant treatment effect was detected in the one-way analysis, I then performed Tukey's HSD post-hoc analyses on the within-subject groups (pre vs. drug). For categorization of cells as bicoding, timing, count or neither, a chi-squared analysis was performed on all cells based on sex or spike width categorization. Anatomical comparisons were performed by mixed-effects two-way ANOVAS. Significance is reported as $p < 0.05$.

Results

NCM has both sex and cell type differences in firing frequency and auditory responses

We first examined firing parameters in NCM for potential sex differences under aCSF (i.e., non-drug) conditions. In the analysis of multiunit data, I found males and females had similar spontaneous firing rates (male 11.49 ± 1.44 and female 9.37 ± 1.21 ; $t(52) = 1.13$, $p = 0.27$), stimulus evoked firing rates (male 26.05 ± 3.03 and female 21.33 ± 2.4 ; $t(52) = 1.22$, $p = 0.23$), auditory responsiveness (zscore; male 0.40 ± 0.043 and female 0.46 ± 0.053 ; $t(52) = -0.68$; $p = 0.50$), timing accuracy (male 0.75 ± 0.033 and female 0.66 ± 0.03 ; $t(51) = 1.83$, $p = .073$), and count accuracy (male 0.44 ± 0.022 and female 0.41 ± 0.023 ; $t(51) = 0.74$, $p = 0.46$; data not shown). In contrast, when I examined data from the population of isolated single NCM units, sex differences emerged (Figures, 19C and Figure 20).

For single units in NCM, neurons in females had an overall higher firing frequency than did males ($F(1,112) = 5.88$, $p = 0.017$; Figure 20A). For stimulus-evoked firing NCM neurons in females also had a higher firing frequency ($F(1,112) = 6.6$;

$p=0.01$; Figure 20A) and a trend for spontaneous firing ($F(1,112)=3.34$; $p=0.07$; Figure 20A). Despite differences in firing frequency, both sexes were similar in overall normalized auditory responsiveness (zscore; $F(1,112)=1.86$; $p=0.18$; data not shown). I next evaluated the cell-type dependent auditory response properties in males and females. NS cells had a higher auditory responsiveness than BS ($F(1,112)=7.16$, $p=0.009$, data not shown), and there was a trending interaction between sex and cell type for zscore ($F(1,112)=2.84$; $p=0.09$) although with low statistical power (0.39). Therefore, I evaluated the cell-type dependent auditory response properties in individual tests fixing the factor sex to determine if the cell type main effect was driven by one sex. This analysis revealed that NS cells in males were more responsive than BS cells ($t(55)=-3.58$; $p=0.001$) but also that NS and BS cells in females were similarly responsive ($t(57)=-0.63$; $p=0.53$; Figure 20B). Therefore, NCM neurons differed between males and females in terms of firing rates as well as the auditory response properties of NS vs. BS neurons.

Males and females did not differ in how accurately NCM neurons represent individual stimuli via rate-based coding (count) ($F(1,112)=0.18$; $p=0.67$) and there was trend for timing coding (timing) $F(1,112)=3.47$, $p=0.065$) with females having slightly higher accuracy scores (i.e., compare females to males in Fig. 20D). For both count and timing accuracy, narrow cells were more accurate than broad cells in both sexes (count: $F(1,112)=22.66$, $p<0.001$; timing: $F(1,112)=38.25$; $p<0.001$; Figure 20D&E). Timing accuracy was higher than count accuracy ($F(1,112)=99.3$, $p<0.001$;) for both sexes and cell types.

Cells were categorized into four groups based on the results of the trial shuffling analysis (see Methods section): bicoding (both timing and count), timing, count, and neither, similar to Caras et al., (2015). Cells that code for stimulus types using timing accuracy (timing and bicoding cells) made up 87.1% of all cells collected. Timing-only cells were 21.6% of the population and count-only cells were 4.3%. Bicoding cells were the majority of cells with 65.5%. Remarkably, males and females did not significantly differ in the proportion of stimulus coding types ($\chi^2=6.02$; $p=0.11$; Figure 20C). Combining both sexes, NS cells significantly differed in the proportion of cell types ($\chi^2=17.08$; $p=0.001$; Figure 20C). This effect was likely driven by the proportion of bicoding cells which constituted 87.2% of all NS cells, but 50.7% of all BS cells. In summary, NCM cells for both sexes preferentially used timing as opposed to count information coding, meaning that the timing of individual action potentials during the auditory stimulus provided more consistent and reliable coding for that stimulus type as compared to a purely rate-based code (count). NS cells in particular utilized both kinds of information (bicoding) in greater proportion than did BS cells.

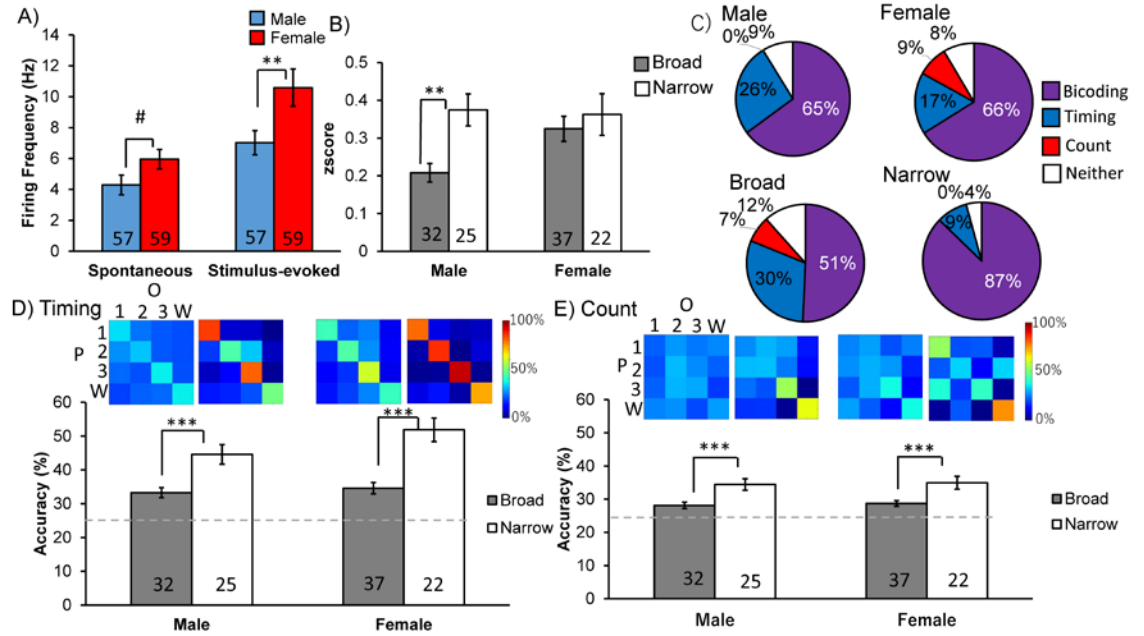


Figure 20: The firing rates of NCM neurons differ between males and females, and the coding properties of NCM neurons differ by cell type classification.

All panels depict bar graphs representing means and SEM for the average of all three conspecific song types and white noise. A) Stimulus-evoked firing frequency (song) is higher than baseline firing frequency (no song) for both sexes. Female cells (n=59) have a higher firing frequency than male cells (n=57) for both spontaneous and stimulus-evoked conditions. B) Male narrow single units (n=25) have a higher normalized auditory responsiveness (zscore) than broad units (n=32). Female broad (n=37) and narrow units (n=22) have similar auditory responsiveness. C) Percentage of cells that are categorized as four coding types: bicoding (purple), count (red), timing (blue), and neither (white) separated by sex (top) or cell type (bottom). Males and females have a similar distribution of coding types, but broad vs. narrow units have significantly different distribution of coding types ($\chi^2=17.08$; $p=0.001$). Specifically, narrow cells have more bicoding cells (87%) than broad cells (51%). D and E) Means and standard errors for the average timing accuracy (D) and count accuracy (E) of the correctly assigned sound types. Broad cells (grey) have lower count and timing accuracy than narrow cells (white) for both males and females. Confusion matrices above each bar depict a representative cell of the mean for that group. O: observed, P: predicted, 1: conspecific song 1, 2: conspecific song 2, 3: conspecific song 3, W; white noise. Colors on confusion matrix are a heat map of accuracy from 0-100%. Gray dotted line represents chance level decoding accuracy (25%). $p=0.07^{\#}$, $p<0.05^*$, $p<0.01^{**}$.

GPER1 is necessary for auditory responsiveness and coding, in a sex- and cell type- specific manner

GPER1 expression occurs in NCM (Acharya and Veney, 2012), and conventional nuclear estrogen receptor agonists do not mimic the rapid actions of estrogens on NCM auditory processing (Remage-Healey et al., 2013). I confirmed the expression of GPER1 in dorsal and ventral NCM using immunofluorescence (Figure 21).

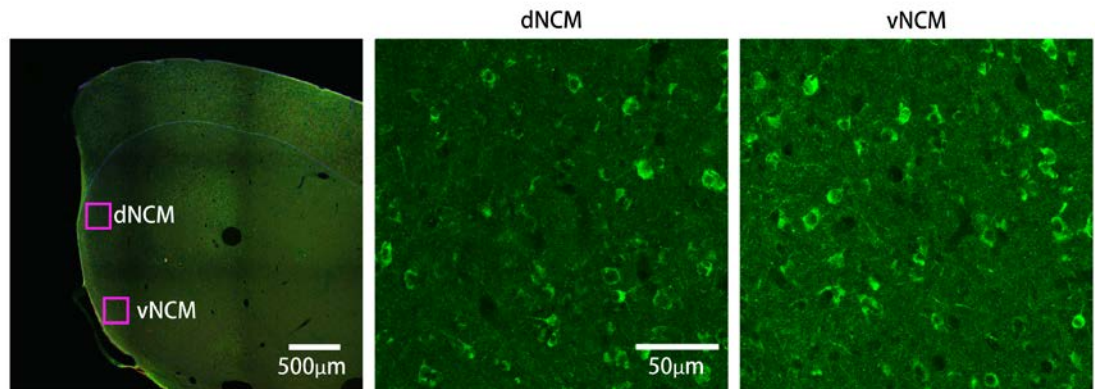


Figure 21: Representative images of GPER1 expression in dorsal and ventral NCM.

GPER1 is expressed in regions that are targeted for recording. Left panel: 10x magnification 3x3 stitch of medial section of forebrain (sagittal plane). Boxes indicate regions from which subsequent images were taken. Middle and right panels: Each image was taken at 60x magnification within respective NCM regions. Images are z-stack maximum projections with 15μm thickness.

I then tested the extent to which GPER1 activation is necessary for sex differences in auditory responsiveness and coding by administering G36, a GPER1 antagonist, via retrodialysis coupled to extracellular recordings in adult males and females. For auditory responsiveness in males, I detected a significant effect of treatment ($F(2,20)=7.32$, $p=0.004$) and a treatment by cell type interaction

($F(2,20)=10.59$, $p=0.001$; Figure 22A left). NS cells in males showed a G36-dependent decrease in auditory responsiveness ($F(2,8)=23.28$; $p<0.001$; pre vs. G36 $t(8)=6.74$, $p=.0036$, Figure 22A left), but BS cells were unaffected ($F(2,12)=0.471$, $p=0.64$; Figure 22A left). In females, I did not detect a significant effect of treatment ($F(2,20)=0.003$, $p=0.997$; Figure 22A right) or treatment by cell type interaction ($F(2,20)=1.25$, $p=0.31$; Figure 22A right). During the pre-aCSF condition, NS cells had a higher zscore than BS cells in males ($t(8.353)=-2.743$, $p=0.024$) but not in females ($t(10)=-0.755$, $p=0.468$), re-confirming the sex difference reported in the overall analyses above.

I next sought to determine whether the GPER1-dependent decrease in auditory-responsiveness could be explained by changes in firing frequency in both the spontaneous period and stimulus-evoked period. I found a significant interaction between firing frequency and sex ($F(1,40)=9.48$, $p=0.006$) and a trend for firing frequency and cell type interaction ($F(1,40)=3.31$, $p=0.08$). I fixed the factor for sex and examined how cell types within sexes exhibited changes in firing rate with drug administration for spontaneous and stimulus-evoked firing frequencies. GPER1 inactivation decreased spontaneous and stimulus-evoked firing in male NS cells (stimulus-evoked main effect: $F(2,8)=9.78$, $p=0.0071$, pre vs. G36 $t(8)=4.93$, $p=0.020$, Figure 22A left inset and; spontaneous main effect: $F(2,8)=5.76$, $p=0.028$, pairwise pre vs. G36 $t(8)=4.01$, $p=0.052$ Figure 22A left inset). All other comparisons were not significant (male BS spontaneous: $F(2,12)=1.36$, $p=0.29$; male BS stimulus-evoked: $F(2,12)=1.27$, $p=0.32$, Figure 22A left inset; female BS spontaneous: $F(2,14)=0.87$, $p=0.44$; female BS stimulus-evoked: $F(2,14)=0.44$, $p=0.65$; female NS spontaneous:

($F(2,6)=0.44$, $p=0.66$; female NS stimulus-evoked: $F(2,6)=0.19$, $p=0.83$, Figure 22A right inset).

I then reasoned that GPER1 inactivation should also change the coding properties of NCM single units respective of sex. Accordingly, I found that, in males, both count and timing accuracy showed a significant effect of G36 treatment (count: $F(2,18)=18.68$, $p<0.001$; timing: $F(2,18)=13.73$, $p<0.001$) and a significant cell type by treatment interaction (count: $F(2,18)=18.31$, $p<0.001$, Fig. 22C left; timing: $F(2,18)=13.0$, $p<0.001$, Figure 22B left). NS neurons in males exhibited a G36-dependent decrease in both count and timing accuracy (count: $F(2,6)=15.20$; $p=0.0045$, pre vs. G36t(6)=6.18, $p=0.011$, Figure 22C; timing: $F(2,6)=10.83$, $p=0.010$, pre vs. G36t(6)=4.82, $p=0.033$, Figure 22B left) during GPER1 inactivation, however there was not a detectable effect in BS cells (count: $F(2,12)=0.26$, $p=0.78$, Figure 22C right; timing: $F(2,12)=1.11$, $p=0.36$ Figure 22B right). In females, there was an overall treatment effect for count and timing accuracy (count: $F(2,20)=3.89$, $p=0.037$, timing: $F(2,20)=5.21$, $p=0.015$) but not a cell type by treatment interaction (count: $F(2,20)=0.95$, $p=0.40$; timing: $F(2,20)=2.41$, $p=0.33$). Moreover, when examining pairwise comparisons for each trial for all female cells, I did not detect an effect of G36 application (count: pre vs. g36 t(20)=1.09, $p=0.53$, Figure 22C right, timing: pre vs. g36 t(20)=1.30, $p=0.63$, Figure 22B right).

In summary, during inactivation of GPER1 I observed a rapid and robust decrease in firing frequency, zscore, and coding accuracy specifically in NS NCM cells in males. Together, these results are consistent with the hypothesis that acute membrane

estrogen receptor signaling is key to auditory responsiveness and coding in a specific population of narrow-spiking neurons in the NCM of males.

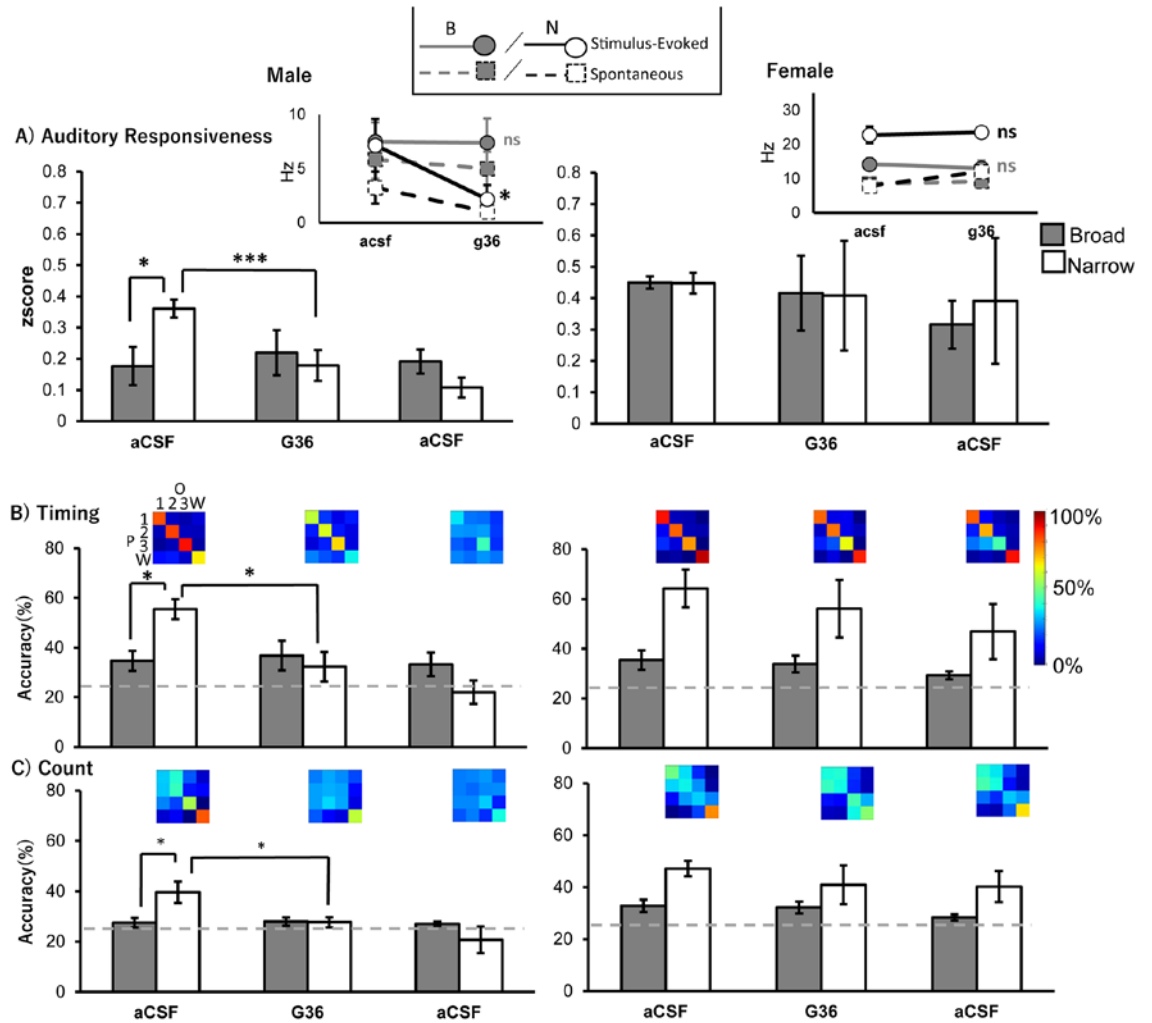


Figure 22: Auditory responsiveness and coding are suppressed during GPER1 inactivation in narrow cells in the NCM of males only.

A) Left: Male auditory responsiveness (zscore) with broad cells (grey, n=7) and narrow cells (white, n=5). Inset: Firing frequency (Hz) of single units for males for acsf and G36 trials. Dashed lines are spontaneous activity (no song) and solid lines are stimulus-evoked activity (songs). Grey lines are broad cells and black lines are narrow cells. There is not a significant difference in firing frequency between the pre-aCSF and G36 for broad cells (ns) but there is a significant difference for narrow cells in stimulus-evoked firing(*). Right: Female auditory responsiveness (zscore) with broad cells (grey, n=8) and narrow cells (white, n=4). Inset: Firing frequency (Hz) of single units for females for aCSF and G36 trials. Dashed lines are spontaneous activity (no song) and solid lines are stimulus-evoked activity (songs). Grey lines are broad cells and black lines are narrow cells. B & C) Bar graphs for timing accuracy (B) and count accuracy (C) for broad (grey) and narrow (white) units in males (left) and females (right). Confusion matrices are representative examples of the means of the narrow single units only. Colors on confusion matrix are a heat map of accuracy from 0-100%. O: observed, P: predicted, 1: conspecific song 1, 2: conspecific song 2, 3: conspecific song

3, W; white noise. Male broad (n=7), male narrow (n=5), female broad (n=8), and female narrow (n=4) Bar graphs depict means and standard errors. Gray dotted line represents chance level decoding accuracy (25%). $p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$.

GPER1 activation is not sufficient to enhance auditory responsiveness or coding accuracy of NCM neurons

To test whether GPER1 activation is sufficient to enhance auditory responsiveness in NCM, akin to native estradiol (e.g., (Remage-Healey et al., 2010; 2011), I tested two doses of the selective GPER1 agonist G1 (100 nM and 100 μ M referred to as low and high dose respectively, below). The high dose did not alter single unit auditory responsiveness ($F(2,36)=1.31$, $p=0.28$, Figure 23A), count accuracy ($F(2,38)=0.006$, $p=0.99$; data not shown), or timing accuracy ($F(2,38)=1.61$, $p=0.21$; data not shown). Additionally, there were no significant interactions for sex by treatment (zscore: $F(2,38)=1.98$, $p=0.15$; count: $F(2,38)=2.00$, $p=0.15$; timing: $F(2,38)=0.004$, $p=0.60$) or cell type by treatment (zscore: $F(2,38)=0.47$, $p=0.63$; count: $F(2,38)=0.091$, $p=0.91$; timing: $F(2,38)=0.64$, $p=0.53$) for the high dose. Notably, at high doses ($>10 \mu$ M), G1 can have nonselective binding and weak antagonism (Dennis et al., 2011) which may mask GPER1-specific effects. Therefore, I selected a lower dose of G1 (100nM) in a new set of experiments to test GPER1 sufficiency. However, similar to the high-dose, I again observed that the low dose did not alter auditory responsiveness ($F(2,66)=3.14$, $p=0.05$; pre vs. G1 low $t(66)=0.44$, $p=0.95$, Fig. 23B), count accuracy ($F(2,66)=0.839$, $p=0.44$; Fig. 24B), or timing accuracy ($F(2,66)=1.98$, $p=0.15$ Fig. 24A). There was also no significant sex by treatment interaction for the low dose (zscore: $F(2,66)=2.42$, $p=0.09$; count: $F(2,66)=2.08$, $p=0.13$, timing: $F(2,66)=1.53$, $p=0.23$). Cell type by treatment interactions in zscore ($F(2,66)=2.70$, $p=0.075$) and count accuracy ($F(2,66)=0.18$, $p=0.83$) were also not significant; however, there was a significant interaction in timing accuracy ($F(2,66)=5.22$, $p=0.007$). BS cells were

unaffected by G1 ($F(2,32)=0.70$, $p=0.50$), but there was a significant treatment effect in NS cells ($F(2,36)=5.14$, $p=0.011$) in which G1 caused a significant decrease in timing accuracy from trial 1 acsf ($t(36)=4.53$, $p=0.0078$; data not shown). When I fixed the analysis by the factor sex, I did not observe significant effects of G1 (males: $F(2,22)=2.45$, $p=0.11$, Figure 24A left; females: $F(2,12)=3.28$, $p=0.07$, Figure 24A right). The effect size for this G1 treatment was moderate ($d=0.62$) in contrast to the substantial effect size of G36 observed for male NS cells ($d=1.19$). In summary, although I observed that GPER1 is key for both auditory-responsiveness and coding stimulus information in narrow-spiking neurons in male NCM, the experiments with G1 indicated that GPER1 activation alone is not sufficient to mimic the rapid effects of estradiol on NCM neurons in either sex.

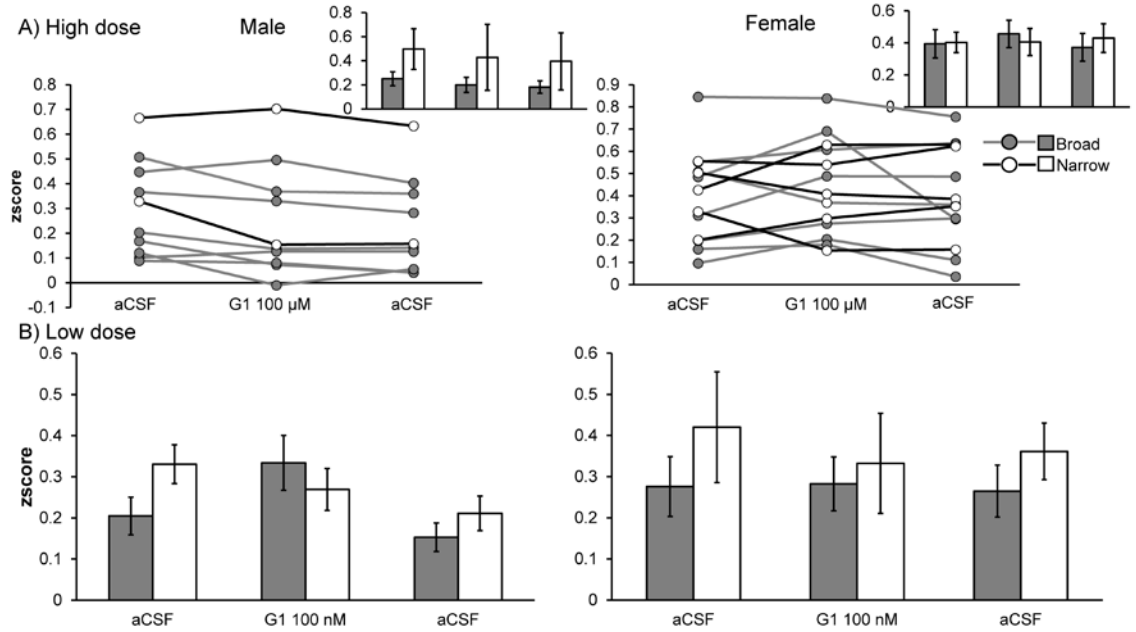


Figure 23: No changes in auditory responsiveness with GPER1 activation.

A) Individual single units depicted across the three treatment trials for the high dose of G1 (100μM) for both males (left) and females (right). Individual data are depicted because of the low number of male narrow cells (n=2) Insets: Descriptive bar graphs of means and standard errors for visual comparison to other figures. B) Means and standard errors for the low dose of G1 (100 nM) for males (left) and females (right) All bar graphs depict means and SEM of conspecific song auditory responsiveness. Broad cells are depicted by grey bars and narrow cells by white bars. High dose: male broad (n=8), male narrow (n=2), female broad (n=8), female narrow (n=5). Low dose: male broad (n=9), male narrow (n=12), female broad (n=11), female narrow (n=9).

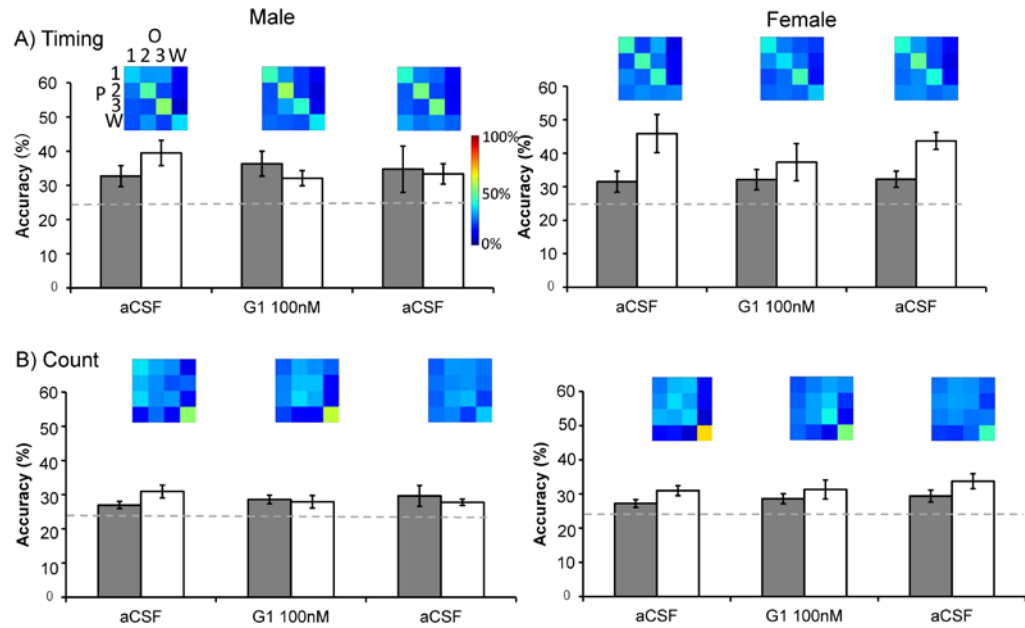


Figure 24: No changes in auditory coding accuracy with GPER1 activation.

All bar graphs depict means and SEM for the average accuracy across all correctly assigned sound types for both broad (grey) and narrow (white) units. Confusion matrices are representative examples of the means of the narrow single units only. Colors on confusion matrix are a heat map of accuracy from 0-100%. O: observed, P: predicted, 1: conspecific song 1, 2: conspecific song 2, 3: conspecific song 3, W; white noise. Data are from the lower dose of G1 (100 nM) experiment and the higher dose has the same relationship. A) G1 application decreased timing accuracy averaged across both sexes but not for each individual sex. B) Count accuracy was not affected for either sex or cell type by G1 application. Low dose: male broad (n=9), male narrow (n=12), female broad (n=11), female narrow (n=9). Gray dotted line represents chance level decoding accuracy (25%).

GPER1 is expressed in inhibitory neurons in NCM but the expression is not sexually dimorphic

Since I observed a sex specific effect of GPER1 inactivation on auditory responsiveness and coding accuracy, I hypothesized that this could be due to differences in GPER1 expression between male and female NCM. Because G36 specifically modulated NS cells in males and prior work has putatively identified NS cells as inhibitory interneurons, I performed co-labeling immunofluorescence for GPER1 and the putative GABAergic neuronal marker, GAD67. First, I observed that ventral NCM had higher expression of GPER1 and GAD67 than dorsal NCM (GPER1: $F(1,10)=9.32$, $p=0.012$, GAD67: $F(1,10)=5.18$, $p=0.046$). Second, I observed that approximately 20% of GAD67-positive cells in NCM were also positive for GPER1, consistent with the hypothesis that estrogens act in part to shape inhibition in NCM (Fig. 25) and that coexpression of GPER1 and GAD67 was similar between regions ventral and dorsal NCM ($F(1,10)=0.32$, $p=0.59$). Third, I also found that in both dorsal and ventral NCM, GPER1 expression and GAD67 expression were not different between the sexes (DNM: GPER1 $F(1,12)=0.11$, $p=0.75$; GAD67: $F(1,12)=2.91$, $p=0.12$, VNCM: GPER1 $F(1,11)=0.21$, $p=0.66$; GAD67: $F(1,11)=0.19$, $p=0.68$). Lastly, I also did not find sex differences in GPER1-GAD67 co-labeled cells (DNM: $F(1,12)=0.14$, $p=0.71$; VNCM: $F(1,11)=1.19$, $p=0.30$). Together these results indicate that GPER1 protein expression alone cannot explain sex differences observed in auditory physiology in NCM. (Figure 25).

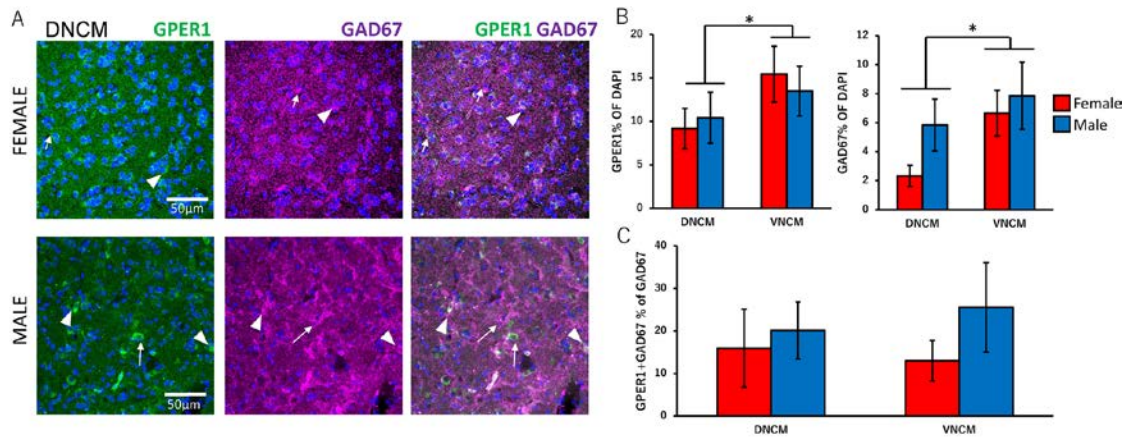


Figure 25: GPER1 expression and colocalization in GABAergic neurons are each not sexually dimorphic.

A) Representative images of dorsal NCM for labeling of GPER1 (green) and GAD67 (magenta). Each image was taken at 60x magnification within respective regions. Images are z-stack maximal projections with 15 μ m thickness. White triangles point to examples of a cell that express both GPER1 and GAD67. White arrows point to examples of a single label. B) Means and SEM error bars for GPER1-positive neurons (left) and GAD67-positive neurons (right) as a percentage of DAPI. C) Means and SEM error bars for GPER1/GAD67-positive neurons as a percentage of GAD67 cells. Red bars are females (DNCM n=6; VNCM n=6) and blue bars are males (DNCM n=7, VNCM n=6). $p < 0.05^*$.

Discussion

I observed several novel sex differences in neurophysiological parameters in the songbird auditory forebrain. First, female single units identified from extracellular recordings had a higher firing frequency. Second, male single unit auditory responsiveness was elevated in narrow-spiking (NS) as compared to broad-spiking (BS) cells. I also found that GPER1 was critical for NCM song responses and coding, depending on sex and cell type. GPER1 inactivation caused decreases in auditory responsiveness and coding accuracy in male NS cells, however it was not effective in

either cell type in females. Furthermore, when responses to stimuli were computationally decoded, spike timing and count classification accuracies were higher in NS than BS in NCM.

The findings indicate that brain regions that are similar in morphology and cytoarchitecture between males and females can exhibit marked sex differences in the physiology of single neurons. Prior work on the songbird NCM and caudomedial mesopallium (CMM) reported sex differences in auditory-evoked immediate early gene expression (Avey et al., 2005; Tomaszycski et al., 2006; Gobes et al., 2009) and multiunit response magnitude (Yoder et al., 2015). Other regions of the songbird auditory network also exhibit sex differences in single-neuron call responses and tone amplitude coding (Caras et al., 2015; Giret et al., 2015). This work builds on this foundation to show that separate classes of NCM neurons (NS and BS) can carry information about conspecific songs in a sex-specific way.

Male zebra finches sing learned vocalizations during courtship, and females do not ordinarily sing (Zann, 1996). Therefore, this report of sex differences in song responsiveness and coding in NCM could reflect the divergent role(s) for song in auditory salience, valence, and life history in males vs. females. Outside of the auditory system, sex differences in the single-unit representation of sensory stimuli have been most clearly delineated for sex-specific pheromonal responses in reptiles (Huang et al., 2006) and rodents (Bergan et al., 2014). Interestingly, these differences may also be associated with estrogen-dependent signaling mechanisms, such as shown here (Bergan et al., 2014; Bergan, 2015) .

I used a pattern classifier similar to Caras et al. (2015) to determine how NCM units discriminate among conspecific songs and whether this differed between the sexes and cell types. Despite sex-differences in neuronal firing profiles, pattern classifier coding was similarly accurate for recordings from male and female NCM. The analysis also indicated that NCM cells, in general, were more temporally consistent when responding to the same conspecific song. When characterizing cells as count, timing, or bicoding, there was a strong bias toward cells that exhibited timing accuracy significantly above chance, and not count accuracy alone. This was consistent with Caras et al. (2015) who found that in Field L, a primary auditory region, and CMM, a secondary auditory region, a greater proportion of neurons were categorized as timing or bicoding for tones presented at different amplitudes. I now report cell-type specific differences in auditory coding of individual songs in NCM, and that this feature is not different between males and females.

Prior studies of BS and NS in songbird auditory cortex have focused exclusively on males. BS cells contribute to background-invariant coding of vocalizations (Schneider and Woolley, 2013), selectivity to songs after tutoring (Yanagihara and Yazaki-Sugiyama, 2016), and sensitivity to song sequence (Ono et al., 2016). This study in both sexes showed that BS and NS neurons differentially responded to auditory stimuli in two specific ways. First, NS cells responded to conspecific song more strongly in males only. Second, NS cells exhibited higher timing and rate coding accuracy, indicating that NS are better at distinguishing among stimuli. Previous findings showed that BS neurons in NCM had greater selectivity for specific song types after tutoring (Yanagihara and Yazaki-Sugiyama, 2016). Taken together, BS neurons

may be selective for specific songs, while NS neurons have greater coding accuracy and favor a bicoding computational mode. Although NS cells are putatively inhibitory interneurons, this assignment has not been validated in NCM (e.g., narrow, fast spiking cells can be excitatory neurons in primate cortex (Vigneswaran et al., 2011)). NCM is also thought to be a source of auditory memory (Bolhuis and Gahr, 2006; Bolhuis and Moorman, 2015; Yanagihara and Yazaki-Sugiyama, 2016), so the cell-type specific coding of conspecific songs in NCM can now be explored to understand the neural basis of perception and individual recognition.

This study is the first to demonstrate a role for GPER1 in sensory processing. I found that blocking GPER1 signaling led to decreases in firing rate, auditory responsiveness, timing accuracy and count accuracy in male NS neurons, revealing that for this sex and cell type, GPER1 is critical for auditory activation and coding of stimuli. Sex-specific mechanisms of rapid estradiol signaling have been described in the mammalian hippocampus (Meitzen et al., 2012; Vierk et al., 2012). Similar to the modulation of NCM by neuroestrogens (Ramage-Healey et al., 2010; Ramage-Healey et al., 2012; Ramage-Healey and Joshi, 2012), neuroestrogens also alter hippocampal memory formation and consolidation (Boulware et al., 2013; Kramar et al., 2013; Frick et al., 2015; Kim et al., 2016; Tuscher et al., 2016). Despite similarities between the sexes in expression of GPER1 and aromatase (Tabatadze et al., 2014; Waters et al., 2015), there are noted sex differences in physiological responses to estrogen in the hippocampus (Huang and Woolley, 2012; Tabatadze et al., 2015; Oberlander and Woolley, 2016). This study now shows that GPER1 actions are also important for sensory processing and are sex-specific in the avian brain.

Although our findings implicate GPER1 in auditory processing, the signaling mechanism may be independent of the previously-established role of estradiol (E2) in the songbird forebrain. The agonist for GPER1, G1, did not mimic the overall enhancement of auditory processing by E2 in either sex (Ramage-Healey et al., 2010; 2012). One resultant hypothesis is that E2 acts through multiple membrane estrogen receptors concurrently, since classical nuclear estrogen receptor agonists do not enhance auditory responsiveness in males (Ramage-Healey et al., 2013). Future work should consider concurrent activation of multiple estrogen receptor subtypes for auditory processing in NCM. There is evidence for a multi-estrogen receptor mechanism in synaptic hippocampal neurotransmission (Kumar et al., 2015), and while GPER1 and E2 can enhance hippocampal-dependent object recognition, estradiol depends on MEK-ERK signaling while GPER1 depends on the JNK pathway (Kim et al., 2016) indicating that there are non-estradiol actions of GPER1 activation.

Prior work in songbirds has also identified other sex specific differences in auditory coding that may be mediated by estrogens. Previous findings in Field L and CMM demonstrated that bicoding cells in females are sensitive to breeding season (when peripheral estrogen levels are high) where both timing accuracy and temporal resolution increased compared to the non-breeding season. This effect of season does not occur in males (Caras et al., 2015). I find that GPER1 inhibition decreased auditory responsiveness and coding for both timing and count classification accuracy, but only in males. While the previous study infers that these effects are mediated via hormonal actions, our study suggests a receptor mechanism that is directly affecting coding in the auditory forebrain in a sex-specific way.

The electrophysiology results led to the hypothesis that a sex difference in GPER1 receptor expression occurs in subregions of NCM. I found that GPER1-positive cell number was similar between males and females, and I established that approximately 20% of GAD67-positive cells in NCM are also positive for GPER1, consistent with the hypothesis that estrogens act in part to shape inhibition in NCM (Fig. 25). However, I did not observe sex differences in the number of cells coexpressing GPER1 and GAD67, and there was a lack of coexpression with interneuron markers parvalbumin or calbindin. Other inhibitory interneuron subtypes such as somastatin and VIP, which are important auditory cell types in mammalian cortex (Pi et al., 2013; Chen et al., 2015; Phillips and Hasenstaub, 2016), could be further explored to account for the sex differences observed here. In addition, local network connections between GPER1-positive cells and other NCM cell subtypes now become an active area of interest in this work.

The temporal cortex of humans can produce estradiol locally, including at synaptic terminals (Yague et al., 2006) much like the zebra finch NCM (Saldanha et al., 2000; Peterson et al., 2005). Although there has not been an anatomical description of GPER1 in the human brain, GPER1 has been shown to be expressed in primate cortex associated with synaptic densities (Crimins et al., 2016). The findings suggest that sex is a fundamental factor when examining mechanisms of audition, and that it is worthwhile to explore neuroestrogen signaling and GPER1 in particular within the primate auditory cortex, especially when considering sex as a biological factor. In context of life history and reproductive strategies for the sex and species of interest, brain areas that process

sensory information should be further explored for sex differences in mechanisms, and in particular considering neuromodulatory mechanisms of estrogens.

CHAPTER V

DISCUSSION

In this dissertation, I addressed whether rapid estrogen signaling in the zebra finch auditory forebrain is sexually differentiated. To address this hypothesis, I tested three major questions: 1) whether interneuron subtypes of estrogen-producing and estrogen-responsive neurons differ by region and sex, 2) whether acute endogenous estradiol production is necessary for auditory responsiveness in both sexes, and 3) whether the membrane estrogen receptor, GPER1, is necessary and sufficient for full auditory responsiveness in both sexes. I concluded from the results that males depend more on active endogenous estradiol synthesis as well as GPER1 activation to encode neuronal responsiveness to songs. These sex differences in auditory responsiveness exist despite similarities in neurochemical identities of aromatase and GPER1 cell types I characterized. I propose that males and females utilize different mechanisms for auditory responsiveness, and I outline several future pursuits to determine how estrogens are influencing audition through these potential mechanisms. In this discussion, I outline how sex differences in rapid estrogen signaling of the auditory lobule may impact auditory coding of several brain regions. I also discuss how differences in auditory activity via inhibition of estradiol production may be evidence of compensatory mechanisms between the sexes. These compensatory mechanisms may be differences in neural production, membrane receptors, or intracellular signaling cascades that differ between males and females. Together, these mechanisms are likely shaped by the heterogeneity found in auditory subregions like NCM, and these cell-type specific differences may be properties of auditory microcircuits. Finally, I make an

argument for sex as a biological variable and how understanding differences in mechanisms can inform translational studies in primates including humans.

Sex differences in sensory encoding

Sex differences in sensory systems have been described in insects (Rideout and Goodwin, 2008), amphibians (Shen et al., 2011; Hall et al., 2016), reptiles (Huang et al., 2006; Sampedro et al., 2008), and mammals (McFadden et al., 2006; Bakker et al., 2010; Wang et al., 2010; Washington and Kanwal, 2012; Bessinis et al., 2013; Bergan et al., 2014) including humans (Cowan et al., 2000; Beech and Beauvois, 2006; Vanneste et al., 2012; Liu et al., 2013; Wisniewski et al., 2014). Depending on the sexual strategies of a species, sex differences are necessary for reproductive fitness, mate selection, and parental roles. Over 90% of birds are monogamous (Black, 1996), and for songbirds, complex, learned vocalizations (songs) are used for mate selection (Grant and Grant, 1997; Verzijden et al., 2012), defense of territory (Goodwin and Podos, 2014), mimicry (Goodale and Kotagama, 2006; Flower et al., 2014), and tutoring of juveniles for future song development (Roberts et al., 2012; Chen et al., 2016). For these reasons, birds such as the zebra finch, in which males sing and females do not, are powerful models of studying sex differences in neuroanatomy and underlying physiology of auditory responses.

For the anatomy and estrogen production studies (Chapter 2 and 3), I used histology as a broad approach to examine multiple auditory forebrain regions such as: caudomedial nidopallium (NCM), caudomedial mesopallium (CMM), Field L, and HVC (proper name) shelf. There are some known functional differences between these regions. Field L is analogous to mammalian primary auditory cortex (A1) because it is

tonotopic and receives inputs directly from the thalamus (Kelley and Nottebohm, 1979; Vates et al., 1996). NCM, CMM, and HVC shelf are all considered analogous to secondary auditory cortex and association areas like Wernicke's area (Gobes and Bolhuis, 2007; Bolhuis et al., 2010). These regions receive inputs from Field L, and have activity-dependent changes to higher-order cognitive processing of sounds such as context of song. NCM and CMM have some unique distinctions in responsiveness to song types. CMM has stronger representation of familiar sounds such as mate's call in female zebra finches (Giret et al., 2015) and mate song in both sexes of parrots (Eda-Fujiwara et al., 2016). It also responds stronger to directed songs (males singing to a female rather than alone) in females (Woolley and Doupe, 2008). NCM has a stronger response to unfamiliar or novel songs as measured by immediate early genes (Woolley and Doupe, 2008), but when measuring auditory-evoked activity of extracellular recordings in this area, NCM is non-selective (Remage-Healey and Joshi, 2012). Using a pattern classifier, I demonstrated that there are temporal properties of NCM firing that are specific to individual conspecific songs as measured by timing accuracy. While narrow-spiking (NS) neurons were more accurate than broad-spiking (BS) neurons, males and females were similar in accuracy and accuracy cell types (bicoding, timing, etc). This work indicates that NCM likely has a role in discrimination of individual conspecific songs that is encoded within single neurons, especially NS putative interneurons. This finding will need to be followed by cell-specific targeting (such as optogenetics) to test whether this cell type is involved in behavioral song discrimination.

Sex differences in auditory responsiveness to song have been described across these forebrain regions. Some studies have found differences in immediate-early gene (Egr-1) expression depending on familiarity with the song (Terpstra et al., 2004; 2006) and whether birds had been exposed to song during development (Tomaszycki et al., 2006). When using electrophysiology, a sex difference has been described in the response magnitude of multiunit recordings in the NCM to novel, conspecific song (Yoder et al., 2015). Previous publications have reported sex differences in auditory-evoked activity to tones in Field L and CMM (Caras et al., 2015) and calls in Field L and caudolateral mesopallium (CLM; Giret et al. 2015) of single units. In this dissertation, I provide neurophysiological evidence for sex differences from single units responding to complex vocalizations (songs) in NCM, demonstrating that there are fundamental differences across parameters such as a higher firing frequency of single units in females, and differences in zscore for NS and BS neurons in males, suggesting alternative network connectivity and mechanisms for coding auditory signals between the sexes. NCM may have sex differences in connectivity or cell types that were not tested in the studies that could contribute to these differences in physiology.

I found a novel sex difference in aromatase expression and estradiol-mediated auditory responsiveness in the posterior HVC shelf. Females had more aromatase and were less affected by fadrozole administration in the posterior HVC shelf as compared to males. This is the first description of a sex difference in HVC shelf. Little is known about the shelf and its involvement in audition. The shelf projects to the sexually dimorphic sensorimotor nucleus, HVC, which is necessary for successful song production in males (Nottebohm et al., 1976) and is significantly smaller in females of

songbird species who lack song-production abilities (Nottebohm and Arnold, 1976; Williams, 1985; Hamaide et al., 2017). This work indicates that further study of the shelf and estrogenic actions in auditory responsiveness and behavior of neurons is worth exploring. More importantly, sex should be carefully considered when analyzing anatomy and mechanism in this region.

When examining multiple auditory regions, I stained for Egr-1 as a marker for neuronal activity and induced the expression by exposing birds to song. Egr-1 has other functional roles in cells, specifically in neurons where it targets genes that are involved in the maintenance and regulation of synapses (Davis et al., 2003; Knapaska and Kaczmarek, 2004; Veyrac et al., 2013). Egr-1 is thought to be an important transcription factor for cellular memory (Moorman et al., 2011) and Egr-1 knockout mice have memory deficits (Han et al., 2014). In the context of birdsong, Clayton (2000) describes Egr-1 as the “genomic action potential”, hypothesizing that this transcription factor plays a role in laying down long-lasting “memories” for auditory experiences that are critical such as in juvenile development where males are in the process of learning a tutor’s song. This study suggests that in adulthood, acute estrogen production is necessary for this response, although its function on adult memories is unclear. Juveniles also can synthesize estradiol in these regions (Chao et al., 2015), and there may be a role for estradiol in consolidation of long-term memories of tutor song, especially for males. It has been proposed that NCM is the storage region for tutor song memory, with evidence supported by lesions (Gobes and Bolhuis, 2007), neural habituation (Chew et al., 1995; 1996), and selectivity after tutoring (Yanagihara and Yazaki-Sugiyama, 2016). Females also may need early exposure to song to form

preferences for high-quality song (Lauay et al., 2005), but the role that song memory has in females is mostly unexplored. Future work in juveniles comparing the sexes will need to determine how the sexes are forming these memories, whether they utilize similar brain regions, and whether estradiol and Egr-1 are involved in memory formation.

Neuroestrogens and steroid production in the brain: compensation hypothesis

The brain's abilities to synthesize and produce steroids has been well established across vertebrates, and aromatase expression and estradiol content is more abundant in brain regions outside of the hypothalamus in non-mammals (Callard et al., 1978). 1978). The zebra finch has been a model to study neuroestrogen production, primarily because of the high estradiol content in regions such as NCM (Saldanha et al., 2000) as well as the brain's ability to upregulate aromatase expression in injured glial cells (Mehos et al., 2016). Both males and females have the capacity to synthesize estrogens in the brain; however, there are some indications that there might be activity dependent differences in estradiol synthesis.

I confirmed that endogenous estradiol was necessary for full auditory responsiveness of neurons in the auditory forebrain of several subregions. Prior work has shown that estradiol rapidly increases overall content when birds hear song (Ramage-Healey et al., 2008; 2012) and that estradiol application onto auditory neurons enhances auditory responsiveness (Ramage-Healey et al., 2010; 2012). There has also been evidence that auditory-induced Egr-1 is sensitive to exogenous estradiol through estradiol implants (Maney et al., 2006) and that this auditory-induced expression is different regionally (Sanford et al., 2010). This dissertation adds to this literature,

connecting for the first time that estradiol synthesized during song-exposure is promoting the auditory-induction of Egr-1 expression in neurons of several secondary auditory regions.

In chapter 3, I found that more auditory regions in males are dependent on acute endogenous estradiol production than females. I examined several auditory regions and the regions that exhibited this sex difference were dorsal NCM and posterior HVC shelf. In both sexes, anterior HVC shelf had a reduction in auditory-induced Egr-1 for fadrozole treated subjects. I quantified somatic aromatase expression in these regions, and found that males and females were similar except for posterior HVC shelf, which had the biggest sex differences between male and female fadrozole-treated animals. Overall, these results revealed that somatic aromatase expression cannot explain all the sex differences I observed. One hypothesis is that differential expression of aromatase in terminals vs. soma may explain these sex differences since somatic aromatase activity may have a different function than terminal expression. Terminal expression is likely regulated by fast, neuron dependent activity (Ramage-Healey et al., 2011). While activity dependent regulation is also true for somatic expression (aromatase activity in microsomes; Comito et al., 2016), since estradiol is a steroid that can freely pass through membrane, dense somatic expression may bathe the local region in estradiol content. It is unknown how estradiol is being degraded centrally. These results replicated prior work that showed that adult males and females had similar aromatase expression in the NCM (Chapter 2; Saldanha et al., 2000). However, males had more fiber expression (Saldanha et al., 2000) and more aromatase activity in synaptosomes than females in this region (Peterson et al., 2005). Dorsal NCM is a region where I saw

a significant decrease in auditory-induced Egr-1 with fadrozole in males but not females. Higher fiber expression and terminal aromatase activity in NCM of males may represent a higher dependency of active estradiol synthesis during auditory stimuli presentations, explaining the sex difference in fadrozole-treated animals in NCM. To date, fiber expression of aromatase cells in HVC shelf alone has not been quantified, so it is unclear whether males and females differ in axonal/terminal expression or activity of this region. Rohmann et al. (2007) report more male aromatase activity in tissue from posterior telencephalon that contains the shelf, but this fraction also contains other aromatase rich regions such as NCM. Since females had more somatic expression of aromatase, this may indicate that E2 content in this region in females could have a higher baseline making them less dependent on active E2 synthesis. To test this hypothesis, direct measurements of the shelf and other aromatase expressing regions (ie. NCM) would need to be compared in males and females using a similar paradigm of fadrozole administration as described in Chapter 3.

The results reported in this dissertation reveal that there are convincing sex differences in how endogenous estradiol production influences auditory physiology, especially in previously underreported regions like the HVCshelf. There is evidence that separate regions of the zebra finch brain (hypothalamus, hippocampus, and nidopallium) have different activity-dependent (ATP, Ca²⁺, Mg²⁺, etc.) regulation of aromatase activity (Comito et al., 2015). I detected region-dependent differences in aromatase cell identity (ie. Parvalbumin⁺, Chapter 2), and there may be differences in regulation of the enzyme based on cell type or subregion. In general, somatic aromatase expression and cell identity is similar between the sexes, but this does not indicate that

differences in activity and expression in other parts other parts of neurons (i.e. terminals) may reflect sex differences in auditory activation of neurons. Sex differences in regulation of neuronal aromatase activity should be further explored in auditory subregions.

I observed somatic clustering of aromatase cells throughout auditory subregions, and performed a thorough analysis of cluster size within NCMv. There was a stark sex difference, where females had more aromatase cells that were unclustered, and males had more aromatase cells found in clusters and more cases of large cluster sizes (5+ aromatase cells found in a cluster). I performed a random simulation of likelihood of aromatase cells touching given the spatial dimension of our images and found both males and females were significantly different than chance in cluster size distribution (Chapter 2). Although I did not detect any sex differences in NCMv in Egr-1 expression, I think this is likely due to a floor effect since I detected the least number of Egr-1+ nuclei, and despite having aromatase expression, I did not see an effect of fadrozole treatment (Chapter 3). Although clustering was not quantified in regions where I detected a decrease in Egr-1 expression, I observed clustering in other subregions (NCMd, HVCshelf). I hypothesize that cell clustering may be a form of synchronizing communication, whether it be through gap-junctions (unconfirmed) or synaptic communication. Since males have more clustering of aromatase cells, it is possible that they could coordinate activity-dependent aromatase regulation more so than females. This may be an explanation of why more regions in males had a stronger decrease in auditory-induced Egr-1. If aromatase cell activity is coordinated through clustering, then it is possible that estradiol production could be coordinated as well.

Future work will need to determine the function of somatic clusters, such as filling aromatase cells found in clusters with gap-junction permeable dyes.

Another hypothesis that may explain these results is that adult females have compensation from estradiol synthesized in the ovaries. I did not detect differences in estradiol content from whole trunk blood between the aromatase inhibitor treated birds and the control group, indicating that the drug treatment was not sufficient to decrease global peripheral supplies, which was intended. This is consistent with previous work where acute peripheral administration of fadrozole is not sufficient to drive down E2 content in blood (Prior et al., 2014). This means that females likely have estradiol from their ovaries circulating. Measurements of brain-derived estradiol (which may be a mix of neuronal and peripheral estradiol) in adulthood showed that males and females had similar estradiol content as measured by microdialysis (Ramage-Healey et al., 2012) and both increased estradiol production when hearing song (Ramage-Healey et al., 2008; 2012), although the magnitude of song-induced increases have never been compared in the same study. The lack of sex differences in E2 content in adulthood contrasts with younger animals where at baseline (no song exposure) males had higher estradiol content during the subadult period (60-80dph) (Chao et al., 2015) which is a period before sexual maturity (90 dph). This is the only period a detectable sex difference in estradiol either in the periphery or centrally has been detected in the zebra finch. This difference may indicate a change in activity or expression of neuronal aromatase at this timeframe, but this has not been examined at this age.

The sex difference of E2 detected in subadulthood also might reflect compensation of peripheral estrogens (i.e. from the ovaries) in females that could be

occurring by the time females reach adulthood. Adult females have large detectable estrogens that can be measured from the ovary and telencephalon (Schlinger and Arnold, 1991, 1992); however, how these sources interact is unknown. Interestingly, males and females do not have detectably different concentrations of estradiol in the blood during development (up to day 50) or adulthood (Adkins-Regan et al., 1990). When adult zebra finches are gonadectomized, estradiol measured from blood dramatically increased in both sexes but more so in males (Adkins-Regan et al., 1990). This finding indicates a capacity for males to produce more estrogens from the only other source of detectable estrogen content known, the brain (Schlinger and Arnold, 1991, 1992), illustrating a lower capacity for females to produce estrogens in the absence of their gonads as opposed to males from the brain. This gonadectomy effect does not occur in young juveniles (12-19dph; Adkins-Regan et al., 1990). To date there has not been any paper to report peripheral estrogen levels during 60-80dph of zebra finches, which is when this detectable sex difference in neural estradiol was reported (Chao et al., 2015). Since this period is just before birds reach sexual maturity, this may be a period of increasing gonadal hormones, reflecting a difference in sub adulthood. One hypothesis is that when birds reach adulthood, compensatory mechanisms become activated, leveling E2 levels in males and females. Some evidence related to gonad size and primary follicle development in females may indicate that there are differences in hormone production from the gonads during this period. Male testis growth is logarithmic, reaching maturity between 59-70 dph, where female ovarian growth is linear, with captive females not producing their largest follicle until 100dph and ovary weight not reaching maximal adulthood mass until 250dph (Zann, 1996).

However, given that I did detect a significant decrease in the anterior HVC shelf in fadrozole-treated females, and there were modest but not significant decreases of Egr-1 in regions such as dorsal NCM, I think that it is unlikely that there is a full protection of the auditory response from the peripheral estradiol alone. If this were the case, it is likely that there would not be regional differences; however other factors may be at play. The mechanism by which estradiol is metabolized is still unknown in the brain. There may be region dependent metabolism, or receptor sensitivity that is saturated under control conditions, but when there is less estradiol being produced, what little is around in females may have region dependent expressions and sensitivities. One way to test whether peripheral estradiol contributes to central content in females is to gonadectomize females to remove ovarian sources of estradiol and compare responsiveness to males.

Membrane estrogen receptors

With the discovery of rapid estrogen signaling (Szego and Davis, 1967), membrane estrogen receptors were characterized as being mediators of rapid changes in cellular responses. The classic estrogen receptors (ER α and ER β) have DNA binding segments to act as transcription factors; however, post translational modifications such as palmitoylation (Meitzen et al., 2013) can insert these proteins into the membrane where they associate with metabotropic glutamate receptors (mGluRs; Meitzen and Mermelstein, 2011). Other membrane bound receptors were discovered, including g-protein estrogen receptor 1 (Revankar et al., 2005), which is a g-protein coupled receptor can also modulate fast-action changes in neurons via E2 binding.

The zebra finch auditory forebrain has been showed to express all three types of receptors (Metzdorf et al., 1999; Fusani et al., 2000; Saldanha and Coomaringam, 2005; Jeong et al., 2011; Acharya and Veney, 2012; Horton et al., 2014). It is unclear whether ER α and ER β expression is only nuclear or in the cytoplasm and membrane because most studies to date have characterized mRNA only (see previous citations), with the exception of one that reports ER α protein expression in the nucleus (Saldanha and Coomaringam, 2005). There is evidence in the quail brain for rapid estradiol actions mediated via ER β and mGluRs (Seredynski et al., 2015); however, there has not been definitive evidence for this mechanism in zebra finch auditory regions. GPER1 protein is found throughout the posterior portions of the forebrain, and its expression in adulthood is similar between males and females (Acharya and Veney, 2012). I replicated this finding described in Chapter 2, where I found no detectable differences between males in females in GPER1 cell number in any auditory region analyzed.

There has been extensive evidence that estrogens can have actions on auditory responsiveness (Maney et al., 2006; Ramage-Healey et al., 2010; 2012; Ramage-Healey and Joshi, 2012); however, a mechanism has not been described until now. I addressed whether GPER1 is necessary and sufficient for auditory-responsiveness in the NCM, specifically. Rather than measuring IEGs, I performed a more dynamic approach recording extracellularly from neurons in the NCM and used microdialysis to administer drugs in a within-subject paradigm to determine how GPER1 affects neuromodulation of single units in males and females. I found that in males only, inactivating the GPER1 receptor decreased auditory-responsiveness of NS neurons. I also showed that both timing and count accuracy decreased in these cells, indicating that

not only is responsiveness affected, but auditory coding as well. This is likely attributed to the decrease in firing frequency in male NS neurons. I did not detect any changes for either cell type in females with GPER1 inactivation, indicating that males and females may be using different membrane estrogen receptors to mediate auditory-evoked firing.

When I administered G1 agonist, I did not detect any changes to auditory responsiveness or accuracy for either sex or cell type, except for a small decrease in timing accuracy in male narrow units, contrary to my predictions. I used two doses of G1 to assure that this lack of effect was not dose dependent, and did not find sufficient enhancement of auditory-evoked firing for either sex or cell types. I conclude that while GPER1 is necessary for responsiveness in narrow cells of males, G1 is not sufficient to enhance auditory responsiveness as has been previously reported with estradiol administration (Ramage-Healey et al., 2010; 2012). Prior work from our lab has used ER α and ER β agonists in males in the NCM under a similar paradigm, and did not find sufficiency in the auditory response (Ramage-Healey et al., 2013). One explanation for a lack of sufficiency of known receptors is E2 could be binding to multiple receptors, and there needs to be concurrent activation of more than just one receptor to enhance auditory responsiveness. This hypothesis could be tested by infusing a cocktail of estrogen receptor agonists and measuring changes to auditory-evoked firing. Previous work in the mammalian hippocampus has shown multi-estrogen receptor mechanism for synaptic transmission (Kumar et al., 2015). There are some gaps in the literature concerning how ER α and ER β agonist might influence female auditory responsiveness of NCM neurons, since only males were investigated (Ramage-Healey et al., 2013). Work from Oberlander and Woolley (2016) showed that there are sex and receptor

specific differences in E2 potentiation of hippocampal neurons. This study illustrated that males and females differ in which of the three receptor types mediate pre-and-post synaptic E2 events. A similar process may also be occurring in the zebra finch NCM as well, and a more thorough analysis of specific agonists and antagonists for all receptor types must first be used to have a complete picture of how E2-enhancement of auditory-firing is occurring in this species and brain region.

Sex-specific intracellular signaling cascades

Membrane estrogen receptors can alter cellular process like neuron firing and gene transcription through signal transduction pathways such as the MEK-ERK and JNK pathways. Within songbird audition, there is compelling evidence that auditory responsiveness is mediated by the MEK-ERK pathway, as reviewed in Chapter 1 (Krentzel and Ramage-Healey, 2015). Briefly, prior work has shown that MEK phosphorylation of ERK is required for auditory-inducible Egr-1 expression within NCM (Cheng and Clayton, 2004) and that E2 can alter Egr-1 expression (Maney et al., 2006). Estradiol is capable of increasing expression of Egr-1 within mammalian tissues (Suva et al., 1991; Pratt et al., 1998), and this is mediated via the MEK-ERK signaling pathway (de Jager et al., 2001; Chen et al., 2004; Kim et al., 2011). Within the brains of songbirds, an acute injection of estradiol alters phosphorylation patterns of several proteins (Heimovics et al., 2012), demonstrating compelling but not direct evidence for estradiol modulation of auditory-induced Egr-1 induction.

I found that acute, endogenous inhibition of estradiol production decreased auditory-induced Egr-1 expression, but more so in subregions of males as opposed to females. This is the first paper testing whether endogenous estradiol is necessary for

Egr-1 expression, and also the first paper to describe a sex difference in auditory-induced Egr-1 dependence on estradiol synthesis in songbirds. There could be several factors contributing to the sex difference I found. Earlier, it was discussed how compensation for other sources of estradiol production may be at play (Section: Neuroestrogens). Intracellular signaling pathways also may contribute to this sex difference. The necessity of pERK in Egr-1 induction was only tested in male animals (Cheng and Clayton, 2004). Female animals could utilize alternative pathways that may be promoting Egr-1 expression in the absence of acute estradiol synthesis. Some mammalian literature indicates sex differences in E2-mediated intracellular signaling pathways of various brain regions (reviewed in Chapter 1, Krentzel and Ramage-Healey, 2015). There is a notable gap in the songbird literature concerning signaling pathways for female auditory processes, and future work will need to include both males and females for comparison.

Coinciding with measurements of Egr-1, I also measured pCREB, a transcription factor of Egr-1 (Knapska and Kaczmarek, 2004; Moorman et al., 2011), to determine if inhibition of endogenous estradiol synthesis also altered phosphorylation patterns of the proposed MEK-ERK pathway, as CREB can be targeted for phosphorylation by this pathway. I hypothesized that I would see a decrease in phosphorylation of this transcription factor in similar directions as Egr-1 if indeed it was also sensitive to estradiol signaling. I also hypothesized that there would be sex differences in fadrozole treated animals, since in mammalian hippocampus, there is sex specific activation of pCREB when cells are administered estradiol (Boulware et al., 2005; Grove-Strawser et al., 2010; Meitzen et al., 2012). In chapter 3, I did not detect

changes in pCREB expression affected by aromatase inhibition or sex differences in expression patterns. There could be a few explanations for not detecting estradiol effects. I could have missed the timeframe to detect this response, or there are differences in overall CREB expression that are masking the effects that are undetectable with the anti-pCREB antibody. There are several other candidates to regulate Egr-1 expression via estradiol signaling, such as Elk-1 which is targeted by similar kinase pathways (Chen et al. 2004).

To address timeframe, I tested whether pCREB was song-inducible, based on the paper by Cheng and Clayton (2004) that found that pERK expression increased within a shorter timeframe and for more acute song exposure. I tested birds in silence, exposed to 85 seconds of triplicate song, and 15 minutes of triplicate song. In either sex, I did not detect any changes to pCREB expression in the NCM for any stimuli duration. I determined that unlike pERK, pCREB is not song-inducible. There are some limitations to this study. First, unlike Cheng and Clayton (2004) I was using immunohistochemistry and cell counting rather than western blot for protein quantification to determine expression patterns. I chose this method for parallelism with serial sections of Egr-1 expression and pCREB from the aromatase inhibition study. Chang and Clayton performed a more global measure by sampling from the entire auditory lobule (which contains many regions). With immunohistochemistry, I targeted specific subregions of the auditory lobule. The changes in auditory-induced phosphorylation may be represented throughout the auditory lobule in overall protein content, rather than a change in immunoreactive cell number in specific subregions. To determine whether pCREB is definitively not regulated similar to pERK by song

playback, I would need to replicate Chang and Clayton's methods to rule out global changes in overall protein content.

Intracellular signaling mechanisms may also explain why I only found that GPER1 inactivation decreased auditory-evoked neuron firing but activation was not sufficient to enhance it as shown with estradiol (Ramage-Healey et al., 2010; 2012). One hypothesis is that GPER1 activity may not always reflect estradiol activity. Although other endogenous ligands have not been found to activate the GPER1 receptor, there are cases of E2 and GPER1 acting through separate pathways to exert their cellular actions. In the mammalian hippocampus, both estradiol and GPER1 enhance memory consolidation (Briz et al., 2015; Kumar et al., 2015; Waters et al., 2015; Kim et al., 2016); however, while estradiol acts through ERK signaling, GPER1 activation instead initiates phosphorylation of the JNK pathway (Kim et al., 2016). This indicates that while measuring behavior, estradiol and GPER1 activation may have similar endpoints, GPER1 could be acting independently of estradiol. To test specificity of the GPER1 receptor in auditory firing, I would need to administer the antagonist and see if it blocks the enhancement effect of estradiol. Future experiments using western blots for pERK could determine if E2 and a GPER1 agonist can increase the phosphorylation of this known auditory pathway. Finally, I only found GPER1 specific effects in narrow cells of males. GPER1 inactivation or activation had no effects in females, even though estradiol enhancement of auditory-evoked firing occurs in both sexes (Ramage-Healey et al., 2010; 2012). If GPER1 is acting through independent mechanisms of estradiol, the sex difference I report may be a clue into these

independent actions, especially since both males and females equally express GPER1 receptor at similar densities in NCM (Chapter 2).

Cell type specificity in auditory cortical regions

Many of the sex differences I report are dependent on cell types or regional differences within the auditory forebrain. Specifically, I found sex differences in cell types when neurons are defined on physiological parameters (ie. action potential width; Chapter 4), but not neurochemical markers. I also detected region-specific sex differences of aromatase inhibition on auditory-induced Egr-1 expression. In chapter 2, I characterized estrogen-producing (aromatase-positive) and estrogen-responsive (GPER1-positive) cell types in the auditory lobule, with a specific focus on coexpression with markers for GABAergic cell types comparing males and females. I did not detect any sex differences in either aromatase or GPER1 cell density or coexpression patterns of these cells with any of the GABAergic markers I tested. These neurochemical markers for estrogen-producing (aromatase) and estrogen-receiving (GPER1) cells had differential expression based on regions within the auditory lobule (Chapter 2). Given that I have shown physiological sex differences of how cell types are representing songs, responsiveness to GPER1 inactivation, and dependency on acute estradiol production, this anatomy similarity reveals that the sex differences I have reported are likely not due to differences in overall expression of the cell types I examined. These sex differences instead could be explained by intracellular signaling, other cell types, or larger network properties of cellular communication.

I did detect several regional differences with aromatase, GPER1, calbindin, and parvalbumin. Many of the regions I analyzed have been previously unreported for these

markers, and this study informs the field on the expected densities of these cell types and where they can be found. A notable regional difference was the expression pattern of GPER1 as compared to aromatase. I find aromatase primarily in auditory regions of the brain, such as NCM and HVC shelf. Exceptions to this are CMM and Field L, which are absent of somatic aromatase expression. However, GPER1 is expressed in both regions. GPER1 has previously been reported in regions that are involved in motor production of song such as HVC and the arcopallium which contains RA (both nuclei are sexually dimorphic in size; Acharya and Veney, 2012). I confirmed this through visual observation of these regions; however, I did not quantify the density of expression since this was outside the scope of this study. I did however quantify the expression of GPER1 in auditory region Field L and CMM. Considering that I do not find somatic aromatase expression in these regions, estradiol signaling can come from two sources: 1) peripheral estradiol or 2) terminal aromatase. Electron microscopy studies would be ideal to determine if aromatase is expressed in terminals that synapse with GPER1 expressing dendrites and somas in these regions. Alternatively, tracing studies can also be performed to determine if these regions receive aromatase expressing projections from the auditory regions like NCM and HVC shelf. Most regions of the auditory lobule have reciprocal connections with each other (Vates et al., 1996), however, the identities of these projections are largely unknown. Given the results I report in chapter 2, where CMM did not have considerable changes to auditory-induced Egr-1 expression when given fadrozole, I think it is unlikely that terminal aromatase activity is participating in a significant way to CMM auditory physiology. E2 in CMM does not alter auditory properties of CMM or upstream HVC selectivity

(Remage-Healey and Joshi, 2012), indicating that CMM has limited capacity of rapid estradiol synthesis or responses, although there is some evidence for changes with season hormones (Caras et al., 2015).

My findings of GPER1 expression are largely consistent with a previous characterization of GPER1 message and protein in the zebra finch forebrain (Acharya and Veney, 2012). In chapter 2, I attempted to characterize GPER1 neurons by examining coexpression with interneuron subtypes parvalbumin and calbindin, yet I could not find coexpression in any region. When I turned to GAD67, which is a more general marker for GABAergic neurons (although notably this antibody was not tagging all GABAergic cells), I found modest coexpression. Generally, this study has ruled out many potential candidates of what types of cells express GPER1. For aromatase, I found modest expression with parvalbumin in some subregions and no coexpression with calbindin in any subregion. There are other interneuron subtypes that have been characterized in the mammalian cortex that have functional significance in encoding different auditory phenomenon such as somatostatin and VIP (Pi et al., 2013; Chen et al., 2015; Phillips and Hasenstaub, 2016). I chose these markers specifically because of the coexpression seen with aromatase in the human temporal cortex (Yague et al., 2006), which is analogous to some of the areas I analyzed; however, further exploration into other interneuron markers may reveal sex differences in GPER1 expression. While I focused on interneurons because of the specificity of GPER1 effects I found in NS neurons of males (putative inhibitory neurons), excitatory cell types may reveal underlying sex differences in the neuroanatomy of auditory forebrain regions.

In general, cell types were an important predictor for sex differences in auditory responsiveness, encoding, and response to neuromodulation when I defined cells based on physiological parameters such as action potential shape and action potential width (Chapter 4). For the recordings, I found a clear delineation of the neurons, where broad-spiking (BS) cells ($>0.5\text{ms}$) had a more unipolar, long peak shape and narrow-spiking (NS) cells ($<0.5\text{ms}$) maintained the more canonical action potential shape with a clear peak and a trough. Both cell types were auditory-responsive, and I detected sex differences in zscore of these cell types, where male NS neurons had higher auditory responsiveness than BS cells, but females cell types were similarly responsive at the level of male NS neurons.

BS cells and NS cells (defined by action potential width) are putatively excitatory projection neurons and interneurons, respectively (Atencio and Schreiner, 2008), although the reliability width for identification can depend on neuron size and distance (Henze et al., 2000; Gonzalez-Burgos et al., 2005; Gold et al., 2006). I have some competing evidence of whether these identifications fit with previous literature. One measure, latency to fire after stimulus, was found to be higher in BS neurons of NCM (Ono et al., 2016). I used the first high-amplitude note of all the songs I presented to the animals, and measured the latency for that cell to fire after the onset of that note. BS cells had a significantly higher latency than NS cells, supporting the hypothesis that these are excitatory projection neurons. However, previous work in NCM has identified that broad cells had a lower firing frequency than narrow cells (Schneider and Woolley, 2013; Ono et al., 2016; Yanagihara and Yazaki-Sugiyama, 2016). It is thought that narrow cells are fast-spiking interneurons; but from my work, narrow cells were

consistently lower in firing frequency than broad cells for spontaneous firing rate, and NS and BS cells did not differ in stimulus-evoked firing rate. None of the previous papers used females in their recordings, and I found the largest difference in spontaneous firing in cells recorded from females. I also have a smaller cell number than previous groups, as I was using single electrode recordings rather than multielectrode arrays. The difference in mean spontaneous firing rate is quite small in other papers (NCM-NS, 3.85 ± 3.36 Hz, NCM-BS 1.95 ± 2.06 Hz, Ono et al. 2016), so it is possible that for males, if I collected more neurons then I might have been able to detect this difference. There are other examples of inconsistencies in firing rate predicting excitatory/inhibitory cell identity. In rhesus macaque cortical neurons, fast-spiking cells (higher firing frequency) have also been found to be excitatory (Vigneswaran et al., 2011). Future work will need to determine the exact identity of NS and BS cells within NCM, and I propose that sex is an important factor when clarifying these identities given the magnitude difference I described between males and females in spontaneous firing rate and the sex differences in auditory responsiveness.

I also determined that NS neurons, for both sexes, had higher classifier accuracy for discriminating between novel, conspecific songs. This is the first report of cell specific differences in accuracy coding for conspecific songs, although previous reports have found that this is true for tones (Caras et al., 2015) and calls (Giret et al., 2015) in Field L and CMM. I also found that NCM neurons overall have more consistent temporal firing (timing accuracy) to distinguish between the song types than firing frequency (count accuracy). For NS neurons, most of the cells were significant than chance for both timing accuracy and count accuracy, which I labeled as bicoding cells

(87%, Chapter 4). This is consistent with Caras et al. (2015) which reported that activity of Field L/CMM neurons in response to different amplitudes for tones, were more biased towards timing accuracy than count accuracy and also identified bicoding neurons. Field L and CMM both send projections to NCM (Vates et al., 1996). This consistency may reflect an inherent property of auditory neurons in the forebrain or results from projections from Field L/CMM.

I also determined that NS cells of NCM in males were sensitive to GPER1 inactivation. Application of the antagonist, G36, decreased NS neuron firing rate, auditory responsiveness (zscore), timing accuracy, and counting accuracy. I propose two main hypotheses that may contribute to this sex by cell type difference. One is that GPER1 maybe differentially expressed on inhibitory interneurons. I ruled out some candidates that could explain this (parvalbumin, calbindin, and the subtype marked by the anti-GAD67 antibody), however, this did not encompass the entirety of inhibitory cell types. Since I was infusing the antagonist into the brain, there was no cell specific modulation occurring in the preparation. The sex by cell type difference I found in GPER1 inactivation, may be a result of local network connections within NCM, where neighboring cells that were not recorded had changes in their firing properties that passed down and selectively changed NS neurons in males. This connectivity may be absent in females or uninfluenced by GPER1. Schneider and Woolley (2013) propose a feedforward inhibition model for auditory encoding in NCM, where both BS and NS neurons receive projections from Field L, but NS (presuming these are inhibitory) can synapse onto BS neurons as a brake mechanism. In this electrophysiology study, I observed that males had a decrease in firing frequency with GPER1 inactivation.

GPER1 may be serving a role in maintaining firing rate and auditory coding of NS cells either through expression on these cell types or excitatory neurons that synapse onto NS neurons.

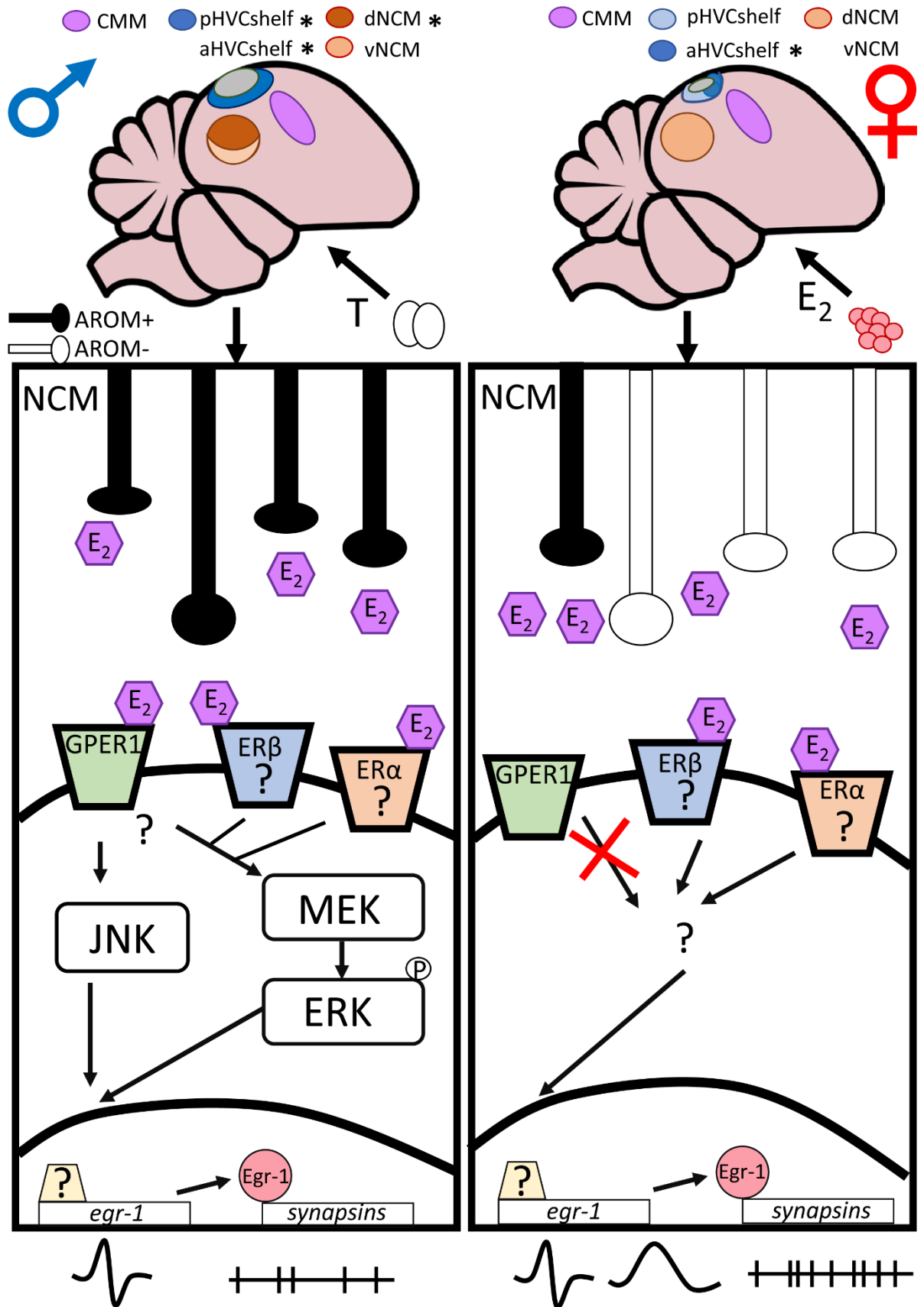


Figure 26: Auditory processing and rapid estrogen modulation of auditory neurons have sex differences in the zebra finch brain.

Mechanistic models for how estrogen is modulating auditory responsiveness in the brain for both sexes. Male is the left panel represented by the blue Mars symbol (♂), and female is the right panel represented by the red Venus symbol (♀). Sagittal views of zebra finch brain have representative auditory brain regions of interest color coded (purple=CMM, blues=HVCshelf, oranges=NCM, gray=HVC). Darker colors are representing region with a significant difference during fadrozole treatment from Chapter 3. These are also marked by an asterisks (*). Testes and ovaries are depicted outside of the brain with either testosterone (T) or estradiol (E2) entering the brain via the periphery. Each box represents a view of a hypothetical synapse. Terminals are color coded as black for aromatase-positive and white for aromatase-negative. Within the cell membrane, proposed receptor types that could be mediating audition are depicted. Question marks (?) represent gaps in the literature. Black arrows are depicting downstream pathways. Within the nucleus, upregulation of Egr-1 is depicted by an unknown transcription factor. Egr-1 is expressed and acts as a transcription factor on the synapsin genes. These synapsins can then be increased or decreased depending on the role Egr-1 has as a regulator. Synapsins are involved in memory formation and plasticity in the brain. Finally, each post-synaptic cell has a waveform that depicts the NS and BS cell types I found in extracellular recordings (Chapter 4), as well as a spike train to depict sex differences in overall firing frequency of auditory neurons. Together, the figure depicts that males have more brain regions that have significant deficits in auditory responsiveness with inhibition of aromatase. This may be due to more dynamic synthesis via abundant aromatase expressing fibers. GPER1 is also included in the male model as a receptor that is key in audition; which kinase pathway it is acting through is unknown. ER α and ER β are included in both the male and female model with question marks because of the proposed hypothesis of multiple ERs enhancing audition and the unknown nature of how either plays a role. GPER1 is excluded from the female model by a red X because of the lack of effects observed in Chapter 3. Intracellular signaling pathways are also excluded because these have not been investigated in females. Finally, both NS and BS cell types are included in the female model because of the similar auditory responsiveness (zscore) of both cell types for females, and the higher firing frequency female cells are depicted by the spike train. For the male model, only NS cell type is depicted because of NS cells having higher auditory responsiveness than BS in males, as well as a lack of an effect of GPER1 in BS cells in males, where GPER1 inactivation have robust effects in male NS cells. Brain outlines made by Dan Vahaba.

Translational implications

Vocal learning has evolved independently in birds (songbirds, parrots, and hummingbirds) and mammals (humans, bats, elephants, whales and dolphins). Many comparisons have been made between the neuroanatomical structure and function of songbird auditory and motor production pathways and human equivalents (Jarvis, 2006; Bolhuis et al., 2010; Petkov and Jarvis, 2012). In this dissertation, I primarily focused on structures analogous to secondary auditory cortex and Wernicke's area of the human temporal cortex. Aromatase has been described in the human cerebral cortex from fetuses (Montelli et al., 2012) and adults (Yague et al., 2006). The human temporal cortex contains aromatase in neurons of all cortical layers (I-VI). This expression exists in somas, fibers, and terminal boutons (Yague et al., 2006). Although most of the aromatase neurons were pyramidal, there was some coexpression with parvalbumin and calbindin interneurons. I also found expression of aromatase with parvalbumin (~15% in NCMv) but no coexpression with calbindin, indicating species similarities and differences between songbirds and humans in aromatase cell types. To date there has not been any sex comparisons of aromatase protein expression in the human brain; however, PET imaging gives some insights into the location of large aromatase densities. Using radioactive vorozole (an inhibitor of aromatase), the highest signal is in the brain of human males, and in the ovulating ovary and brain of human females (Biegon et al., 2015). Given the specific sex differences I find in auditory induced Egr-1 expression after fadrozole treatment as well as sex differences in aromatase clustering, exploring how aromatase is expressed differently between males and females of the human brain is worth pursuing.

Although it is unclear the role that rapid estrogen signaling plays in human audition and language processing, it is known that the human brain is capable of widespread steroidogenesis (Stoffel-Wagner, 2003) and human hearing and speech development is sensitive to changes in hormones. Hormone replacement therapy has been found to be useful in offsetting the degradation of hearing in aging (Charitidi et al., 2009). Unbound, bioactive estradiol in infancy correlates with predictors of later language development (Wermke et al., 2014; Quast et al., 2016). Testosterone administered in adulthood (which can be converted into estradiol) also has associations with strengthening the functional connectivity between Wernicke's and Broca's area (Hahn et al., 2016). Membrane estrogen receptor expression has not been characterized in the human brain although it has in other tissues (Srivastava and Evans, 2013); however, GPER1 expression is described in primate cortex of rhesus macaques (Crimins et al., 2017). The human brain likely has the capabilities of rapid estradiol modulation of auditory-related events in cortical neurons; however, this will need to be further studied.

Although single unit recordings of human cortex are rare, there has been one study that examined cells from the primary auditory cortex (A1) of two subjects (Ossmy et al., 2015). Using similar pattern classification methods that I described in Chapter 4, Ossmy et al. (2015) found that A1 single units have unique spike patterns for individual words. I found that in zebra finch auditory neurons of NCM, there are cell type differences in auditory accuracy of conspecific song and GPER1 inactivation decreased accuracy encoding of songs in males specifically. GPER1 may play a similar role in

auditory encoding of learned vocalizations in the human cortex as well, although further exploration will need to characterize the cytoarchitecture in the human temporal cortex.

Sex as a biological factor in neuroscience

This dissertation provides an example of the importance of studying sex as a biological variable within basic science. There are still biases within neuroscience and biology where either males are exclusively studied or sex is not reported (Beery and Zucker, 2011). I demonstrate that in sensory regions of the brain, where both males and females have similar gross neuroanatomy and cellular identities, there are sex differences in the mechanisms by which auditory activity is regulated via rapid estradiol signaling. I also have demonstrated that despite an anatomical similarity, auditory-evoked activity can be sex dependent on physiological parameters to characterize cells (ie. Action potential width). These findings broadly demonstrate the importance of using electrophysiological techniques and measuring activity-dependent proteins to determine whether sex differences exist.

This dissertation also provides a framework to address sex differences from a mechanistic perspective. By determining the mechanism by which endogenous estrogens are impacting auditory processing and using sex as a biological variable within these questions, I determined critical similarities and differences in males and females. These have generated several hypotheses, challenging the understanding of how auditory physiology is maintained in the adult brain and the estrogen receptors that mediate this neuromodulation.

The NIH has recently called on basic science researchers to include males and females in all studies unless explicitly justified otherwise (Clayton and Collins, 2014). I

argue that when considering songbirds as a model for complex auditory processing that resembles human processing of language, both males and females need to be considered. There is a large bias within the songbird field that favors male zebra finches over females because males exclusively sing, and females do not. However, both males and females need to hear and discriminate songs, and arguably females are an excellent auditory model because zebra finch song is used exclusively in courtship to which the females are selective. I provide evidence for sex differences not only in cell type responsiveness to song, but also mechanisms by which estradiol is changing song responsiveness in the brain. When considering how brain-derived estrogens and rapid estrogen signaling mechanisms may impact human interpretation of sounds and higher order language processing, the evidence provided here gives a strong argument to consider sex as a biological variable. This dissertation is an example of how considering sex within basic science research may inform future studies and hypotheses in translational models – in particular sensory perception and encoding.

More broadly, this dissertation also argues that gross anatomical investigations are not sufficient to deem males and females “similar” in terms of neuronal mechanisms and function. Despite not detecting sex differences in cell identity of estrogen-producing and estrogen-receiving cell populations (Chapter 2); when perturbing these cell types via inhibitors (Chapters 3&4), I demonstrated robust decreases in auditory responsiveness in males as opposed to females. While there have been robust sex differences in anatomical descriptions of the human brain (Ritchie et al., 2017), there is controversy over the significance of these anatomical differences. I propose that ruling out sex difference from anatomy alone is ignoring mechanism, such as

neuromodulation, that may underlie neuronal communication. Importantly, sex differences in mechanism does not always reflect a sex difference in behavior. Some sex differences are developed to constrain males and females to be more like each other in behavior, and the brain needs to utilize differentiated mechanisms as a response to sex differences in genes or hormones (De Vries, 2004). Analyzing sex related to brain regions that do not have explicit sex differences in behavior may still be a valuable pursuit for understanding the underlying biology.

I encourage other researchers to consider sex when investigating activity dependent processes in sensory regions of cortical or cortical-like structures in their model species. Although sensory regions are not traditionally considered “reproductive centers” as nuclei in the hypothalamus which control sex behavior, the interactions males and females have within reproductive contexts depend on several sensory regions: whether it is olfaction in rodents, audition and vision in birds, or somatosensation across animals.

Conclusions

As more is understood concerning the neuromodulatory actions of molecules like estradiol that influence neuronal firing and signal transduction in different brain areas, sex is a critical factor when disentangling the mechanisms facilitating these processes. While much work has encapsulated rapid actions in reproduction regions such as the hypothalamus and memory areas like the hippocampus, this dissertation shows how sexually-differentiated mechanisms are impacting sensory encoding of complex signals such as learned vocalizations. It appears that local microcircuitry and cell-specific mechanisms are important in the contribution to auditory responsiveness,

and that males and females may be differentiated in how these network and cell types connect to represent complex sensory information.

BIBLIOGRAPHY

- Abraham IM, Herbison AE (2005) Major sex differences in non-genomic estrogen actions on intracellular signaling in mouse brain in vivo. *Neuroscience* 131:945-951.
- Abraham IM, Todman MG, Korach KS, Herbison AE (2004) Critical in vivo roles for classical estrogen receptors in rapid estrogen actions on intracellular signaling in mouse brain. *Endocrinology* 145:3055-3061.
- Acharya KD, Veney SL (2012) Characterization of the G-protein-coupled membrane-bound estrogen receptor GPR30 in the zebra finch brain reveals a sex difference in gene and protein expression. *Dev Neurobiol* 72:1433-1446.
- Adkins-Regan E, Abdelnabi M, Mobarak M, Ottinger MA (1990) Sex steroid levels in developing and adult male and female zebra finches (*Poephila guttata*). *Gen Comp Endocrinol* 78:93-109.
- Adkins-Regan E, Leung CH (2006) Sex steroids modulate changes in social and sexual preference during juvenile development in zebra finches. *Horm Behav* 50:772-778.
- Adkins-Regan E, Mansukhani V, Seiwert C, Thompson R (1994) Sexual differentiation of brain and behavior in the zebra finch: critical periods for effects of early estrogen treatment. *J Neurobiol* 25:865-877.
- Almey A, Milner TA, Brake WG (2016) Estrogen receptor alpha and G-protein coupled estrogen receptor 1 are localized to GABAergic neurons in the dorsal striatum. *Neurosci Lett* 622:118-123.
- Anthoni H et al. (2012) The aromatase gene CYP19A1: several genetic and functional lines of evidence supporting a role in reading, speech and language. *Behav Genet* 42:509-527.
- Armstead WM, Kiessling JW, Riley J, Kofke WA, Vavilala MS (2011) Phenylephrine infusion prevents impairment of ATP- and calcium-sensitive potassium channel-mediated cerebrovasodilation after brain injury in female, but aggravates impairment in male, piglets through modulation of ERK MAPK upregulation. *J Neurotrauma* 28:105-111.
- Arnold AP (1997) Experimental analysis of sexual differentiation of the zebra finch brain. *Brain Res Bull* 44:503-507.
- Arnold AP, Xu J, Grisham W, Chen X, Kim YH, Itoh Y (2004) Minireview: Sex chromosomes and brain sexual differentiation. *Endocrinology* 145:1057-1062.

- Aronica SM, Kraus WL, Katzenellenbogen BS (1994) Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. *Proc Natl Acad Sci U S A* 91:8517-8521.
- Atencio CA, Schreiner CE (2008) Spectrotemporal processing differences between auditory cortical fast-spiking and regular-spiking neurons. *J Neurosci* 28:3897-3910.
- Avey MT, Phillmore LS, MacDougall-Shackleton SA (2005) Immediate early gene expression following exposure to acoustic and visual components of courtship in zebra finches. *Behav Brain Res* 165:247-253.
- Bailey DJ, Wade J (2003) Differential expression of the immediate early genes FOS and ZENK following auditory stimulation in the juvenile male and female zebra finch. *Brain Res Mol Brain Res* 116:147-154.
- Bailey DJ, Wade J (2005) FOS and ZENK responses in 45-day-old zebra finches vary with auditory stimulus and brain region, but not sex. *Behav Brain Res* 162:108-115.
- Bakker J, Pierman S, Gonzalez-Martinez D (2010) Effects of aromatase mutation (ArKO) on the sexual differentiation of kisspeptin neuronal numbers and their activation by same versus opposite sex urinary pheromones. *Horm Behav* 57:390-395.
- Ball GF, Wade J (2013) The value of comparative approaches to our understanding of puberty as illustrated by investigations in birds and reptiles. *Horm Behav* 64:211-214.
- Balthazart J, Baillien M, Ball GF (2001a) Rapid and reversible inhibition of brain aromatase activity. *J Neuroendocrinol* 13:63-73.
- Balthazart J, Baillien M, Ball GF (2001b) Phosphorylation processes mediate rapid changes of brain aromatase activity. *J Steroid Biochem Mol Biol* 79:261-277.
- Balthazart J, Baillien M, Ball GF (2006) Rapid control of brain aromatase activity by glutamatergic inputs. *Endocrinology* 147:359-366.
- Balthazart J, Cornil CA, Charlier TD, Taziaux M, Ball GF (2009) Estradiol, a key endocrine signal in the sexual differentiation and activation of reproductive behavior in quail. *J Exp Zool A Ecol Genet Physiol* 311:323-345.
- Barabas K, Szego EM, Kaszas A, Nagy GM, Juhasz GD, Abraham IM (2006) Sex differences in oestrogen-induced p44/42 MAPK phosphorylation in the mouse brain in vivo. *J Neuroendocrinol* 18:621-628.

- Balthazart J, Tlemcani O, Ball GF (1996) Do sex differences in the brain explain sex differences in the hormonal induction of reproductive behavior? What 25 years of research on the Japanese quail tells us. *Horm Behav* 30:627-661.
- Beech JR, Beauvois MW (2006) Early experience of sex hormones as a predictor of reading, phonology, and auditory perception. *Brain Lang* 96:49-58.
- Beery AK, Zucker I (2011) Sex bias in neuroscience and biomedical research. *Neurosci Biobehav Rev* 35:565-572.
- Bell BA, Phan ML, Vicario DS (2015) Neural responses in songbird forebrain reflect learning rates, acquired salience, and stimulus novelty after auditory discrimination training. *J Neurophysiol* 113:1480-1492.
- Benoit AM, Inskoop EK, Dailey RA (1992) Effect of a nonsteroidal aromatase inhibitor on in vitro and in vivo secretion of estradiol and on the estrous cycle in ewes. *Domest Anim Endocrinol* 9:313-327.
- Bergan JF (2015) Neural Computation and Neuromodulation Underlying Social Behavior. *Integr Comp Biol* 55:268-280.
- Bergan JF, Ben-Shaul Y, Dulac C (2014) Sex-specific processing of social cues in the medial amygdala. *Elife* 3:e02743.
- Bessinis DP, Dalla C, Kokras N, Pitychoutis PM, Papadopoulou-Daifoti Z (2013) Sex-dependent neurochemical effects of environmental enrichment in the visual system. *Neuroscience* 254:130-140.
- Biegon A, Alexoff DL, Kim SW, Logan J, Pareto D, Schlyer D, Wang GJ, Fowler JS (2015) Aromatase imaging with [N-methyl-11C]vorozole PET in healthy men and women. *J Nucl Med* 56:580-585.
- Bitan T, Lifshitz A, Breznitz Z, Booth JR (2010) Bidirectional connectivity between hemispheres occurs at multiple levels in language processing but depends on sex. *J Neurosci* 30:11576-11585.
- Black JM (1996) *Partnerships in Birds: The Study of Monogamy*. USA: Oxford University Press.
- Blaustein JD, Lehman MN, Turcotte JC, Greene G (1992) Estrogen receptors in dendrites and axon terminals in the guinea pig hypothalamus. *Endocrinology* 131:281-290.
- Bolhuis JJ, Gahr M (2006) Neural mechanisms of birdsong memory. *Nat Rev Neurosci* 7:347-357.

- Bolhuis JJ, Moorman S (2015) Birdsong memory and the brain: in search of the template. *Neurosci Biobehav Rev* 50:41-55.
- Bolhuis JJ, Zijlstra GG, den Boer-Visser AM, Van Der Zee EA (2000) Localized neuronal activation in the zebra finch brain is related to the strength of song learning. *Proc Natl Acad Sci U S A* 97:2282-2285.
- Bosma PT, Blazquez M, Collins MA, Bishop JD, Drouin G, Priede IG, Docherty K, Trudeau VL (1999) Multiplicity of glutamic acid decarboxylases (GAD) in vertebrates: molecular phylogeny and evidence for a new GAD paralog. *Mol Biol Evol* 16:397-404.
- Boulware MI, Heisler JD, Frick KM (2013) The memory-enhancing effects of hippocampal estrogen receptor activation involve metabotropic glutamate receptor signaling. *J Neurosci* 33:15184-15194.
- Boulware MI, Kordasiewicz H, Mermelstein PG (2007) Caveolin proteins are essential for distinct effects of membrane estrogen receptors in neurons. *J Neurosci* 27:9941-9950.
- Boulware MI, Weick JP, Becklund BR, Kuo SP, Groth RD, Mermelstein PG (2005) Estradiol activates group I and II metabotropic glutamate receptor signaling, leading to opposing influences on cAMP response element-binding protein. *J Neurosci* 25:5066-5078.
- Brainard MS, Doupe AJ (2000) Auditory feedback in learning and maintenance of vocal behaviour. *Nat Rev Neurosci* 1:31-40.
- Braun K, Scheich H, Heizmann CW, Hunziker W (1991) Parvalbumin and calbindin-D28K immunoreactivity as developmental markers of auditory and vocal motor nuclei of the zebra finch. *Neuroscience* 40:853-869.
- Breedlove SM, Arnold AP (1983) Hormonal control of a developing neuromuscular system. I. Complete Demasculinization of the male rat spinal nucleus of the bulbocavernosus using the anti-androgen flutamide. *J Neurosci* 3:417-423.
- Brenowitz EA, Ramage-Healey L (2016) It takes a seasoned bird to be a good listener: communication between the sexes. *Curr Opin Neurobiol* 38:12-17.
- Briz V, Liu Y, Zhu G, Bi X, Baudry M (2015) A novel form of synaptic plasticity in field CA3 of hippocampus requires GPER1 activation and BDNF release. *J Cell Biol* 210:1225-1237.
- Callard GV, Petro Z, Ryan KJ (1978) Phylogenetic distribution of aromatase and other androgen-converting enzymes in the central nervous system. *Endocrinology* 103:2283-2290.

- Caras ML, Sen K, Rubel EW, Brenowitz EA (2015) Seasonal plasticity of precise spike timing in the avian auditory system. *J Neurosci* 35:3431-3445.
- Chao A, Paon A, Ramage-Healey L (2015) Dynamic variation in forebrain estradiol levels during song learning. *Dev Neurobiol* 75:271-286.
- Charitidi K, Meltser I, Tahera Y, Canlon B (2009) Functional responses of estrogen receptors in the male and female auditory system. *Hear Res* 252:71-78.
- Chen CC, Lee WR, Safe S (2004) Egr-1 is activated by 17beta-estradiol in MCF-7 cells by mitogen-activated protein kinase-dependent phosphorylation of ELK-1. *J Cell Biochem* 93:1063-1074.
- Chen IW, Helmchen F, Lutcke H (2015) Specific Early and Late Oddball-Evoked Responses in Excitatory and Inhibitory Neurons of Mouse Auditory Cortex. *J Neurosci* 35:12560-12573.
- Cheng HY, Clayton DF (2004) Activation and habituation of extracellular signal-regulated kinase phosphorylation in zebra finch auditory forebrain during song presentation. *J Neurosci* 24:7503-7513.
- Chew SJ, Mello C, Nottebohm F, Jarvis E, Vicario DS (1995) Decrements in auditory responses to a repeated conspecific song are long-lasting and require 2 periods of protein-synthesis in the songbird forebrain. *Proc Natl Acad Sci U S A* 92:3406-3410.
- Chew SJ, Vicario DS, Nottebohm F (1996) A large-capacity memory system that recognizes the calls and songs of individual birds. *Proc Natl Acad Sci U S A* 93:1950-1955.
- Clayton DF (2000) The genomic action potential. *Neurobiol Learn Mem* 74:185-216.
- Clayton JA, Collins FS (2014) Policy: NIH to balance sex in cell and animal studies. *Nature* 509:282-283.
- Cohen J (1988) *Statistical Power Analysis for Behavioral Sciences*, 2nd Edition: Lawrence Erlbaum Associates.
- Comito D, Pradhan DS, Karleen BJ, Schlinger BA (2015) Region-Specific Rapid Regulation of Aromatase Activity in Zebra Finch Brain. *J Neurochem*.
- Cornez G, ter Haar SM, Cornil CA, Balthazart J (2015) Anatomically discrete sex differences in neuroplasticity in zebra finches as reflected by perineuronal nets. *PLoS One* 10:e0123199.

- Cornil CA, Seredynski AL, de Bournonville C, Dickens MJ, Charlier TD, Ball GF, Balthazart J (2013) Rapid control of reproductive behaviour by locally synthesised oestrogens: focus on aromatase. *J Neuroendocrinol* 25:1070-1078.
- Cornil CA, Taziaux M, Baillien M, Ball GF, Balthazart J (2006) Rapid effects of aromatase inhibition on male reproductive behaviors in Japanese quail. *Horm Behav* 49:45-67.
- Corpechot C, Robel P, Axelson M, Sjovall J, Baulieu EE (1981) Characterization and measurement of dehydroepiandrosterone sulfate in rat brain. *Proc Natl Acad Sci U S A* 78:4704-4707.
- Cowan RL, Frederick BB, Rainey M, Levin JM, Maas LC, Bang J, Hennen J, Lukas SE, Renshaw PF (2000) Sex differences in response to red and blue light in human primary visual cortex: a bold fMRI study. *Psychiatry Res* 100:129-138.
- Crimins JL, Wang AC, Yuk F, Puri R, Janssen WG, Hara Y, Rapp PR, Morrison JH (2016) Diverse Synaptic Distributions of G Protein-coupled Estrogen Receptor 1 in Monkey Prefrontal Cortex with Aging and Menopause. *Cereb Cortex*.
- Cross E, Roselli CE (1999) 17beta-estradiol rapidly facilitates chemoinvestigation and mounting in castrated male rats. *Am J Physiol* 276:R1346-1350.
- Dagostin AA, Mello CV, Leao RM (2012) Increased bursting glutamatergic neurotransmission in an auditory forebrain area of the zebra finch (*Taenopygia guttata*) induced by auditory stimulation. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* 198:705-716.
- Davis S, Bozon B, Laroche S (2003) How necessary is the activation of the immediate early gene *zif268* in synaptic plasticity and learning? *Behavioural Brain Research* 142:17-30.
- Day NF, Terleski KL, Nykamp DQ, Nick TA (2013) Directed functional connectivity matures with motor learning in a cortical pattern generator. *J Neurophysiol* 109:913-923.
- de Jager T, Pelzer T, Muller-Botz S, Imam A, Muck J, Neyses L (2001) Mechanisms of estrogen receptor action in the myocardium. Rapid gene activation via the ERK1/2 pathway and serum response elements. *J Biol Chem* 276:27873-27880.
- De Vries GJ (2004) Minireview: Sex differences in adult and developing brains: compensation, compensation, compensation. *Endocrinology* 145:1063-1068.

- Dickens MJ, de Bournonville C, Balthazart J, Cornil CA (2014) Relationships between rapid changes in local aromatase activity and estradiol concentrations in male and female quail brain. *Horm Behav* 65:154-164.
- Dennis MK, Field AS, Burai R, Ramesh C, Petrie WK, Bologna CG, Oprea TI, Yamaguchi Y, Hayashi S, Sklar LA, Hathaway HJ, Arterburn JB, Prossnitz ER (2011) Identification of a GPER/GPR30 antagonist with improved estrogen receptor counterselectivity. *J Steroid Biochem Mol Biol* 127:358-366.
- Dong L, Wang W, Wang F, Stoner M, Reed JC, Harigai M, Samudio I, Kladde MP, Vyhlidal C, Safe S (1999) Mechanisms of transcriptional activation of bcl-2 gene expression by 17beta-estradiol in breast cancer cells. *J Biol Chem* 274:32099-32107.
- Duan R, Xie W, Li X, McDougal A, Safe S (2002) Estrogen regulation of c-fos gene expression through phosphatidylinositol-3-kinase-dependent activation of serum response factor in MCF-7 breast cancer cells. *Biochem Biophys Res Commun* 294:384-394.
- Eda-Fujiwara H, Satoh R, Hata Y, Yamasaki M, Watanabe A, Zandbergen MA, Okamoto Y, Miyamoto T, Bolhuis JJ (2016) Sex differences in behavioural and neural responsiveness to mate calls in a parrot. *Sci Rep* 6:18481.
- Enard W, Przeworski M, Fisher SE, Lai CS, Wiebe V, Kitano T, Monaco AP, Paabo S (2002) Molecular evolution of FOXP2, a gene involved in speech and language. *Nature* 418:869-872.
- Erlander MG, Tillakaratne NJ, Feldblum S, Patel N, Tobin AJ (1991) Two genes encode distinct glutamate decarboxylases. *Neuron* 7:91-100.
- Fan L, Zhao Z, Orr PT, Chambers CH, Lewis MC, Frick KM (2010) Estradiol-induced object memory consolidation in middle-aged female mice requires dorsal hippocampal extracellular signal-regulated kinase and phosphatidylinositol 3-kinase activation. *J Neurosci* 30:4390-4400.
- Fernandez SM, Lewis MC, Pechenino AS, Harburger LL, Orr PT, Gresack JE, Schafe GE, Frick KM (2008) Estradiol-induced enhancement of object memory consolidation involves hippocampal extracellular signal-regulated kinase activation and membrane-bound estrogen receptors. *J Neurosci* 28:8660-8667.
- Filardo EJ, Quinn JA, Bland KI, Frackelton AR, Jr. (2000) Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. *Mol Endocrinol* 14:1649-1660.

- Flower TP, Gribble M, Ridley AR (2014) Deception by Flexible Alarm Mimicry in an African Bird. *Science* 344:513-516.
- Foidart A, Tlemcani O, Harada N, Abe-Dohmae S, Balthazart J (1995) Pre- and post-translational regulation of aromatase by steroidal and non-steroidal aromatase inhibitors. *Brain Res* 701:267-278.
- Forger NG, de Vries GJ (2010) Cell death and sexual differentiation of behavior: worms, flies, and mammals. *Curr Opin Neurobiol* 20:776-783.
- Forlano PM, Deitcher DL, Bass AH (2005) Distribution of estrogen receptor alpha mRNA in the brain and inner ear of a vocal fish with comparisons to sites of aromatase expression. *J Comp Neurol* 483:91-113.
- Fortress AM, Fan L, Orr PT, Zhao Z, Frick KM (2013) Estradiol-induced object recognition memory consolidation is dependent on activation of mTOR signaling in the dorsal hippocampus. *Learn Mem* 20:147-155.
- Freking F, Ramachandran B, Schlinger BA (1998) Regulation of aromatase, 5 alpha- and 5 beta-reductase in primary cell cultures of developing zebra finch telencephalon. *J Neurobiol* 36:30-40.
- Frick KM (2013) Epigenetics, oestradiol and hippocampal memory consolidation. *J Neuroendocrinol* 25:1151-1162.
- Frick KM, Gresack JE (2003) Sex differences in the behavioral response to spatial and object novelty in adult C57BL/6 mice. *Behav Neurosci* 117:1283-1291.
- Frick KM, Kim J, Tuscher JJ, Fortress AM (2015) Sex steroid hormones matter for learning and memory: estrogenic regulation of hippocampal function in male and female rodents. *Learn Mem* 22:472-493.
- Fusani L, Van't Hof T, Hutchison JB, Gahr M (2000) Seasonal expression of androgen receptors, estrogen receptors, and aromatase in the canary brain in relation to circulating androgens and estrogens. *J Neurobiol* 43:254-268.
- Gahr M, Garcia-Segura LM (1996) Testosterone-dependent increase of gap-junctions in HVC neurons of adult female canaries. *Brain Res* 712:69-73.
- Giret N, Menardy F, Del Negro C (2015) Sex differences in the representation of call stimuli in a songbird secondary auditory area. *Front Behav Neurosci* 9:290.
- Gobes SM, Bolhuis JJ (2007) Birdsong memory: a neural dissociation between song recognition and production. *Curr Biol* 17:789-793.

- Gobes SM, Ter Haar SM, Vignal C, Vergne AL, Mathevon N, Bolhuis JJ (2009) Differential responsiveness in brain and behavior to sexually dimorphic long calls in male and female zebra finches. *J Comp Neurol* 516:312-320.
- Gold C, Henze DA, Koch C, Buzsaki G (2006) On the origin of the extracellular action potential waveform: A modeling study. *J Neurophysiol* 95:3113-3128.
- Gong A, Freking FW, Wingfield J, Schlinger BA, Arnold AP (1999) Effects of embryonic treatment with fadrozole on phenotype of gonads, syrinx, and neural song system in zebra finches. *Gen Comp Endocrinol* 115:346-353.
- Gonzalez-Burgos G, Krimer LS, Povysheva NV, Barrionuevo G, Lewis DA (2005) Functional properties of fast spiking interneurons and their synaptic connections with pyramidal cells in primate dorsolateral prefrontal cortex. *J Neurophysiol* 93:942-953.
- Goodale E, Kotagama SW (2006) Context-dependent vocal mimicry in a passerine bird. *Proc Biol Sci* 273:875-880.
- Goodwin SE, Podos J (2014) Team of rivals: alliance formation in territorial songbirds is predicted by vocal signal structure. *Biol Lett* 10:20131083.
- Grant PR, Grant BR (1997) Mating patterns of Darwin's finch hybrids determined by song and morphology. *Biol J Linn Soc* 60:317-343.
- Gresack JE, Schafe GE, Orr PT, Frick KM (2009) Sex differences in contextual fear conditioning are associated with differential ventral hippocampal extracellular signal-regulated kinase activation. *Neuroscience* 159:451-467.
- Grisham W, Arnold AP (1994) Distribution of GABA-like immunoreactivity in the song system of the zebra finch. *Brain Res* 651:115-122.
- Grisham W, Lee J, McCormick ME, Yang-Stayner K, Arnold AP (2002) Antiandrogen blocks estrogen-induced masculinization of the song system in female zebra finches. *J Neurobiol* 51:1-8.
- Grisham W, Lee J, Park SH, Mankowski JL, Arnold AP (2008) A dose-response study of estradiol's effects on the developing zebra finch song system. *Neurosci Lett* 445:158-161.
- Grove-Strawser D, Boulware MI, Mermelstein PG (2010) Membrane estrogen receptors activate the metabotropic glutamate receptors mGluR5 and mGluR3 to bidirectionally regulate CREB phosphorylation in female rat striatal neurons. *Neuroscience* 170:1045-1055.

- Gurney ME, Konishi M (1980) Hormone-induced sexual differentiation of brain and behavior in zebra finches. *Science* 208:1380-1383.
- Hahn A, Kranz GS, Sladky R, Kaufmann U, Ganger S, Hummer A, Seiger R, Spies M, Vanicek T, Winkler D, Kasper S, Windischberger C, Swaab DF, Lanzenberger R (2016) Testosterone affects language areas of the adult human brain. *Hum Brain Mapp* 37:1738-1748.
- Hahnloser RH, Kotowicz A (2010) Auditory representations and memory in birdsong learning. *Curr Opin Neurobiol* 20:332-339.
- Hall IC, Woolley SM, Kwong-Brown U, Kelley DB (2016) Sex differences and endocrine regulation of auditory-evoked, neural responses in African clawed frogs (*Xenopus*). *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* 202:17-34.
- Halpern DF, Benbow CP, Geary DC, Gur RC, Hyde JS, Gernsbacher MA (2007) The Science of Sex Differences in Science and Mathematics. *Psychol Sci Public Interest* 8:1-51.
- Hamaide J, De Groof G, Van Steenkiste G, Jeurissen B, Van Audekerke J, Naeyaert M, Van Ruijssevelt L, Cornil C, Sijbers J, Verhoye M, Van der Linden A (2017) Exploring sex differences in the adult zebra finch brain: In vivo diffusion tensor imaging and ex vivo super-resolution track density imaging. *Neuroimage* 146:789-803.
- Han S, Hong S, Mo J, Lee D, Choi E, Choi JS, Sun W, Lee HW, Kim H (2014) Impaired Extinction of Learned Contextual Fear Memory in Early Growth Response 1 Knockout Mice. *Mol Cells* 37:24-30.
- Hara E, Rivas MV, Ward JM, Okanoya K, Jarvis ED (2012) Convergent differential regulation of parvalbumin in the brains of vocal learners. *PLoS One* 7:e29457.
- Heimovics SA, Prior NH, Ma C, Soma KK (2016) Rapid Effects of an Aggressive Interaction on Dehydroepiandrosterone, Testosterone and Oestradiol Levels in the Male Song Sparrow Brain: a Seasonal Comparison. *J Neuroendocrinol* 28:12345.
- Heimovics SA, Prior NH, Maddison CJ, Soma KK (2012) Rapid and widespread effects of 17beta-estradiol on intracellular signaling in the male songbird brain: a seasonal comparison. *Endocrinology* 153:1364-1376.
- Henze DA, Borhegyi Z, Csicsvari J, Mamiya A, Harris KD, Buzsaki G (2000) Intracellular features predicted by extracellular recordings in the hippocampus in vivo. *J Neurophysiol* 84:390-400.

- Herlitz A, Rehnman J (2008) Sex Differences in Episodic Memory. *Current Directions in Psychological Science* 17:52-56.
- Holloway CC, Clayton DF (2001) Estrogen synthesis in the male brain triggers development of the avian song control pathway in vitro. *Nat Neurosci* 4:170-175.
- Horton BM, Hudson WH, Ortlund EA, Shirk S, Thomas JW, Young ER, Zinzow-Kramer WM, Maney DL (2014) Estrogen receptor alpha polymorphism in a species with alternative behavioral phenotypes. *Proc Natl Acad Sci U S A* 111:1443-1448.
- Huang GZ, Woolley CS (2012) Estradiol acutely suppresses inhibition in the hippocampus through a sex-specific endocannabinoid and mGluR-dependent mechanism. *Neuron* 74:801-808.
- Huang GZ, Zhang JJ, Wang D, Mason RT, Halpern M (2006) Female snake sex pheromone induces membrane responses in vomeronasal sensory neurons of male snakes. *Chem Senses* 31:521-529.
- Ikeda MZ, Krentzel AA, Oliver TS, Scarpa GB, Ramage-Healey L (2017) Clustered organization and region-specific identities of estrogen-producing neurons in the zebra finch (*Taeniopygia guttata*) forebrain. *J Comp Neurology*. in submission.
- Ikeda MZ, Jeon SD, Cowell RA, Ramage-Healey L (2015) Norepinephrine Modulates Coding of Complex Vocalizations in the Songbird Auditory Cortex Independent of Local Neuroestrogen Synthesis. *J Neurosci* 35:9356-9368.
- Itoh Y, Kampf K, Arnold AP (2006) Comparison of the chicken and zebra finch Z chromosomes shows evolutionary rearrangements. *Chromosome Res* 14:805-815.
- Jacobs EC, Arnold AP, Campagnoni AT (1996) Zebra finch estrogen receptor cDNA: cloning and mRNA expression. *J Steroid Biochem Mol Biol* 59:135-145.
- Jarvis ED (2004) Learned birdsong and the neurobiology of human language. *Ann N Y Acad Sci* 1016:749-777.
- Jarvis ED, Mello CV, Nottebohm F (1995) Associative learning and stimulus novelty influence the song-induced expression of an immediate early gene in the canary forebrain. *Learn Mem* 2:62-80.
- Jarvis ED, Nottebohm F (1997) Motor-driven gene expression. *Proc Natl Acad Sci U S A* 94:4097-4102.

- Jeong JK, Terleph TA, Burrows K, Tremere LA, Pinaud R (2011) Expression and rapid experience-dependent regulation of type-A GABAergic receptors in the songbird auditory forebrain. *Dev Neurobiol* 71:803-817.
- Kelley DB, Nottebohm F (1979) Projections of a telencephalic auditory nucleus-field-1 in canary. *J Comp Neurol* 183:455-469.
- Kelly MJ, Moss RL, Dudley CA (1976) Differential sensitivity of preoptic-septal neurons to microelectrophoresed estrogen during the estrous cycle. *Brain Res* 114:152-157.
- Kim J, Szinte JS, Boulware MI, Frick KM (2016) 17beta-Estradiol and Agonism of G-protein-Coupled Estrogen Receptor Enhance Hippocampal Memory via Different Cell-Signaling Mechanisms. *J Neurosci* 36:3309-3321.
- Kim JH, Jeong IY, Lim Y, Lee YH, Shin SY (2011) Estrogen receptor beta stimulates Egr-1 transcription via MEK1/Erk/Elk-1 cascade in C6 glioma cells. *BMB Rep* 44:452-457.
- Knapska E, Kaczmarek L (2004) A gene for neuronal plasticity in the mammalian brain: Zif268/Egr-1/NGFI-A/Krox-24/TIS8/ZENK? *Prog Neurobiol* 74:183-211.
- Kochak GM, Mangat S, Mulagha MT, Entwistle EA, Santen RJ, Lipton A, Demers L (1990) The pharmacodynamic inhibition of estrogen synthesis by fadrozole, an aromatase inhibitor, and its pharmacokinetic disposition. *J Clin Endocrinol Metab* 71:1349-1355.
- Konishi M (2004) The role of auditory feedback in birdsong. *Ann N Y Acad Sci* 1016:463-475.
- Konishi M, Akutagawa E (1988) A critical period for estrogen action on neurons of the song control system in the zebra finch. *Proc Natl Acad Sci U S A* 85:7006-7007.
- Kramar EA, Babayan AH, Gall CM, Lynch G (2013) Estrogen promotes learning-related plasticity by modifying the synaptic cytoskeleton. *Neuroscience* 239:3-16.
- Krentzel AA, Ramage-Healey L (2015) Sex differences and rapid estrogen signaling: A look at songbird audition. *Front Neuroendocrinol* 38:37-49.
- Krentzel AA, Ramage-Healey L (2014) Song-inducible gene expression in the songbird auditory forebrain: evidence for sex differences, regional differences, and a role for estrogen synthesis. 565.08/VV47 *Neuroscience 2014 Abstracts Online*.

- Kudo K, Qiao CX, Kanba S, Arita J (2004) A selective increase in phosphorylation of cyclic AMP response element-binding protein in hippocampal CA1 region of male, but not female, rats following contextual fear and passive avoidance conditioning. *Brain Res* 1024:233-243.
- Kumar A, Bean LA, Rani A, Jackson T, Foster TC (2015) Contribution of estrogen receptor subtypes, ERalpha, ERbeta, and GPER1 in rapid estradiol-mediated enhancement of hippocampal synaptic transmission in mice. *Hippocampus* 25:1556-1566.
- Lauay C, Gerlach NM, Adkins-Regan E, Devoogd TJ (2004) Female zebra finches require early song exposure to prefer high-quality song as adults. *Anim. Behav.* 68:1249-1255.
- Laredo SA, Villalon Landeros R, Trainor BC (2014) Rapid effects of estrogens on behavior: environmental modulation and molecular mechanisms. *Front Neuroendocrinol* 35:447-458.
- Leitner S, Voigt C, Metzdorf R, Catchpole CK (2005) Immediate early gene (*ZENK*, *Arc*) expression in the auditory forebrain of female canaries varies in response to male song quality. *J Neurobiol* 64:275-284.
- Liu P, Chen Z, Jones JA, Wang EQ, Chen S, Huang D, Liu H (2013) Developmental sex-specific change in auditory-vocal integration: ERP evidence in children. *Clin Neurophysiol* 124:503-513.
- London SE, Boulter J, Schlinger BA (2003) Cloning of the zebra finch androgen synthetic enzyme CYP17: a study of its neural expression throughout posthatch development. *J Comp Neurol* 467:496-508.
- London SE, Clayton DF (2008) Functional identification of sensory mechanisms required for developmental song learning. *Nat Neurosci* 11:579-586.
- London SE, Clayton DF (2010) Genomic and neural analysis of the estradiol-synthetic pathway in the zebra finch. *BMC Neurosci* 11:46.
- London SE, Monks DA, Wade J, Schlinger BA (2006) Widespread capacity for steroid synthesis in the avian brain and song system. *Endocrinology* 147:5975-5987.
- Luine V, Frankfurt M (2013) Interactions between estradiol, BDNF and dendritic spines in promoting memory. *Neuroscience* 239:34-45.
- Maekawa F, Tsukahara S, Kawashima T, Nohara K, Ohki-Hamazaki H (2014) The mechanisms underlying sexual differentiation of behavior and physiology in mammals and birds: relative contributions of sex steroids and sex chromosomes. *Front Neurosci* 8:242.

- Maney DL (2012) Whither the gonads? (Comment on DOI 10.1002/bies.201200081). *Bioessays* 34:1008.
- Maney DL, Cho E, Goode CT (2006) Estrogen-dependent selectivity of genomic responses to birdsong. *Eur J Neurosci* 23:1523-1529.
- Maney DL, Goode CT, Lange HS, Sanford SE, Solomon BL (2008) Estradiol modulates neural responses to song in a seasonal songbird. *J Comp Neurol* 511:173-186.
- Maney DL, Pinaud R (2011) Estradiol-dependent modulation of auditory processing and selectivity in songbirds. *Front Neuroendocrinol* 32:287-302.
- Mangiamele LA, Gomez JR, Curtis NJ, Thompson RR (2017) GPER/GPR30, a membrane estrogen receptor, is expressed in the brain and retina of a social fish (*Carassius auratus*) and colocalizes with isotocin. *J Comp Neurol* 525:252-270.
- McCarthy MM (2010) How it's made: organisational effects of hormones on the developing brain. *J Neuroendocrinol* 22:736-742.
- McCarthy MM, Arnold AP (2011) Reframing sexual differentiation of the brain. *Nat Neurosci* 14:677-683.
- McCarthy MM, Arnold AP, Ball GF, Blaustein JD, De Vries GJ (2012) Sex differences in the brain: the not so inconvenient truth. *J Neurosci* 32:2241-2247.
- McCarthy MM, Konkle AT (2005) When is a sex difference not a sex difference? *Front Neuroendocrinol* 26:85-102.
- McFadden D, Pasanen EG, Raper J, Lange HS, Wallen K (2006) Sex differences in otoacoustic emissions measured in rhesus monkeys (*Macaca mulatta*). *Horm Behav* 50:274-284.
- Mehos CJ, Nelson LH, Saldanha CJ (2016) A Quantification of the Injury-Induced Changes in Central Aromatase, Oestrogenic Milieu and Steroid Receptor Expression in the Zebra Finch. *J Neuroendocrinol* 28:12348.
- Meitzen J, Grove DD, Mermelstein PG (2012) The organizational and aromatization hypotheses apply to rapid, nonclassical hormone action: neonatal masculinization eliminates rapid estradiol action in female hippocampal neurons. *Endocrinology* 153:4616-4621.
- Meitzen J, Luoma JI, Boulware MI, Hedges VL, Peterson BM, Tuomela K, Britson KA, Mermelstein PG (2013) Palmitoylation of estrogen receptors is essential for neuronal membrane signaling. *Endocrinology* 154:4293-4304.

- Meitzen J, Mermelstein PG (2011) Estrogen receptors stimulate brain region specific metabotropic glutamate receptors to rapidly initiate signal transduction pathways. *J Chem Neuroanat* 42:236-241.
- Mello CV (2014) The zebra finch, *Taeniopygia guttata*: an avian model for investigating the neurobiological basis of vocal learning. *Cold Spring Harb Protoc* 2014:1237-1242.
- Mello CV, Clayton DF (1994) Song-induced ZENK gene expression in auditory pathways of songbird brain and its relation to the song control system. *J Neurosci* 14:6652-6666.
- Mello CV, Vicario DS, Clayton DF (1992) Song presentation induces gene expression in the songbird forebrain. *Proc Natl Acad Sci U S A* 89:6818-6822.
- Metzdorf R, Gahr M, Fusani L (1999) Distribution of aromatase, estrogen receptor, and androgen receptor mRNA in the forebrain of songbirds and nonsongbirds. *J Comp Neurol* 407:115-129.
- Micevych PE, Wong AM, Mittelman-Smith MA (2015) Estradiol Membrane-Initiated Signaling and Female Reproduction. *Compr Physiol* 5:1211-1222.
- Milner TA, McEwen BS, Hayashi S, Li CJ, Reagan LP, Alves SE (2001) Ultrastructural evidence that hippocampal alpha estrogen receptors are located at extranuclear sites. *J Comp Neurol* 429:355-371.
- Montelli S, Peruffo A, Zambenedetti P, Rossipal E, Giacomello M, Zatta P, Cozzi B (2012) Expression of aromatase P450(AROM) in the human fetal and early postnatal cerebral cortex. *Brain Res* 1475:11-18.
- Mooney R (2009) Neural mechanisms for learned birdsong. *Learn Mem* 16:655-669.
- Mooney R, Prather JF (2005) The HVC microcircuit: the synaptic basis for interactions between song motor and vocal plasticity pathways. *J Neurosci* 25:1952-1964.
- Moore AK, Wehr M (2013) Parvalbumin-expressing inhibitory interneurons in auditory cortex are well-tuned for frequency. *J Neurosci* 33:13713-13723.
- Moorman S, Mello CV, Bolhuis JJ (2011) From songs to synapses: molecular mechanisms of birdsong memory. Molecular mechanisms of auditory learning in songbirds involve immediate early genes, including zenk and arc, the ERK/MAPK pathway and synapsins. *Bioessays* 33:377-385.

- Mouterde SC, Elie JE, Mathevon N, Theunissen FE (2017) Single neurons in the avian auditory cortex encode individual identity and propagation distance in naturally degraded communication calls. *J Neurosci*.
- Naftolin F, Ryan KJ (1975) The metabolism of androgens in central neuroendocrine tissues. *J Steroid Biochem* 6:993-997.
- Naurin S, Hansson B, Hasselquist D, Kim YH, Bensch S (2011) The sex-biased brain: sexual dimorphism in gene expression in two species of songbirds. *BMC Genomics* 12:37.
- Naurin S, Hasselquist D, Bensch S, Hansson B (2012) Sex-biased gene expression on the avian Z chromosome: highly expressed genes show higher male-biased expression. *PLoS One* 7:e46854.
- Nordeen KW, Nordeen EJ, Arnold AP (1986) Estrogen establishes sex differences in androgen accumulation in zebra finch brain. *J Neurosci* 6:734-738.
- Nottebohm F, Arnold AP (1976) Sexual dimorphism in vocal control areas of the songbird brain. *Science* 194:211-213.
- Nottebohm F, Stokes TM, Leonard CM (1976) Central control of song in canary, *serinus-canarius*. *J Comp Neurol* 165:457-486.
- O'Malley BW, Means AR (1974) Female steroid hormones and target cell nuclei. *Science* 183:610-620.
- Oberlander JG, Woolley CS (2016) 17beta-Estradiol Acutely Potentiates Glutamatergic Synaptic Transmission in the Hippocampus through Distinct Mechanisms in Males and Females. *J Neurosci* 36:2677-2690.
- Olkowicz S, Kocourek M, Lucan RK, Portes M, Fitch WT, Herculano-Houzel S, Nemeč P (2016) Birds have primate-like numbers of neurons in the forebrain. *Proc Natl Acad Sci U S A* 113:7255-7260.
- Ono S, Okanoya K, Seki Y (2016) Hierarchical emergence of sequence sensitivity in the songbird auditory forebrain. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* 202:163-183.
- Ossmy O, Fried I, Mukamel R (2015) Decoding speech perception from single cell activity in humans. *Neuroimage* 117:151-159.
- Pawlisch BA, Remage-Healey L (2015) Neuroestrogen signaling in the songbird auditory cortex propagates into a sensorimotor network via an 'interface' nucleus. *Neuroscience* 284:522-535.

- Peterson RS, Yarram L, Schlinger BA, Saldanha CJ (2005) Aromatase is pre-synaptic and sexually dimorphic in the adult zebra finch brain. *Proc Biol Sci* 272:2089-2096.
- Petkov CI, Jarvis ED (2012) Birds, primates, and spoken language origins: behavioral phenotypes and neurobiological substrates. *Front Evol Neurosci* 4:12.
- Pfenning AR et al. (2014) Convergent transcriptional specializations in the brains of humans and song-learning birds. *Science* 346:1256846.
- Phan A, Gabor CS, Favaro KJ, Kaschack S, Armstrong JN, MacLusky NJ, Choleris E (2012) Low doses of 17beta-estradiol rapidly improve learning and increase hippocampal dendritic spines. *Neuropsychopharmacology* 37:2299-2309.
- Phan ML, Pytte CL, Vicario DS (2006) Early auditory experience generates long-lasting memories that may subserve vocal learning in songbirds. *Proc Natl Acad Sci U S A* 103:1088-1093.
- Phillips EA, Hasenstaub AR (2016) Asymmetric effects of activating and inactivating cortical interneurons. *Elife* 5.
- Phoenix CH, Goy RW, Gerall AA, Young WC (1959) Organizing action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig. *Endocrinology* 65:369-382.
- Pi HJ, Hangya B, Kvitsiani D, Sanders JI, Huang ZJ, Kepecs A (2013) Cortical interneurons that specialize in disinhibitory control. *Nature* 503:521-524.
- Pinaud R, Fortes AF, Lovell P, Mello CV (2006) Calbindin-positive neurons reveal a sexual dimorphism within the songbird analogue of the mammalian auditory cortex. *J Neurobiol* 66:182-195.
- Pinaud R, Tremere LA (2012) Control of central auditory processing by a brain-generated oestrogen. *Nat Rev Neurosci* 13:521-527.
- Pinaud R, Velho TA, Jeong JK, Tremere LA, Leao RM, von Gersdorff H, Mello CV (2004) GABAergic neurons participate in the brain's response to birdsong auditory stimulation. *Eur J Neurosci* 20:1318-1330.
- Pradhan DS, Yu Y, Soma KK (2008) Rapid estrogen regulation of DHEA metabolism in the male and female songbird brain. *J Neurochem* 104:244-253.
- Pratt MA, Satkunaratanam A, Novosad DM (1998) Estrogen activates raf-1 kinase and induces expression of Egr-1 in MCF-7 breast cancer cells. *Mol Cell Biochem* 189:119-125.

- Prior NH, Yap KN, Soma KK (2014) Acute and chronic effects of an aromatase inhibitor on pair-maintenance behavior of water-restricted zebra finch pairs. *Gen Comp Endocrinol* 196:62-71.
- Quast A, Hesse V, Hain J, Wermke P, Wermke K (2016) Baby babbling at five months linked to sex hormone levels in early infancy. *Infant Behav Dev* 44:1-10.
- Rauske PL, Shea SD, Margoliash D (2003) State and neuronal class-dependent reconfiguration in the avian song system. *J Neurophysiol* 89:1688-1701.
- Remage-Healey L, Coleman MJ, Oyama RK, Schlinger BA (2010) Brain estrogens rapidly strengthen auditory encoding and guide song preference in a songbird. *Proc Natl Acad Sci U S A* 107:3852-3857.
- Remage-Healey L, Dong S, Maidment NT, Schlinger BA (2011) Presynaptic control of rapid estrogen fluctuations in the songbird auditory forebrain. *J Neurosci* 31:10034-10038.
- Remage-Healey L, Dong SM, Chao A, Schlinger BA (2012) Sex-specific, rapid neuroestrogen fluctuations and neurophysiological actions in the songbird auditory forebrain. *J Neurophysiol* 107:1621-1631.
- Remage-Healey L, Jeon SD, Joshi NR (2013) Recent evidence for rapid synthesis and action of oestrogens during auditory processing in a songbird. *J Neuroendocrinol* 25:1024-1031.
- Remage-Healey L, Joshi NR (2012) Changing neuroestrogens within the auditory forebrain rapidly transform stimulus selectivity in a downstream sensorimotor nucleus. *J Neurosci* 32:8231-8241.
- Remage-Healey L, London SE, Schlinger BA (2010a) Birdsong and the neural production of steroids. *J Chem Neuroanat* 39:72-81.
- Remage-Healey L, Maidment NT, Schlinger BA (2008) Forebrain steroid levels fluctuate rapidly during social interactions. *Nat Neurosci* 11:1327-1334.
- Rensel MA, Ellis JM, Harvey B, Schlinger BA (2015) Sex, estradiol, and spatial memory in a food-caching corvid. *Horm Behav* 75:45-54.
- Rensel MA, Salwiczek L, Roth J, Schlinger BA (2013) Context-specific effects of estradiol on spatial learning and memory in the zebra finch. *Neurobiol Learn Mem* 100:41-47.
- Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER (2005) A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* 307:1625-1630.

- Rhen T, Crews D (2000) Organization and activation of sexual and agonistic behavior in the leopard gecko, *Eublepharis macularius*. *Neuroendocrinology* 71:252-261.
- Rideout EJ, Goodwin SF (2008) Sexual dimorphism: can you smell the difference? *Curr Biol* 18:R425-427.
- Riebel K (2000) Early exposure leads to repeatable preferences for male song in female zebra finches. *Proc Biol Sci* 267:2553-2558.
- Riebel K, Smallegange IM, Terpstra NJ, Bolhuis JJ (2002) Sexual equality in zebra finch song preference: evidence for a dissociation between song recognition and production learning. *Proc Biol Sci* 269:729-733.
- Ritchie SJ, Cox SR, Shen X, Lombardo MV, Reus LM, Alloza C, Harris MA, Alderson H, Hunter S, Neilson E, Liewald DC, Auyeung B, Whalley HC, Lawrie SM, Gale CR, Bastin ME, McIntosh AM, Deary IJ (2017) Sex Differences In The Adult Human Brain: Evidence From 5,216 UK Biobank Participants. *bioRxiv*.
- Roepke TA, Ronnekleiv OK, Kelly MJ (2011) Physiological consequences of membrane-initiated estrogen signaling in the brain. *Front Biosci (Landmark Ed)* 16:1560-1573.
- Rohmann KN, Schlinger BA, Saldanha CJ (2007) Subcellular compartmentalization of aromatase is sexually dimorphic in the adult zebra finch brain. *Dev Neurobiol* 67:1-9.
- Saldanha CJ, Coomaralingam L (2005) Overlap and co-expression of estrogen synthetic and responsive neurons in the songbird brain--a double-label immunocytochemical study. *Gen Comp Endocrinol* 141:66-75.
- Saldanha CJ, Schlinger BA, Micevych PE, Horvath TL (2004) Presynaptic N-methyl-D-aspartate receptor expression is increased by estrogen in an aromatase-rich area of the songbird hippocampus. *J Comp Neurol* 469:522-534.
- Saldanha CJ, Tuerk MJ, Kim YH, Fernandes AO, Arnold AP, Schlinger BA (2000) Distribution and regulation of telencephalic aromatase expression in the zebra finch revealed with a specific antibody. *J Comp Neurol* 423:619-630.
- Sampedro C, Font E, Desfilis E (2008) Size variation and cell proliferation in chemosensory brain areas of a lizard (*Podarcis hispanica*): effects of sex and season. *Eur J Neurosci* 28:87-98.
- Sanford SE, Lange HS, Maney DL (2010) Topography of estradiol-modulated genomic responses in the songbird auditory forebrain. *Dev Neurobiol* 70:73-86.

- Schlinger BA, Arnold AP (1991) Brain is the major site of estrogen synthesis in a male songbird. *Proc Natl Acad Sci U S A* 88:4191-4194.
- Schlinger BA, Arnold AP (1992) Circulating estrogens in a male songbird originate in the brain. *Proc Natl Acad Sci U S A* 89:7650-7653.
- Schneider DM, Nelson A, Mooney R (2014) A synaptic and circuit basis for corollary discharge in the auditory cortex. *Nature* 513:189-194.
- Schneider DM, Woolley SM (2013) Sparse and background-invariant coding of vocalizations in auditory scenes. *Neuron* 79:141-152.
- Schwab C, Yu S, Wong W, McGeer EG, McGeer PL (2013) GAD65, GAD67, and GABAT immunostaining in human brain and apparent GAD65 loss in Alzheimer's disease. *J Alzheimers Dis* 33:1073-1088.
- Seredynski AL, Balthazart J, Ball GF, Cornil CA (2015) Estrogen Receptor beta Activation Rapidly Modulates Male Sexual Motivation through the Transactivation of Metabotropic Glutamate Receptor 1a. *J Neurosci* 35:13110-13123.
- Sharma A, Mohammad F, Singh P (2009) Gender differences in a *Drosophila* transcriptomic model of chronic pentylentetrazole induced behavioral deficit. *PLoS One* 4:e8136.
- Shaywitz BA, Shaywitz SE, Pugh KR, Constable RT, Skudlarski P, Fulbright RK, Bronen RA, Fletcher JM, Shankweiler DP, Katz L, et al. (1995) Sex differences in the functional organization of the brain for language. *Nature* 373:607-609.
- Shen P, Schlinger BA, Campagnoni AT, Arnold AP (1995) An atlas of aromatase mRNA expression in the zebra finch brain. *J Comp Neurol* 360:172-184.
- Shen JX, Xu ZM, Yu ZL, Wang S, Zheng DZ, Fan SC (2011) Ultrasonic frogs show extraordinary sex differences in auditory frequency sensitivity. *Nat Commun* 2:342.
- Shors TJ, Pickett J, Wood G, Paczynski M (2009) Acute Stress Persistently Enhances Estrogen Levels in the Female Rat. *Stress* 3:163-171.
- Soma KK, Alday NA, Hau M, Schlinger BA (2004) Dehydroepiandrosterone metabolism by 3beta-hydroxysteroid dehydrogenase/Delta5-Delta4 isomerase in adult zebra finch brain: sex difference and rapid effect of stress. *Endocrinology* 145:1668-1677.
- Srivastava DP, Evans PD (2013) G-protein oestrogen receptor 1: trials and tribulations of a membrane oestrogen receptor. *J Neuroendocrinol* 25:1219-1230.

- Srivastava DP, Waters EM, Mermelstein PG, Kramar EA, Shors TJ, Liu F (2011) Rapid estrogen signaling in the brain: implications for the fine-tuning of neuronal circuitry. *J Neurosci* 31:16056-16063.
- Stack A, Carrier N, Dietz D, Hollis F, Sorenson J, Kabbaj M (2010) Sex differences in social interaction in rats: role of the immediate-early gene *zif268*. *Neuropsychopharmacology* 35:570-580.
- Stoffel-Wagner B (2003) Neurosteroid biosynthesis in the human brain and its clinical implications. *Ann N Y Acad Sci* 1007:64-78.
- Suva LJ, Harm SC, Gardner RM, Thiede MA (1991) In vivo regulation of *Zif268* messenger RNA expression by 17 beta-estradiol in the rat uterus. *Mol Endocrinol* 5:829-835.
- Svec LA, Wade J (2009) Estradiol induces region-specific inhibition of ZENK but does not affect the behavioral preference for tutored song in adult female zebra finches. *Behav Brain Res* 199:298-306.
- Szego CM, Davis JS (1967) Adenosine 3',5'-monophosphate in rat uterus: acute elevation by estrogen. *Proc Natl Acad Sci U S A* 58:1711-1718.
- Szego EM, Barabas K, Balog J, Szilagy N, Korach KS, Juhasz G, Abraham IM (2006) Estrogen induces estrogen receptor alpha-dependent cAMP response element-binding protein phosphorylation via mitogen activated protein kinase pathway in basal forebrain cholinergic neurons in vivo. *J Neurosci* 26:4104-4110.
- Tabatadze N, Huang G, May RM, Jain A, Woolley CS (2015) Sex Differences in Molecular Signaling at Inhibitory Synapses in the Hippocampus. *J Neurosci* 35:11252-11265.
- Tabatadze N, Sato SM, Woolley CS (2014) Quantitative analysis of long-form aromatase mRNA in the male and female rat brain. *PLoS One* 9:e100628.
- Taziaux M, Cornil CA, Balthazart J (2004) Aromatase inhibition blocks the expression of sexually-motivated cloacal gland movements in male quail. *Behav Processes* 67:461-469.
- Tchernichovski O, Schwabl H, Nottebohm F (1998) Context determines the sex appeal of male zebra finch song. *Anim Behav* 55:1003-1010.
- Terpstra NJ, Bolhuis JJ, den Boer-Visser AM (2004) An analysis of the neural representation of birdsong memory. *J Neurosci* 24:4971-4977.

- Terpstra NJ, Bolhuis JJ, Riebel K, van der Burg JM, den Boer-Visser AM (2006) Localized brain activation specific to auditory memory in a female songbird. *J Comp Neurol* 494:784-791.
- Teyler TJ, Vardaris RM, Lewis D, Rawitch AB (1980) Gonadal steroids: effects on excitability of hippocampal pyramidal cells. *Science* 209:1017-1018.
- Thompson JB, Dzubur E, Wade J, Tomaszycski M (2011) The effects of estradiol on 17beta-hydroxysteroid dehydrogenase type IV and androgen receptor expression in the developing zebra finch song system. *Brain Res* 1401:66-73.
- Tomaszycski ML, Sluzas EM, Sundberg KA, Newman SW, DeVoogd TJ (2006) Immediate early gene (ZENK) responses to song in juvenile female and male zebra finches: effects of rearing environment. *J Neurobiol* 66:1175-1182.
- Toran-Allerand CD, Guan X, MacLusky NJ, Horvath TL, Diano S, Singh M, Connolly ES, Jr., Nethrapalli IS, Tinnikov AA (2002) ER-X: a novel, plasma membrane-associated, putative estrogen receptor that is regulated during development and after ischemic brain injury. *J Neurosci* 22:8391-8401.
- Trainor BC, Finy MS, Nelson RJ (2008) Rapid effects of estradiol on male aggression depend on photoperiod in reproductively non-responsive mice. *Horm Behav* 53:192-199.
- Tremere LA, Jeong JK, Pinaud R (2009) Estradiol shapes auditory processing in the adult brain by regulating inhibitory transmission and plasticity-associated gene expression. *J Neurosci* 29:5949-5963.
- Tremere LA, Kovaleski RF, Burrows K, Jeong JK, Pinaud R (2012) Mechanistic basis and functional roles of long-term plasticity in auditory neurons induced by a brain-generated estrogen. *J Neurosci* 32:16478-16495.
- Tuscher JJ, Szinte JS, Starrett JR, Krentzel AA, Fortress AM, Ramage-Healey L, Frick KM (2016) Inhibition of local estrogen synthesis in the hippocampus impairs hippocampal memory consolidation in ovariectomized female mice. *Horm Behav* 83:60-67.
- Vanneste S, Joos K, De Ridder D (2012) Prefrontal cortex based sex differences in tinnitus perception: same tinnitus intensity, same tinnitus distress, different mood. *PLoS One* 7:e31182.
- Vates GE, Broome BM, Mello CV, Nottebohm F (1996) Auditory pathways of caudal telencephalon and their relation to the song system of adult male zebra finches (*Taenopygia guttata*). *J Comp Neurol* 366:613-642.

- Veyrac A, Gros A, Bruel-Jungerman E, Rochefort C, Kleine Borgmann FB, Jessberger S, Laroche S (2013) Zif268/egr1 gene controls the selection, maturation and functional integration of adult hippocampal newborn neurons by learning. *Proc Natl Acad Sci U S A* 110:7062-7067.
- Vierk R, Glassmeier G, Zhou L, Brandt N, Fester L, Dudzinski D, Wilkars W, Bender RA, Lewerenz M, Gloger S, Graser L, Schwarz J, Rune GM (2012) Aromatase inhibition abolishes LTP generation in female but not in male mice. *J Neurosci* 32:8116-8126.
- Vigneswaran G, Kraskov A, Lemon RN (2011) Large identified pyramidal cells in macaque motor and premotor cortex exhibit "thin spikes": implications for cell type classification. *J Neurosci* 31:14235-14242.
- Wade J, Arnold AP (2004) Sexual differentiation of the zebra finch song system. *Ann N Y Acad Sci* 1016:540-559.
- Wang H, Chen B, Wang Y, Zhou Y (2010) The sexual difference of aging-associated functional degradation in visual cortical cells of rats. *Neurosci Lett* 486:179-183.
- Washington SD, Kanwal JS (2012) Sex-dependent hemispheric asymmetries for processing frequency-modulated sounds in the primary auditory cortex of the mustached bat. *J Neurophysiol* 108:1548-1566.
- Waters EM, Thompson LI, Patel P, Gonzales AD, Ye HZ, Filardo EJ, Clegg DJ, Gorecka J, Akama KT, McEwen BS, Milner TA (2015) G-protein-coupled estrogen receptor 1 is anatomically positioned to modulate synaptic plasticity in the mouse hippocampus. *J Neurosci* 35:2384-2397.
- Wermke K, Hain J, Oehler K, Wermke P, Hesse V (2014) Sex hormone influence on human infants' sound characteristics: melody in spontaneous crying. *Biol Lett* 10:20140095.
- Williams H (2004) Birdsong and singing behavior. *Ann N Y Acad Sci* 1016:1-30.
- Wisniewski AB, Espinoza-Varas B, Aston CE, Edmundson S, Champlin CA, Pasanen EG, McFadden D (2014) Otoacoustic emissions, auditory evoked potentials and self-reported gender in people affected by disorders of sex development (DSD). *Horm Behav* 66:467-474.
- Wohlgemuth S, Adam I, Scharff C (2014) FoxP2 in songbirds. *Curr Opin Neurobiol* 28:86-93.
- Woolley SC, Doupe AJ (2008) Social context-induced song variation affects female behavior and gene expression. *PLoS Biol* 6:e62.

- Yague JG, Munoz A, de Monasterio-Schrader P, Defelipe J, Garcia-Segura LM, Azcoitia I (2006) Aromatase expression in the human temporal cortex. *Neuroscience* 138:389-401.
- Yague JG, Wang AC, Janssen WG, Hof PR, Garcia-Segura LM, Azcoitia I, Morrison JH (2008) Aromatase distribution in the monkey temporal neocortex and hippocampus. *Brain Res* 1209:115-127.
- Yanagihara S, Yazaki-Sugiyama Y (2016) Auditory experience-dependent cortical circuit shaping for memory formation in bird song learning. *Nat Commun* 7:11946.
- Yoder KM, Phan ML, Lu K, Vicario DS (2015) He hears, she hears: are there sex differences in auditory processing? *Dev Neurobiol* 75:302-314.
- Zengin-Toktas Y, Woolley SC (2017) Singing modulates parvalbumin interneurons throughout songbird forebrain vocal control circuitry. *PLoS One* 12:e0172944.
- Zann RA (1996) *The zebra finch: a synthesis of field and laboratory studies* Oxford University Press.