ANATOMICAL EXPRESSION AND FUNCTIONAL ROLE OF THE G-PROTEIN COUPLED ESTROGEN RECEPTOR 1 IN THE SONG SYSTEM OF ZEBRA FINCHES (TAENIOPYGIA GUTTATA)

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AVPV	Anteroventral periventricular nucleus
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
CMM	Caudomedial mesopallium
CNS	Central nervous system
DHT	Dihydrotestosterone
DLM	Dorsolateral nucleus of the medial thalamus
E2	Estradiol
ER	Estrogen receptor
FAD	Fadrozole
GPER1	G-protein coupled estrogen receptor 1
HVC	High Vocal Center
LMAN	Lateral magnocellular nucleus of the anterior nidopallium
mRNA	messenger RNA
NCM	Caudomedial nidopallium
NIf	Nucleus interfacialis
nXIIts	Tracheosyringeal portion of the hypoglossal nucleus
oV	Nucleus Ovoidalis
P	Post-hatching
RA	Robust nucleus of arcopallium
SDN-POA	Sexually dimorphic nucleus of the preoptic area

T	Testosterone
TP	Testosterone propionate

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CHAPTER 1

General Introduction

1.1 Sexual Differentiation and the Role of Estradiol

In mammals, sexual differentiation (i.e., development of different phenotypes in males and females (reviewed in Arnold, 2002)) is initiated by expression of the Y-linked testis determining *Sry* gene in males (reviewed in Becker et al., 2002). This gene induces differentiation of the bi-potential gonad into testes and the lack thereof will lead to formation of ovaries early in development **(Figure 1.1)**. Compared to ovaries, which remain quiescent until puberty, testes secrete greater levels of hormones earlier that guide masculine development of the male fetus. More specifically, testosterone (T) along with its metabolite dihydrotestosterone (DHT) promote development of the male body. Not only are peripheral structures significantly masculinized by testicular secretions during early development, but so are some areas of the brain. These developmental brain and peripheral changes are permanent and occur during what is known as the organizational period. Later in life, hormones can intermittently act upon circuitry that was established during early development to activate sex specific behaviors and processes (activational period) (reviewed in Becker et al., 2002).

Although some brain regions in males are masculinized by androgens, in many vertebrates, the majority occurs after T from the periphery is converted in the brain into estradiol (E2) via the enzyme aromatase (reviewed in Wade, 2001). The importance of E2 for brain masculinization is perhaps best demonstrated by a series of studies that were conducted on the

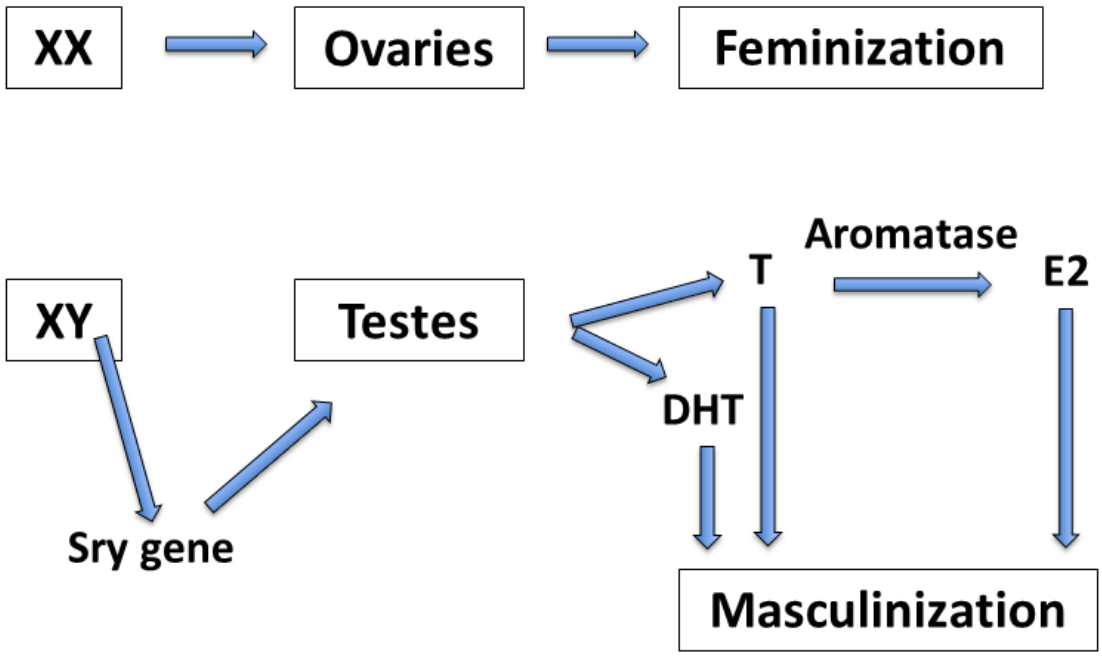

Figure 1.1 Sexual differentiation of the brain and periphery. The *Sry* gene induces formation of testes whose androgenic products drive masculine development of the periphery. E2 aromatized from T drives much of the brain masculinization. Lack of the *Sry* gene in females leads to development of ovaries and feminization of both central and peripheral structures.

sexually dimorphic nucleus of the preoptic area (SDN-POA) in rats. The SDN-POA is a hypothalamic brain region involved in the regulation of copulatory behavior in males, and is larger in this sex as compared to females (reviewed in Morris et al., 2004). Castration of newborn male rats resulted in a significant decrease in the volume of this nucleus when measured in adulthood (Gorski et al., 1978) as well as a significant decrease in male sexual activity (Anderson et al., 1986). This effect on the SDN-POA could be prevented if animals were treated with testosterone propionate (TP) (an aromatizable analog of testosterone) one day following castration (Jacobson et al., 1981). Additionally, perinatal treatment of male rats with the aromatase inhibitor ATD significantly reduced the volume of SDN-POA, which also correlated with reduced male sexual activity and preference for females (Houtsmuller et al., 1994). By comparison, DHT (a non-aromatizable androgen) does not appear to be important for sexual differentiation of this nucleus as treatment of female rats with E2 and T, but not DHT, on the day of birth significantly increased the number of cells within the SDN-POA by post-natal day 12 (Sickel and McCarthy, 2000). Treatment of female rats with TP or E2 after birth also increased their SDN-POA to a volume comparable to that of intact and TP-treated males (Gorski et al., 1978; 1981). Taken together, these findings suggest that natural differences in early E2 exposure are responsible for sexual differentiation of this nucleus.

E2 modulates sexual differentiation of the brain by regulating specific cellular processes. For example, in the SDN-POA of male rats, E2 promotes cell survival resulting in a larger volume of this nucleus as compared to females who lose a considerable portion of their neurons due to a lack of exposure to E2 (reviewed in Morris et al., 2004). E2 can also influence sexual differentiation of the brain by promoting apoptosis. A prominent example is the anteroventral periventricular nucleus (AVPV), a hypothalamic region that controls the ovarian cycle, and is

smaller in males as compared to females. E2 induces death (or prevents survival) of dopaminergic neurons within the AVPV of males as evidenced by a significant increase in the number of these neurons in estrogen receptor α (ERα) deficient male mice (Simerly et al., 1997). In addition, perinatal treatment of female rats with TP significantly decreased the number of dopaminergic neurons within the AVPV (Simerly et al., 1985), and E2 was effective in reducing the volume of AVPV when administered within the first 5 days after birth (Arai et al., 1993). Through similar cell death and survival actions E2 likely influences sexual differentiation of the brain in many other vertebrates.

1.2 The Zebra Finch Song System

In order to better understand how widely applicable the influence of E2 is on brain sexual differentiation many model species have been examined. Among these, the song circuit in zebra finches is one of the most extensively studied. For over 40 years the circuit that controls singing behavior has served as a useful comparative model for understanding how brain sex differences arise **(Figure 1.2A)**. It consists of interconnected pathways of neuronal projections starting at HVC (High Vocal Center, used as a proper name). HVC is indirectly connected to the lateral magnocellular nucleus of the anterior nidopallium (LMAN) via Area X and the dorsolateral nucleus of the medial thalamus (DLM). This pathway is responsible for song learning. A second pathway, which is responsible for song production, consists of HVC directly sending projections to the robust nucleus of arcopallium (RA), which in turn innervates the tracheosyringeal portion of the hypoglossal nucleus (nXIIts). Motoneurons within nXIIts innervate the vocal organ (syrinx) **(Figure 1.2B)** (reviewed in Reiner et al., 2004), which is located at the junction of the trachea and the two bronchi, and is supported internally by the pessulus, a central rigid tissue

providing structural support. Sound is produced during expiration when air flows past the labia, a set of vibrating membranes within the bronchi. The frequency of sound is regulated by several pairs of bilateral syringeal muscles that control the movement of an internal cartilaginous framework, thereby modulating the tension on the labia (reviewed in Goller and Riede, 2013) **(Figure 1.2C)**.

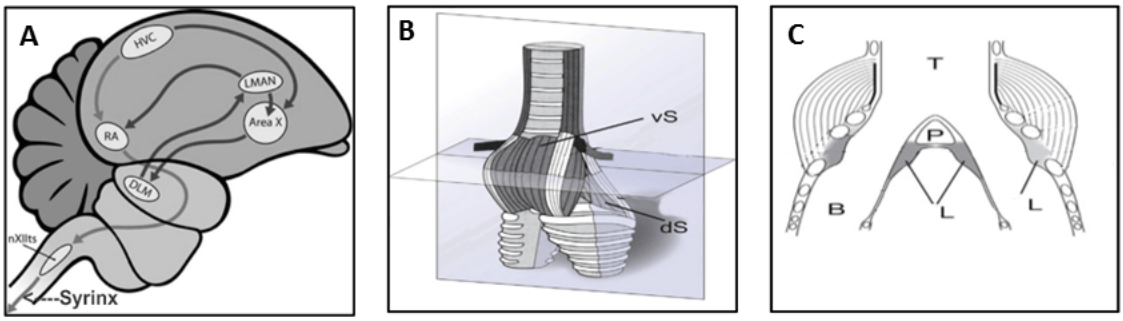

Figure 1.2 The song system in zebra finch brain. Map of the song circuit (A) Light gray arrows depict the connections that make up the major descending pathway responsible for song production. The pathway for song learning is illustrated by the dark gray arrows. **(B) External and (C) coronal views of the syrinx anatomy.** The syrinx is situated at the junction of the trachea and the two bronchi. Several groups of muscles control tension on the labia as air flows through, resulting in production of sound. The largest of these are ventralis and dorsalis. Abbreviations: T, trachea; P, pessulus; L, Labium; B, bronchi; vS, ventralis; dS, dorsalis (figures adapted from Goller and Riede, 2013).

1.3 Sex Differences in the Song System

Extensive sexual dimorphisms have been identified throughout the song circuit. For instance, the volumes of HVC, RA, and nXIIts are larger in males than in females. The number and size of neurons in HVC, RA, and LMAN are greater in males. Axonal projections from HVC to RA are more robust in males. Area X, which is easily identifiable in males, cannot be detected in females using a standard Nissl stain (reviewed in Arnold, 1992). The male syrinx has a greater mass, and the ventralis and dorsalis muscles are larger in males as compared to females (reviewed in Wade and Buhlman, 2000). In total, these male-biased dimorphisms parallel behavior; only male zebra finches normally sing. They acquire song during development by imitating that of a "tutor" (a male conspecific, typically their father) (reviewed in Scharff and Nottebohm, 1991). Juveniles first produce a "sub-song", a poorly structured vocalization that resembles song, as early as post-hatching (P) day 28. Through repeated practice, this gradually develops into a permanent "crystallized" form closer to adulthood (around P80).

1.4 Steroid Hormone Manipulations in the Brain

Since the late 1970s, studies have been conducted to understand the role of steroid hormones in shaping dimorphic features of the song system. Plasma levels of E2, T, and DHT were manipulated in female zebra finches early in development and in general, E2 was most successful in masculinizing several features of the song system (reviewed in Wade, 2001). More specifically, treatment of female hatchlings with E2 implants induced the formation of an Area X, and increased several other measures including soma sizes within HVC, LMAN, and RA, volumes of HVC, RA, and Area X, as well as the number and spacing of RA neurons (Simpson and Vicario, 1991; Adkins-Regan et al., 1994; Jacobs et al., 1995). Interestingly, these E2-treated

females were able to produce a male-like song when T was administered in adulthood to activate singing behavior (Pohl-Apel, 1985; Adkins-Regan and Ascenzi, 1987). Despite its potent masculinizing effects, however, E2 did not result in a complete sex reversal of the examined dimorphisms in any of these studies. In other words, the altered features in females were still significantly less masculine as compared to males. Regardless, since E2 had potent effects on the song system in females, follow-up studies were performed with T with the prediction that they would yield similar results. Surprisingly, they did not. In general, T was less effective than E2 in masculinizing the brain. T implants in hatchling females did increase the volumes of and soma sizes within HVC, LMAN, and RA, but not as greatly as that achieved with E2 treatment. T also did not affect the number of RA neurons or their spacing (Grisham and Arnold, 1995). By comparison, DHT had a very minimal to no masculinizing effect in this system (Nordeen and Nordeen, 1989; Schlinger and Arnold, 1991; Jacobs et al., 1995). Curiously, the role of E2 in sexual differentiation of the male brain are not as clearly defined but some masculinizing effects such as on the size of neurons in HVC and LMAN have been reported (Mathews and Arnold, 1991). Although there are a few unexplainable discrepancies, collectively these data do demonstrate a masculinizing (cell survival) function for E2 in the zebra finch neural song circuit.

1.5 Steroid Hormone Manipulations in the Syrinx

Across vertebrates, steroid hormones not only influence sexual differentiation of the CNS, but there is also evidence for their effect on peripheral structures. A well-studied example is the larynx of the African clawed frog, *Xenopus laevis*. Males have more muscle fibers within this organ, enabling them to generate courtship songs (Kelley and Tobias, 1989) that females are unable to normally produce. To determine the influence of gonadal steroid hormones on sexual

differentiation of this structure, males and females were gonadectomized and females were transplanted with testes in the area where the ovaries normally reside (Marin et al., 1990). Gonadectomy significantly reduced the number of muscle fibers in males. In females, the number of larynx muscle fibers increased following transplantation with testes. Treatment of juvenile male and female frogs with TP significantly increased the number of cells in muscle and cartilage tissues in both sexes but E2 had no effect on these measures (Sassoon et al., 1987). Treatment of females with DHT also significantly increased larynx muscle fiber number (Tobias et al., 1993). Studies such as these point to androgens as a more significant hormone acting on peripheral structures in Xenopus, but do not necessarily make them the only hormones that can serve this function.

For example, the effects of similar hormone manipulations have been investigated on peripheral structures (i.e. the syrinx) in zebra finches. T treatment resulted in a subtle, but significant increase in syrinx mass of females when administered daily for the first three weeks after hatching but this measure still remained significantly lower than that of males. DHT was ineffective in altering syrinx morphology (Wade et al., 2002). Although it is not clear how T caused a slight increase in the weight of the syrinx, given the lack of response to DHT, results do suggest that E2 aromatized from T may be involved in sexual differentiation of this organ. But interestingly, rather than masculinize, several studies have concluded that E2 acts to feminize the syrinx (Takahashi and Noumura, 1987; Wade et al., 2002). For example, male zebra finches treated with E2 for the first three weeks post-hatching experienced a significant decrease in syrinx mass and muscle fiber sizes of dorsalis. Fiber sizes in ventralis were also decreased, but only approached statistical significance (Wade et al., 2002). This same treatment did not affect

syrinx muscle fiber size or weight in females, suggesting that the female syrinx may not be responsive to additional E2 levels above that of normal.

The masculinizing effects of E2 in the brain and its feminizing effect on the syrinx (possibly through apoptotic action) clearly demonstrate its importance in sexual differentiation of the song system. The main question, however, is how does it act to affect this system? To address this the distribution of estrogen receptors has been studied.

1.6 Estrogen Receptors

Many effects of estrogens are mediated by the nuclear estrogen receptors ERα and ERβ (reviewed in Morissette et al., 2008). Both receptors are present in the mammalian and avian forebrain (reviewed in McCarthy, 2008). In general, these receptors have the greatest expression in diencephalic regions, but are also found localized to various areas of the cortex. For instance, in the developing and adult rat brain ERα protein is primarily localized to the preoptic-hypothalamic regions (where its expression is sexually dimorphic) and the amygdala (Yokosuka et al., 1997). In the developing rat brain, ERβ protein is mainly localized to the preoptic, hypothalamic, and limbic areas with scattered distribution in the cortex (Perez et al., 2003) and no sex differences reported. In adults, ERβ protein is sexually dimorphic in areas such as the medial septum, the bed nucleus of the stria terminalis, the medial preoptic area, and the hippocampus (Zhang et al., 2002). In mice, ERβ mRNA has sexually dimorphic localization within the medial basal hypothalamus and preoptic regions in the developing brain (Karolczak and Beyer, 1998) and in the medial preoptic area in adults (Wolfe et al, 2005). Whereas ERα is expressed within these same areas in the developing and adult mouse brain (Mitra et al., 2003), no dimorphisms have been reported for this receptor.

The nuclear ERs have also been identified and characterized in the zebra finch brain, and have been studied for their role in giving rise to sexually dimorphic development of the song system. In general, few neurons expressing ERα have been detected in the song circuit of zebra finches (reviewed in Wade, 2001). The distribution of ERα mRNA was investigated, and within the song circuit, only HVC showed immunohistochemical labeling between P10 and P25 in both sexes. Labeling of cells containing ERα in RA was considerably lower compared to that in HVC and this was only visible at P25. At no age was there a sex difference, and no expression of ERα was detected within LMAN or Area X (Jacobs et al., 1999). By comparison, ERβ mRNA was weakly present in several regions of the telencephalon. However, to date it has not been localized within any song control nuclei (Bernard et al., 1999; Bender and Veney, unpublished data). In the syrinx of males and females at the examined ages of P3, P10 and P17, ERα mRNA was absent, and ERβ mRNA was present exclusively in the chondrocytes of the bronchosyringeal cartilages and the pessulus but with no observed sex difference (Veney and Wade, 2005).

1.7 Estrogen Receptor Manipulations

To further evaluate the importance of E2 in dimorphic development of this system studies have been conducted to block its action at the level of the receptor. Antagonists to the nuclear estrogen receptors were employed but most failed to significantly demasculinize the brain when administered during ages when E2 treatment is known to have the greatest masculinizing effects (reviewed in Konishi and Gurney, 1982; Nordeen et al., 1986). Interestingly, some antagonists even resulted in masculinization or hypermasculinization. One example is Tamoxifen that binds both ERα and ERβ receptors (reviewed in Meyer et al., 2011). Tamoxifen was administered to male and female zebra finches daily for the first 20 days after hatching. This treatment resulted

in increased neuronal size and volumes of HVC, RA and LMAN, thus masculinizing females and hypermasculinizing males (Mathews et al., 1988). Similar results were obtained with additional ER antagonists to both ERα and ERβ (LY117018 and CI628) (reviewed in McEwen and Alves, 1999; reviewed in Bramlett and Burris, 2002). Since these studies were originally conducted, it has been demonstrated that these drugs can act as "partial agonists" and activate E2 receptors instead of completely blocking them (Jackson et al., 1997) thus supporting the idea that the discrepancies in the estrogen receptor-blocking experiments could be attributed to ineffectiveness of some drugs to act as true antagonists. In order to address this limitation, Bender and Veney (2008) used an ER inhibitor (ICI 182,780) with more specific antagonistic properties to both ERα and ERβ (Wade et al., 1993; Van Den Bemd et al., 1999; Alfinito et al., 2008). Daily ICI administration for 25 days post-hatching significantly demasculinized neuron soma size in HVC and RA of both sexes. No effect was observed in LMAN, and Area X was not analyzed. Muscle fiber size in dorsalis and ventralis in both males and females was increased following the same treatment (Martin and Veney, 2008). The overall limited expression and lack of sexual dimorphism in the nuclear estrogen receptors, as well as the less than complete reversal of dimorphic features with ICI treatment, raises the possibility that these receptors may not underlie the full scope of E2 action in this system.

1.8 Estradiol and Auditory Processing

In addition to influencing dimorphisms within the song system, E2 affects auditory function in zebra finches. In these species, the ability to hear song and process auditory stimuli is an important component of song learning. Both males and females learn song from an adult tutor (typically their father), but for slightly different reasons. In males, song is necessary for mate

attraction and nest defense. Females do not sing but use tutor song as a basis for recognizing good quality song for mate selection in adulthood (Clayton, 1988). Related to this, E2 influences the processing of auditory information (Remage-Healey et al., 2008; 2010; Vahaba et al., 2017), but it is unclear how this occurs or what receptors are involved specifically during early juvenile phases of song learning (approximately P25-60) (Chao et al., 2015). Similar to song nuclei, the nuclear estrogen receptors have limited expression in juvenile zebra finch auditory forebrain (reviewed in Maney and Pinaud, 2011) raising the possibility that other receptor mechanisms may be important.

To better understand the role of E2 in dimorphic development of the song system and its influence on auditory processing it is important to identify the receptor mechanism(s) through which it acts in these systems. The G-protein-coupled, membrane bound estrogen receptor 1 (GPER1) is a possible candidate. GPER1 protein is present in the brain (Acharya and Veney, 2012) and syrinx (unpublished observations) of developing zebra finches and its expression is sexually dimorphic within the song system at least at one examined age. At this same age GPER1 is also expressed within a major auditory region in both males and females (Acharya and Veney, 2012). In this dissertation, I more completely describe the distribution of this receptor within the neural song circuit and select auditory regions during early post-hatching ages, and examine the effects of antagonizing this receptor on song nuclei and syrinx dimorphisms.

1.9 Specific Aims of Dissertation

Aim 1. Characterize GPER1 protein expression within song nuclei and auditory regions at select post-hatching ages during early development. Within the neural zebra finch song system and auditory regions ERα and ERβ expression is limited, suggesting that another

receptor(s) plays a major role. GPER1 is a candidate, but to date there is only limited knowledge of its protein expression. My proposed study will be the first to more thoroughly describe receptor localization. This will be important for defining a functional role for this receptor in this model system. GPER1 protein expression will be semi-quantitatively described within song nuclei and select auditory regions at post-hatching days 15, 25, 30, 35, 40, and 45, which represent an age range when the brain is most sensitive to E2, dimorphisms within song control regions are detectable, and birds process auditory stimuli during song learning. **Aim 1 tests the hypothesis that GPER1 is abundantly expressed in song nuclei and auditory areas throughout early development.**

Aim 2. Determine whether intracranial antagonism of GPER1 protein will affect normal dimorphic development of song nuclei and the syrinx in zebra finches. Within the zebra finch song system, E2 appears to masculinize the brain and feminize the syrinx, but the receptor through which it acts is not known. The candidate GPER1 has greater protein expression in male HVC at P21 compared to females (Acharya and Veney, 2012). This receptor is also expressed in the syrinx of males and females at the examined ages of P15-45 (unpublished observation). Collectively these data suggest that GPER1 may influence development of the song circuit. To more directly examine this **Aim 2 tests the hypothesis that antagonism of GPER1 will demasculinize the neural song circuit and masculinize the syrinx in zebra finches.**

Aim 3. Determine whether intramuscular antagonism of GPER1 protein will affect normal dimorphic development of the song system in zebra finches. E2 is an important masculinizing hormone within the brain in zebra finches. Although it is not clear through which receptor it acts

to have effects on the system (a central question that is being investigated in this dissertation), it is also interesting that attempts to block this hormone's production and/or action have historically resulted in varying effects on the song system. More specifically, experiments involving central (directly into the brain) or in vitro manipulations have resulted in very different outcomes compared to those in vivo (via peripheral or systemic changes). This suggests that within this model system, route of drug administration is important. As a first step towards gaining a better appreciation for the importance of this concept, particularly as it relates to an understanding of how GPER1 influences dimorphic development of the song circuit, **Aim 3 hypothesizes that intramuscular injections of G-15 will demasculinize features of the song circuit, but the results from the brain will be less robust than those obtained from intracranical injections of this drug.**

1.10 References

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 72:1433-46.

Adkins-Regan E, Ascenzi M. 1987. Social and sexual behaviour of male and female zebra finches treated with oestradiol during the nestling period. Animal Behav 35:1100-12.

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. Dev Neurobiol 25: 865-877.

Alfinito PD, Chen X, Atherton J, Cosmi S, Deecher DC. 2008. ICI 182,780 penetrates brain and hypothalamic tissue and has functional effects in the brain after systemic dosing. Endocrinology 149:5219-26.

Anderson RH, Fleming DE, Rhees RW, Kinghorn E. 1986. Relationships between sexual activity, plasma testosterone, and the volume of the sexually dimorphic nucleus of the preoptic area in prenatally stressed and non-stressed rats. Brain Res 370:1-0.

Arai Y, Nishizuka M, Murakami S, Miyakawa M, Machida M, Takeuchi H, Sumida H. 1993. Morphological correlates of neuronal plasticty to gonadal steroids: sexual differentiation of the preoptic area. In: The development of sex differences and similarities in behavior. Dordrecht, Springer, pp 311-323.

Arnold AP. 1992. Developmental plasticity in neural circuits controlling birdsong: sexual differentiation and the neural basis of learning. Dev Neurobiol 23:1506-1528.

Arnold AP. 2002. Concepts of genetic and hormonal induction of vertebrate sexual differentiation in the twentieth century, with special reference to the brain. In: Hormones, brain and behavior, pp. 105-135.

Becker JB, Breedlove SM, Crews D, McCarthy MM. 2002. Behavioral endocrinology. Boston, MIT Press, pp. 76-78.

Bender AT, Veney SL. 2008. Treatment with the specific estrogen receptor antagonist ICI 182,780 demasculinizes neuron soma size in the developing zebra finch brain. Brain Res 1246:47-53.

Bernard DJ, Bentley GE, Balthazart J, Turek FW, Ball GF. 1999. Androgen receptor, estrogen receptor α, and estrogen receptor β show distinct patterns of expression in forebrain song control nuclei of european starlings. Endocrinology 140:4633-43.

Bramlett KS, Burris TP. 2002. Effects of selective estrogen receptor modulators (SERMs) on coactivator nuclear receptor (NR) box binding to estrogen receptors. Mol Genet Metab 76:225-33.

Chao A, Paon A, Remage-Healey L. 2015. Dynamic variation in forebrain estradiol levels during song learning. Dev Neurobiol 75:271-86.

Clayton NS. 1988. Song discrimination learning in zebra finches. Animal Behav 36:1016-24.

Goller F, Riede T. 2013. Integrative physiology of fundamental frequency control in birds. J Physiol Paris 107:230-42.

Gorski RA, Gordon JH, Shryne JE, Southam AM. 1978. Evidence for a morphological sex difference within the medial preoptic area of the rat brain. Brain Res 148:333-46.

Gorski RA, Csernus VJ, Jacobson CD. 1981. Sexual dimorphism in the preoptic area. In: Reproduction and Development, pp. 121-130.

Grisham W, Arnold AP. 1995. A direct comparison of the masculinizing effects of testosterone, androstenedione, estrogen, and progesterone on the development of the zebra finch song system. Dev Neurobiol 26:163-170.

Houtsmuller EJ, Brand T, De Jonge FH, Joosten RN, Van De Poll NE, Slob AK. 1994. SDN-POA volume, sexual behavior, and partner preference of male rats affected by perinatal treatment with ATD. Physiol Behav. 56:535-41.

Jacobs EC, Grisham W, Arnold, AP. 1995. Lack of a synergistic effect between estradiol and dihydrotestosterone in the masculinization of the zebra finch song system. Dev Neurobiol 27:513-519.

Jacobs EC, Arnold AP, Campagnoni AT. 1999. Developmental regulation of the distribution of aromatase-and estrogen-receptor-mRNA-expressing cells in the zebra finch brain. Dev Neurosci 21:453-72.

Jacobson CD, Csernus VJ, Shryne JE, Gorski RA. 1981. The influence of gonadectomy, androgen exposure, or a gonadal graft in the neonatal rat on the volume of the sexually dimorphic nucleus of the preoptic area. J Neurosci 1:1142-7.

Jackson TA, Richer JK, Bain DL, Takimoto GS, Tung L, Horwitz KB. 1997. The partial agonist activity of antagonist-occupied steroid receptors is controlled by a novel hinge domain-binding coactivator L7/SPA and the corepressors N-CoR or SMRT. Mol Endocrinol 11:693-705.

Karolczak M, Beyer C. 1998. Developmental sex differences in estrogen receptor-β mRNA expression in the mouse hypothalamus/preoptic region. Neuroendocrinology 68:229-34.

Kelley DB, Tobias ML. 1989. The genesis of courtship song: cellular and molecular control of a sexually differentiated behavior. Perspectives in neural systems and behavior. New York: Wiley, pp. 175-94.

Konishi M, Gurney ME. 1982. Sexual differentiation of brain and behaviour. Trends Neurosci 5:20-3.

Maney D, Pinaud R. 2011. Estradiol-dependent modulation of auditory processing and selectivity in songbirds. Front Neuroendocrinol 32:287-302.

Marin ML, Tobias ML, Kelley DB. 1990. Hormone-sensitive stages in the sexual differentiation of laryngeal muscle fiber number in Xenopus laevis. Development 110:703-11.

Mathews GA, Brenowitz EA, Arnold AP. 1988. Paradoxical hypermasculinization of the zebra finch song system by an antiestrogen. Horm Behav 22:540-551.

Mathews GA, Arnold AP. 1991. Tamoxifen's effects on the zebra finch song system are estrogenic, not antiestrogenic. Dev Neurobiol 22:957-969.

McCarthy MM. Estradiol and the developing brain. 2008. Physiol Rev 88:91-134.

McEwen BS, Alves SE. 1999. Estrogen actions in the central nervous system. Endocr Rev 20:279-307.

Meyer MR, Prossnitz ER, Barton M. 2011. The G protein-coupled estrogen receptor GPER/GPR30 as a regulator of cardiovascular function. Vasc Pharmacol 55:17-25.

Mitra SW, Hoskin E, Yudkovitz J, Pear L, Wilkinson HA, Hayashi S, Pfaff DW, Ogawa S, Rohrer SP, Schaeffer JM, McEwen BS. 2003. Immunolocalization of estrogen receptor β in the mouse brain: comparison with estrogen receptor α. Endocrinology 144:2055-67.

Morris JA, Jordan CL, Breedlove SM. 2004. Sexual differentiation of the vertebrate nervous system. Nat Neurosci 7:1034.

Morissette M, Le Saux M, D'Astous M, Jourdain S, Al Sweidi S, Morin N, Estrada-Camarena E, et al. 2008. Contribution of estrogen receptors alpha and beta to the effects of estradiol in the brain. J Steroid Biochem Mol Biol 108:327-338.

Nordeen KW, Nordeen EJ, Arnold AP. 1986. Estrogen establishes sex differences in androgen accumulation in zebra finch brain. J Neurosci 6:734-8.

Nordeen EJ, Nordeen KW. 1989. Estrogen stimulates the incorporation of new neurons into avian song nuclei during adolescence. Dev Brain Res 49:27-32.

Pérez SE, Chen EY, Mufson EJ. 2003. Distribution of estrogen receptor alpha and beta immunoreactive profiles in the postnatal rat brain. Dev Brain Res 145:117-39.

Pohl-Apel G. 1985. The correlation between the degree of brain masculinization and song quality in estradiol treated female zebra finches. Brain Res 336:381-3.

Reiner A, Perkel DJ, Bruce LL, Butler AB, Csillag A, Kuenzel W, Medina L, Paxinos G, Shimizu T, Striedter G, Wild M. 2004. The avian brain nomenclature forum: terminology for a new century in comparative neuroanatomy. J Comp Neurol 473:E1.

Remage-Healey L, Maidment NT, Schlinger BA. 2008. Forebrain steroid levels fluctuate rapidly during social interactions. Nat Neurosci 11:1327.

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 107:3852-7.

Sassoon DA, Gray GE, Kelley DB. 1987. Androgen regulation of muscle fiber type in the sexually dimorphic larynx of Xenopus laevis. J Neurosci 7:3198-206.

Scharff C, Nottebohm F. 1991. A comparative study of the behavioral deficits following lesions of various parts of the zebra finch song system: implications for vocal learning. J Neurosci 11:2896-913.

Schlinger BA, Arnold AP. 1991. Androgen effects on the development of the zebra finch song system. Brain Res 561:99-105.

Sickel MJ, McCarthy MM. 2000. Calbindin-D28k immunoreactivity is a marker for a subdivision of the sexually dimorphic nucleus of the preoptic area of the rat: developmental profile and gonadal steroid modulation. J Neuroendocrinol 12:397-402.

Simerly RB, Swanson LW, Gorski RA. 1985. The distribution of monoaminergic cells and fibers in a periventricular preoptic nucleus involved in the control of gonadotropin release: immunohistochemical evidence for a dopaminergic sexual dimorphism. Brain Res 330:55-64.

Simerly RB, Zee MC, Pendleton JW, Lubahn DB, Korach KS. 1997. Estrogen receptor-dependent sexual differentiation of dopaminergic neurons in the preoptic region of the mouse. Proc Natl Acad Sci 94:14077-82.

Simpson HB, Vicario DS. 1991. Early estrogen treatment alone causes female zebra finches to produce learned, male-like vocalizations. Dev Neurobiol 22:755-776.

Takahashi MM, Noumura T. 1987. Sexually dimorphic and laterally asymmetric development of the embryonic duck syrinx: effect of estrogen on in vitro cell proliferation and chondrogenesis. Dev Biol 121:417-422.

Tobias ML, Marin ML, Kelley DB. 1993. The roles of sex, innervation, and androgen in laryngeal muscle of Xenopus laevis. J Neurosci 13:324-33.

Vahaba DM, Macedo-Lima M, Remage-Healey L. 2017. Sensory Coding and Sensitivity to Local Estrogens Shift during Critical Period Milestones in the Auditory Cortex of Male Songbirds. eNeuro 4:ENEURO-0317.

Van Den Bemd GJ, Kuiper GG, Pols HA, Van Leeuwen JP. 1999. Distinct effects on the conformation of estrogen receptor α and β by both the antiestrogens ICI 164,384 and ICI 182,780 leading to opposite effects on receptor stability. Biochem Biophys Res Commun 261:1-5.

Veney SL, Wade J. 2005. Post-hatching syrinx development in the zebra finch: an analysis of androgen receptor, aromatase, estrogen receptor α and estrogen receptor β mRNAs. J Comp Physiol 191:97-104.

Wade GN, Blaustein JD, Gray JM, Meredith JM. 1993. ICI 182,780: a pure antiestrogen that affects behaviors and energy balance in rats without acting in the brain. Am J Physiol Regul Integr Comp Physiol 265:R1392-8.

Wade J, Buhlman L. 2000. Lateralization and effects of adult androgen in a sexually dimorphic neuromuscular system controlling song in zebra finches. J Comp Neurol 426:154-64.

Wade J. 2001. Zebra finch sexual differentiation: the aromatization hypothesis revisited. Microsc Res Tech 54:354-363.

Wade J, Buhlman L, Swender D. 2002. Post-hatching hormonal modulation of a sexually dimorphic neuromuscular system controlling song in zebra finches. Brain Res 929:191-201.

Wolfe CA, Van Doren M, Walker HJ, Seney ML, McClellan KM, Tobet SA. 2005. Sex differences in the location of immunochemically defined cell populations in the mouse preoptic area/anterior hypothalamus. Dev Brain Res 157:34-41.

Yokosuka M, Okamura H, Hayashi S. 1997. Postnatal development and sex difference in neurons containing estrogen receptor-α immunoreactivity in the preoptic brain, the diencephalon, and the amygdala in the rat. J Comp Neurol 1997 389:81-93.

Zhang JQ, Cai WQ, Zhou DS, Su BY. 2002. Distribution and differences of estrogen receptor beta immunoreactivity in the brain of adult male and female rats. Brain Res 935:73-80.

CHAPTER 2

Immunohistochemical Characterization of the G-Protein Coupled Estrogen Receptor 1 in the Neural Song System and Auditory Regions of Juvenile Zebra Finches

2.1 Introduction

Estradiol (E2) has potent influences on sexually dimorphic development of the circuit that controls song learning and production in zebra finches. It masculinizes several features of the neural circuit, such as neuron number and size. But in the non-neural portion of the song system, the vocal organ syrinx, E2 feminizes characteristics such as muscle fiber size (Gurney and Konishi, 1980; Gurney, 1982; Mathews and Arnold, 1991; Grisham and Arnold, 1995; Jacobs et al., 1995). In addition to affecting dimorphisms in the brain, E2 is important for auditory processing. In juveniles, auditory function is integral to song learning. Males learn to sing by hearing and imitating the song of an adult male tutor, who is typically their father (reviewed in Zann, 1996). Female zebra finches do not normally sing, but must also learn a tutor's song for the purposes of discriminating between male mates in adulthood (Clayton, 1988). As adults, birds need to hear and process song of conspecifics for social communication and courtship behaviors (reviewed in Zann, 1996). In adults of both sexes, E2 is rapidly synthesized in response to hearing conspecific song primarily within the caudomedial nidopallium (NCM) and enhances several features of auditory processing (Remage-Healey et al.,

2008; 2010; 2011; 2012). Although NCM itself is not structurally dimorphic, select sex differences have been reported in its auditory activity in response to song in adult and sub-adult (60 – 90 days of age) zebra finches. For instance, in adults, firing frequency and auditory responsiveness are dimorphically influenced when exposed to conspecific song (Krentzel et al., 2018). Sub-adult zebra finches exhibit lasting neuronal firing activity (i.e. a form of neuronal memory) in NCM following exposure to a tutor song, which is blocked in males treated with the aromatase inhibitor fadrozole (Yoder et al., 2015). Whereas in females the same treatment does not affect neuronal response to tutor song, it does inhibit their memory for a recently heard conspecific song. These results suggest that E2 has selective effects within NCM towards contextually relevant auditory stimuli. Less is known about the role of E2 in other major auditory regions such as the caudomedial mesopallium (CMM) and Field L. These areas are also not dimorphic, but in contrast to NCM, they are believed to be incapable of synthesizing or directly responding to E2 due to their lack of aromatase and nuclear estrogen receptor expression. However, these regions are hypothesized to be indirectly influenced by E2 via afferent input from E2 sensitive areas such as the cochlea and/or NCM (reviewed in Maney and Pinaud, 2011).

There are three overlapping phases to the song learning process; early sensory (P25 – 35) and sensorimotor phases (P30 – 60) as well as the later sub-adult phase (P60 – 80). Despite the sex differences in auditory processing that have been reported in the sub-adult phase of song learning, it is not known whether similar dimorphisms exist in the sensory and sensorimotor phases. Local infusion of E2 into NCM of male zebra finches during the sensory phase decreased auditory responsiveness following exposure to conspecific song, whereas the same treatment during the sensorimotor period resulted in either an enhanced or dampened auditory activity in a hemisphere-dependent manner. Female zebra finches were not included in this study (Vahaba et

al., 2017). An analysis of baseline E2 concentrations in juvenile NCM revealed a progressive increase in males with age and a significant male-biased sex difference which was only detected in the sub-adult ages but not during the earlier phases of song learning (Chao et al., 2015). Female baseline levels of E2 in NCM remained constant throughout all of these time points. The importance of this is not entirely clear, but in both males and females, E2 may be required for the process of forming a memory of tutor song (reviewed in Jin and Clayton, 1997). During the sensory phase, E2 may be similarly allowing males and females to be more responsive or receptive to song stimulus. Beginning with the sensorimotor phase, the function of E2 could diverge between the sexes: in males, E2 may enable the bird to compare his own vocalizations to his memory of the tutor song as he practices singing. Subsequently, the rise in endogenous E2 concentrations during the sub-adult period may be important for enhanced auditory motor integration just prior to song crystallization in adulthood. Although the field of avian biology includes a sensorimotor phase in female song learning, it does not equally apply to all species. In some species females do produce song, but that is not the case in female zebra finches. Still they may use E2 during this and the sub-adult phases for the further processing and memorization of tutor song.

Despite the potent influences of E2 on dimorphic development of the neural song system its mechanism of action is not well understood. Additionally, clarifying the mechanism by which E2 acts can aid our understanding of its functional effects on juvenile auditory processing. Nuclear estrogen receptors ERα and ERβ facilitate many effects of E2 across several species (reviewed in Morissette et al., 2008) but in zebra finches, these receptors have minimal expression within the neural song system with no reported sex differences (Bernard et al., 1999; reviewed in Wade, 2001; Wade et al., 2002; Veney and Wade, 2005). In the auditory forebrain,

ERα mRNA is expressed in NCM beginning at P2 in males and females (Jacobs et al., 1999) and its mRNA and protein are localized to this region in adults of both sexes (Jacobs et al., 1999; Saldanha and Coomaralingam, 2005). ERβ protein is also expressed in NCM of male and female adult zebra finches but other auditory areas such as Field L and CMM appear to be devoid of both receptors (reviewed in Maney and Pinaud, 2011). Given the limited localization of nuclear estrogen receptors within the song circuit and auditory forebrain, additional forms of estrogen receptor may underlie functions within these two systems.

The G-protein-coupled membrane bound estrogen receptor 1 (GPER1) is a candidate, which is best known for its involvement in rapid, cellular signaling that occurs within minutes to hours (which would be relevant for auditory processing) (Srivastava and Evans, 2013) but it can also mediate long-term, genomic effects within a timeframe of hours to days (which could contribute to dimorphic development of song nuclei) (reviewed in Prossnitz and Barton, 2011). GPER1 is present within zebra finch song nuclei. Specifically, its mRNA was expressed in whole telencephalic tissue of developing birds at ages starting from post-hatching (P) day 3 through 45, and in adults greater than 100 days of age (Acharya and Veney, 2012). Within this period, at P21 and P30, GPER1 gene expression was greater in males as compared to females. As presence of mRNA does not necessarily equate expression of functional protein, GPER1 protein expression was also analyzed within the telencephalon at P21 and in adulthood. In general, GPER1 was widely expressed throughout the telencephalon in both males and females with varying densities. Within the neural song circuit, HVC had the most abundant GPER1 labeling followed by RA. Of note was a male-biased expression in HVC. Immunoreactivity was minimal in LMAN and Area X. A moderate density of GPER1 labeling was detected within NCM. By adulthood, the overall distribution of GPER1 labeling appeared significantly less

robust within these same regions and the sex difference in HVC disappeared. More recently, GPER1 protein expression was analyzed within NCM of adults and whereas it was not dimorphic, local inhibition of the receptor rapidly decreased firing frequency and auditory responsiveness to song in males but not females (Krentzel et al., 2018). Although females were not affected, it is possible that GPER1 does influence these same auditory properties in other contexts, for instance following exposure to the song of a mate.

Taken together, expression of GPER1 within song nuclei and auditory region NCM suggests that this receptor may contribute to development of the song circuit and to auditory function. However, a more extensive analysis of its protein localization during early development is lacking, which would be important for elucidating functional contributions of the receptor. Here we semi-quantitatively describe GPER1 protein expression in male and female zebra finches at select ages during early development, specifically in the major song nuclei and auditory regions. We hypothesize that GPER1 has widespread expression within all examined regions and at all ages.

2.2 Methods

2.2.1 Animals

Breeding pairs of zebra finches were housed in communal aviaries in our facility at Kent State University which is maintained on a 14:10 light:dark cycle. Their diet consisted of ad libitum access to finch seed and water, along with weekly supplements of hard boiled chicken eggs mixed with bread and fresh spinach or oranges. Juveniles used in the experiment were the offspring of these adults and remained in the care of their parents until collection. Adequate measures were taken to minimize pain and discomfort. All procedures were in accordance with

Kent State University's Institutional Animal Use and Care Committee and conformed to NIH national guidelines.

2.2.2 Tissue collection and histology

Experimental birds (n = 3 males and n = 3 females) were captured at post-hatching (P) days 15, 25, 30, 35, 40, and 45. This range was selected for two important reasons. First, it represents snap-shot ages during the developmental period in which E2 is known to have significant effects on emerging song circuit dimorphisms (Nordeen and Nordeen, 1988; Adkins-Regan et al., 1994; reviewed in Zann, 1996). Secondly, this time period represents the sensory and sensorimotor phases of song learning during which E2 may be required for processing of auditory stimuli. Subjects were deeply anesthetized with Equithesin and transcardially perfused with 0.75% saline followed by 30 ml of 4% phosphate buffered formalin (PBF). The sex of each animal was determined at this time by identification of physical dimorphic characteristics (beak color and plumage) and/or examination of gonads. At the conclusion of the perfusion, the brain was removed, post-fixed overnight in 4% PBF, and cryoprotected in 10% sucrose for 2 hours. This was followed by an overnight immersion in 20% sucrose and then 30% sucrose the next night. The post-fixation and sucrose immersion steps all occurred at 4°C. Tissue was then covered with polyvinylpyrrolidone (PVP) (0.15 g/ml H_2O) for cryoprotection and stored at -80°C until use. Brains were coronally cryosectioned at 40 μm into alternating groups, creating three equal series. One set of these sections was thaw-mounted onto gelatin coated slides and stained with Thionin. The remaining two sets were placed into separate vials containing 0.1 M phosphate buffered saline (PBS) to prevent folding of tissue, then transferred into a cryoprotectant solution (PVP, sucrose, and ethylene glycol solution), and stored at -80°C.

2.2.3 Immunohistochemistry (IHC)

All procedures with the exception of the primary antibody step were performed at room temperature on a rotating shaker. On the first day of IHC, one of the two sets of brain sections stored in the cryoprotectant solution was warmed to room temperature, then rinsed 3X in 0.1 M PBS for 10 min each followed by 0.5% H_2O_2 for 20 min to inactivate endogenous peroxidases and rinsing 3X in 0.1 M PBS for 10 min each. In order to block nonspecific binding, sections were placed in 10% goat serum in PBS with 0.3% Triton X-100 (PBS-T) for 1.5 hr. Next, brain slices were incubated for 48 hr at 4°C with an anti-GPER1 rabbit polyclonal antibody (LS-A4268; 1:1500; MBL international) which has been previously validated in zebra finches (Acharya and Veney, 2012). Two days later, tissue was removed from primary antibody and rinsed twice with PBS-T for 10 min each, followed by 1 hr in biotinylated goat anti-rabbit secondary antibody (1:1500; Vector labs). Next, sections were rinsed 3X with PBS-T for 10 min each before incubation in avidin-biotin complex for 1 hr. Brain slices were then briefly rinsed in PBS for 5 min and exposed to diaminobenzidine to detect the immunolabeling. Lastly, sections were float mounted onto gelatin-coated slides and coverslipped for analysis.

2.2.4 IHC analysis

Using CellSens Dimension software, GPER1 immunoreactivity was semi-quantitatively analyzed in song nuclei HVC, RA, LMAN, and Area X (present only in males), as well as auditory regions NCM, and Field L. Sections containing CMM were not included. As immunoreactive cells appeared to be homogeneously distributed throughout each nucleus, one representative section was selected for analysis in approximately the middle of the nucleus where it appeared to have the largest cross-sectional area. An adjacent Nissl-stained section was used to

aid in identifying the borders of each nucleus. To get an estimate of the relative abundance of GPER1+ cells within each chosen IHC section, a rectangular box (0.046 mm^2) was placed over the center of the nucleus. The box served as a frame in which GPER1+ neurons were counted under a 40x magnification. Neuronal labeling was distinguished from glial on the basis of larger size, pale nucleus, and a darkly stained cytoplasm (Kirn and DeVoogd, 1989; Miller and Potempa, 1990). Counting alternated left and right hemispheres across animals. The same procedure was repeated for the corresponding adjacent Nissl section in which the total number of neurons within the counting frame was determined. Dividing the two counts revealed the relative proportion of GPER1+ cells within the defined region of each nucleus.

2.3 Results

2.3.1 GPER1 immunoreactivity in song nuclei

Semi-quantitative data from a single representative section within each of the major song nuclei HVC, RA, LMAN, and Area X are summarized in **Table 2.1**. Analysis in HVC revealed robust GPER1 immunoreactivity in both males **(Figure 2.1A and B)** and females throughout all examined ages. GPER1 labeling was also detected in RA **(Figure 2.1C)**. At P15, density of labeling in males within this nucleus was robust but appeared to drastically decrease after P30. In females, density of staining in RA remained fairly consistent throughout the examined period. LMAN **(Figure 2.1D and E)** also contained GPER1+ cells with densities comparable to that of HVC in males and females from P15 to P30, but in males, labeling completely disappeared in this nucleus starting around P35. Area X was devoid of immunoreactive neurons (females do not have an Area X). Few glial cells seemed to be positive for GPER1 in this nucleus in males **(Figure 2.1D and F)** which were not included since the analysis focused on neuronal labeling.

Overall, P15 animals appeared to have the most consistently robust GPER1 expression between sexes and across song nuclei with the exception of Area X.

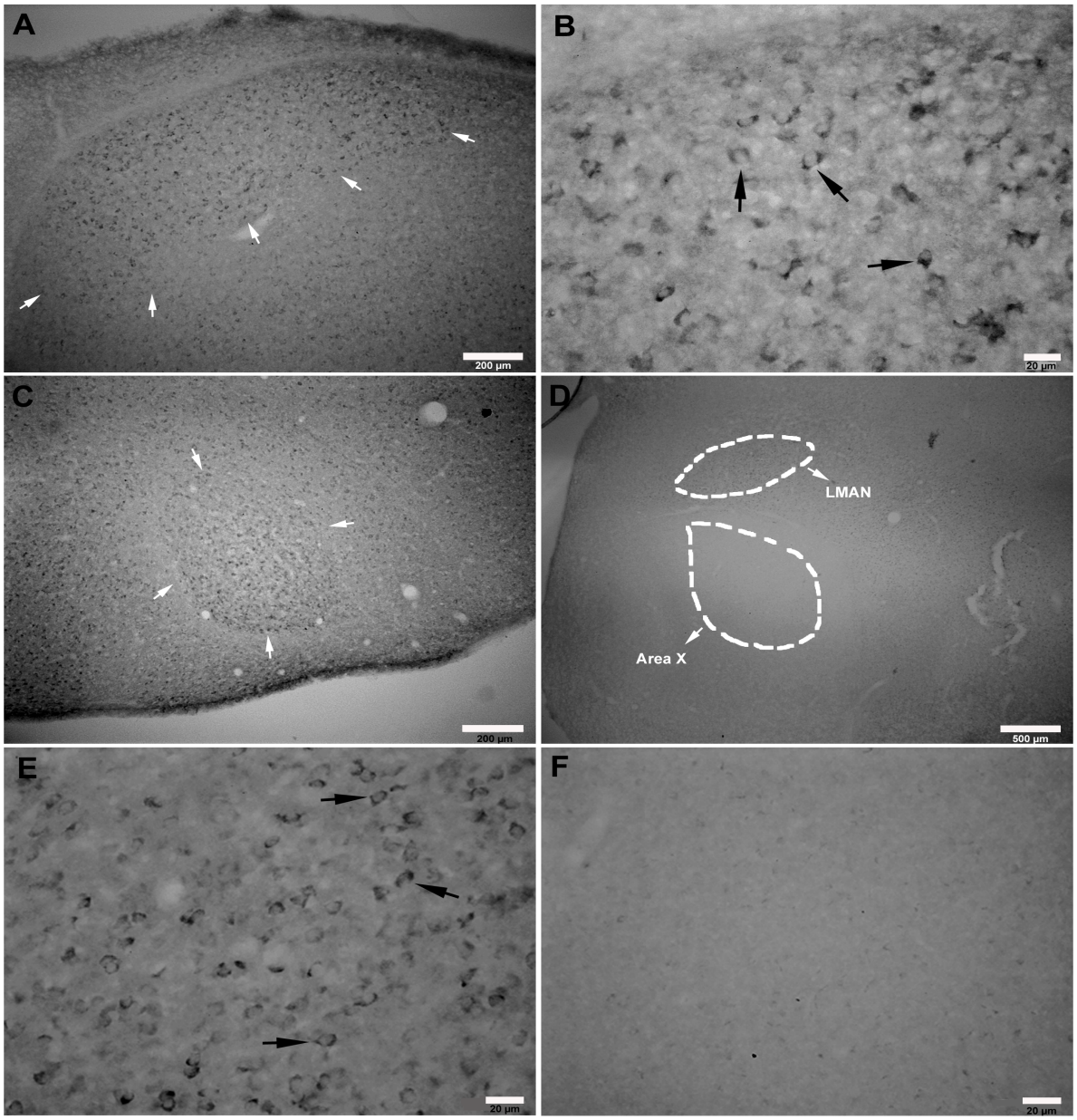

Figure 2.1 Photomicrographs of GPER1 immunoreactivity in song nuclei of a P25 male. (A) represents labeling within HVC with **(B)** depicting its magnified view. GPER1 expression is visible within **(C)** RA and **(D)** LMAN. **(D)** Area X is devoid of labeling. Magnified views of **(E)** LMAN and **(F)** Area X are presented. White arrows and dashed lines delineate nuclear borders (confirmed with Nissl stain). Black arrows depict GPER1+ neurons. Scale bars, 200 µm (A, C), 20 µm (B, E, F), 500 µm (D).

	HVC		RA		LMAN		Area X
	Male	Female	Male	Female	Male	Female	Male
P15	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + +	---
P25	+ + +	+ + +	+ +	+ +	+ + +	+	---
P30	+ + +	+ + +	+ +	+ + +	+ +	+ + + +	---
P35	+ +	+ + +	+	+ + +	---	+ +	---
P40	+ +	+ + + +	+	+ + +	---	+ + +	---
P45	+ +	+ + +	+	+ +	---	+ + + +	---

Table 2.1 Density of GPER1+ neurons within a defined region in a single representative section of each of the major song nuclei: HVC, RA, LMAN and Area X in male and female zebra finches at select developmental ages. Each entry represents the relative ratio of GPER1+ neurons over the total neuron number within the counting frame. ---, complete absence of immunoreactivity; +, fewer than 27% labeling; + +, 28-40% labeling; + + +, 41-59% labeling; + + + +, greater than 60% labeling.

2.3.2 GPER1 immunoreactivity in auditory regions

GPER1 expression was semi-quantitatively evaluated within auditory regions NCM and Field L in a single representative section **(Table 2.2)**. Additionally, robust labeling was detected in an auditory region immediately caudal to Field L resembling the nucleus interfacialis (NIf) **(Figure 2.2D)**. Overall, GPER1 was expressed in all the examined areas throughout development in males and females **(Figure 2.2A-D)**. Within NCM, GPER1+ neurons appeared more abundant in males as compared to females at P25 and P15. In both sexes, GPER1 expression seemed to be elevated at P45 as compared to the earlier examined ages. Within Field L of males, the most abundant GPER1 immunoreactivity was detected at P15 but appeared to decrease by P25 and stay low throughout the later time points. In females, GPER1 immunostaining in Field L tended to fluctuate with age, but at P45 females appeared to have denser labeling as compared to males. NIf also contained abundant GPER1 immunoreactivity in both sexes with relatively consistent density throughout the examined period of development. The majority of GPER1+ cells within this region appeared to be glia judging by their considerably smaller size and opaque nucleus as compared to neurons (Miller and Potempa, 1990) but we exclusively analyzed neuronal GPER1 expression in this region as it was still abundant. Overall, as compared to NIf, immunoreactivity in NCM and Field L appeared less pronounced.

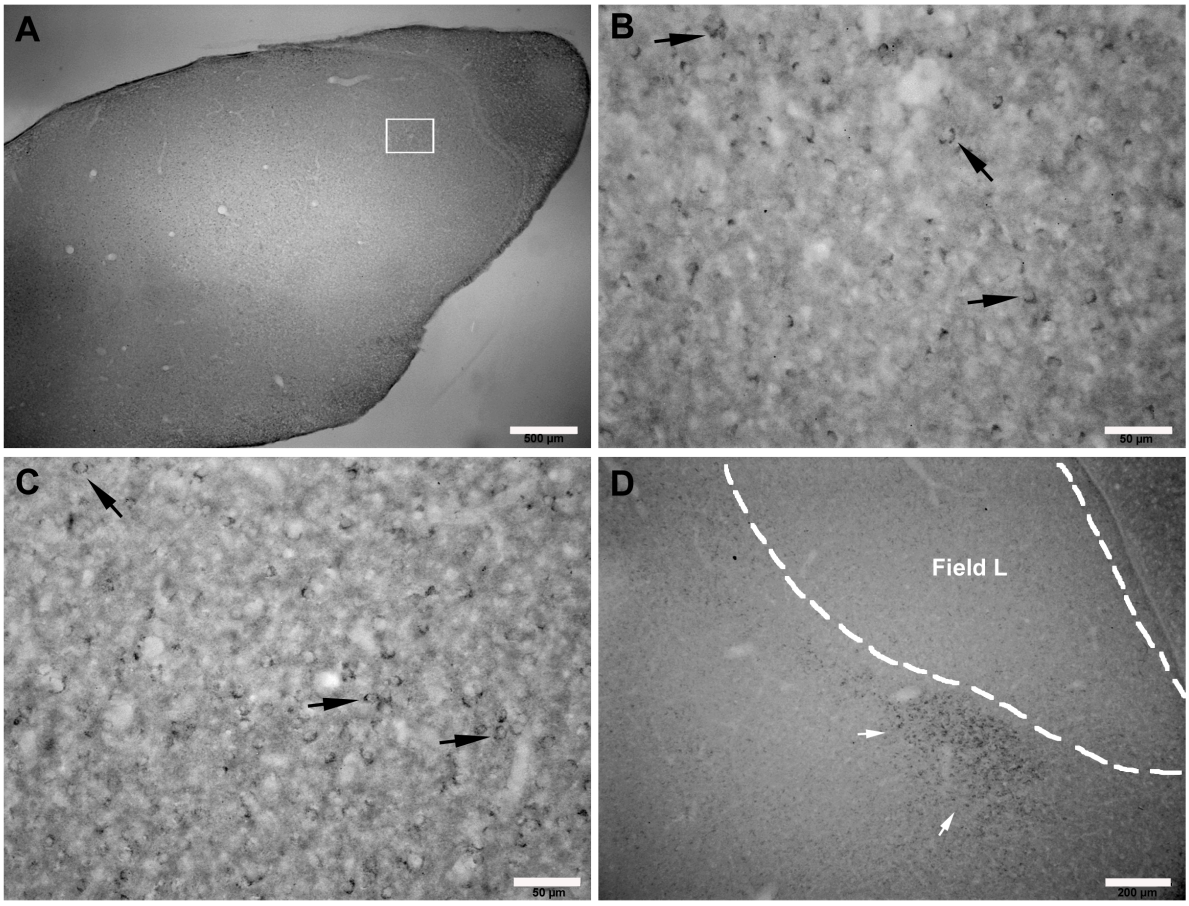

Figure 2.2 Photomicrographs of GPER1 immunoreactivity in auditory regions. (A) the box represents the approximate region within NCM of a P25 female where analysis was performed. **(B)** and **(C)** are magnified views of GPER1 immunoreactivity within NCM of a P25 and a P45 female, respectively. **(D)** depicts labeling within Field L (white dashed lines delineate borders verified with Nissl) and NIf (white arrows). Scale bars, 500 µm (A), 50 µm (B, C), 200 µm (D).

	NCM		Field L		NIf	
	Male	Female	Male	Female	Male	Female
P15	+ +	+	+ + +	+ + + +	+ + +	+ + + +
P25	+ + +	+	+	+	+ + +	+ +
P30	+	+ +	+	+ + +	+ +	+ + + +
P35	+	+	+	+	+ +	+ + + +
P40	+ +	+ +	+	+ +	+ +	+ + + +
P45	+ + + +	+ + + +	+	+ + + +	+ + + +	+ + + +

Table 2.2 Density of GPER1+ neurons within a defined region in a single representative section of auditory regions NCM, Field L and NIf in male and female zebra finches at select developmental ages. Each entry represents the ratio of GPER1+ neurons over the total neuron number within the counting frame. ---, complete absence of immunoreactivity; +, fewer than 27% labeling; + +, 28-40% labeling; + + +, 41-59% labeling; + + + +, greater than 60% labeling.

2.4 Discussion

In this study we semi-quantitatively characterized GPER1 protein expression in the major song nuclei and select auditory regions at specific ages from P15 to P45 in the zebra finch brain. Our analysis revealed GPER1 immuonreactivity within HVC, RA, and LMAN in both males and females at all ages examined. Area X was the only nucleus that lacked neuronal labeling at any of the time points. In RA and LMAN, density of immunoreactive cells appeared to vary considerably with age. Auditory regions NCM and Field L also contained GPER1+ neurons throughout the examined period with varying densities across sex and age. These data support the hypothesis that GPER1 has abundant expression within the neural song system throughout the proposed critical period of E2 action on system dimorphisms (the first 45 days post-hatching) (Konishi and Akutagawa, 1988; Adkins-Regan et al., 1994), and during the sensory and sensorimotor phases of song learning.

Among the song nuclei, HVC appeared to have the most robust GPER1 labeling at all ages, extending previous work that reported more enhanced expression within this nucleus as compared to other song nuclei at P21 (Acharya and Veney, 2012). Whereas Acharya and Veney also reported a male-biased expression of GPER1 at P21, our analysis was semi-quantitative, and therefore it is not clear whether a sex difference exists in HVC at additional ages, or in other song control regions. It is also possible that a sex difference in GPER1 within this nucleus appears prior to P15 and is no longer visible after P21. Regardless, the abundant and consistently elevated expression of GPER1 within HVC across these juvenile ages suggests that E2 may be acting through this receptor to influence development of this nucleus and perhaps other song nuclei indirectly via transsynaptic support mechanisms involving growth factors (Meitzen et al. 2007). One example is Area X which appeared devoid of neuronal GPER1 at all ages in our

analysis. Sexual differentiation of this nucleus may result because of downstream targeted support stemming from HVC (reviewed in Brenowitz and Larson, 2015). Additionally, HVC occupies a crucial node within the song circuit: it is part of the motor pathway for song production, is responsive to auditory stimuli, and is the only motor control nucleus that projects robustly to the pathway for song learning (reviewed in Barclay and Harding, 1990). Therefore, GPER1 within HVC may also help mediate sensorimotor integration within this nucleus throughout early development.

In RA, the density of GPER1+ neurons appeared similar between the sexes at P25 and earlier, but curiously, it decreased considerably in males beginning around P35, and remained relatively low through at least P45, whereas female RA seemed to maintain more elevated levels of expression throughout this timeframe. Interestingly, this difference in expression overlaps with the period when neuron death increases significantly in females, creating the sex difference in cell number within RA, while neuronal number remains constant in males (Kirn and DeVoogd, 1989). Neuronal apoptosis is a major process underlying dimorphic development of song nuclei (Konishi and Akutagawa, 1985; Nordeen et al., 1987). The consistently elevated levels of GPER1 in females after P25 suggest that GPER1 may contribute to the dimorphism in RA neuron number by promoting cell death in this sex. This idea is consistent with the apoptotic functions that have been described for GPER1 in various tissues in rodents such as smooth muscle, bone and brain (Ding et al., 2009; Windahl et al., 2009; Chimento et al., 2010; Wnuk et al., 2017).

Within LMAN, there was a consistent decrease in density of labeling with age in males until it was no longer visible by around P35. In females, GPER1 was robustly expressed throughout the examined period with the exception of P25 when it appeared less abundant as

compared to males. These patterns of expression are concurrent with significant changes in LMAN nuclear volume. Specifically, the volume of LMAN initially increases in both sexes but a male-biased sex difference emerges around P10 and is visible until around P20 (Nixdorf-Bergweiler, 1996). The volume of LMAN then begins to decline significantly in males after this age and in females after P30 which has been attributed to a substantial loss of neurons (Bottjer et al., 1985). The observed greater distribution of GPER1+ cells in LMAN of males as compared to females by P25 suggests that this receptor may contribute to the initial male-biased difference in the volume. But rather than by apoptosis (similar to what may occur in RA) this could be accomplished through neuroprotection. Therefore, the substantial decrease in volume of LMAN in both sexes after P30 could be due to lack of GPER1 receptors in males resulting in loss of neuroprotection, and an increase in receptor distribution in females leading to enhanced apoptosis. The idea of a dual functionality for GPER1 based on sex is not novel and is consistent with rodent studies reporting cell survival and death functions of this receptor in the same organism (Chimento et al., 2010; 2012; reviewed in Srivastava and Evans, 2013) and sexually dimorphic actions within the same tissue (Martensson et al., 2009; Ford et al., 2011; Broughton et al., 2014). Collectively, our data suggest that in zebra finches, GPER1 may contribute to neural song system dimorphisms through similar influences on cell survival and apoptosis.

Our data also support a role for GPER1 in auditory function in juveniles. In the auditory region NCM, GPER1+ neurons were expressed and appeared slightly more abundant in males as compared to females during the sensory phase of song learning (prior to P35). Starting around P35, which approximately marks the beginning of the sensorimotor phase, the pattern of labeling appeared identical in both sexes, showing a gradual increase and reaching the highest expression by P45. The increase in GPER1 receptor expression within NCM also appears consistent with

the endogenous rise in E2 levels in this region that occurs in males throughout this period (Chao et al., 2015). Although E2 levels remain stable in females during this period, GPER1 receptor expression appears to rise in this sex similar to males. One possible explanation is that GPER1 receptors are upregulated in females as a compensatory mechanism in order to maintain comparable responsiveness to E2 across the sexes.

Within Field L, the pattern of GPER1 immunoreactivity seemed to differ noticeably between males and females. Whereas both sexes appeared to have elevated levels at P15, in males labeling seemed to decrease to the lowest levels from P25 to P45, but appeared to remain high in females. Although to our knowledge, sex differences in auditory function or a role for E2 in this process have not been explored in Field L of zebra finches, at least in one other songbird species, Gambel's white-crowned sparrow, E2 affects select neuronal firing properties within this region in a sex-dependent manner (Caras et al., 2015).

Field L is a major relay center in the forebrain that receives auditory input from the thalamic nucleus Ovoidalis (Ov) and projects to several regions within the telencephalon, including NCM, the "shelf" (region immediately medial and ventral to HVC), and the "cup" (area immediately anteroventral to RA) which serve as interfaces between the auditory and song regions (Vates et al., 1996) **(Figure 2.3)**. It is not clear why females have an overall greater GPER1 expression in Field L throughout the sensory and sensorimotor phases, but this could be related to an enhanced role for this receptor in encoding of behaviorally relevant auditory signals in this sex which is hypothesized to occur in this region (Boumans et al., 2008). Future studies should investigate this possibility.

Our semi-quantitative analysis also revealed robust GPER1 expression within a region highly resembling NIf in both males and females at all ages. NIf receives input related to

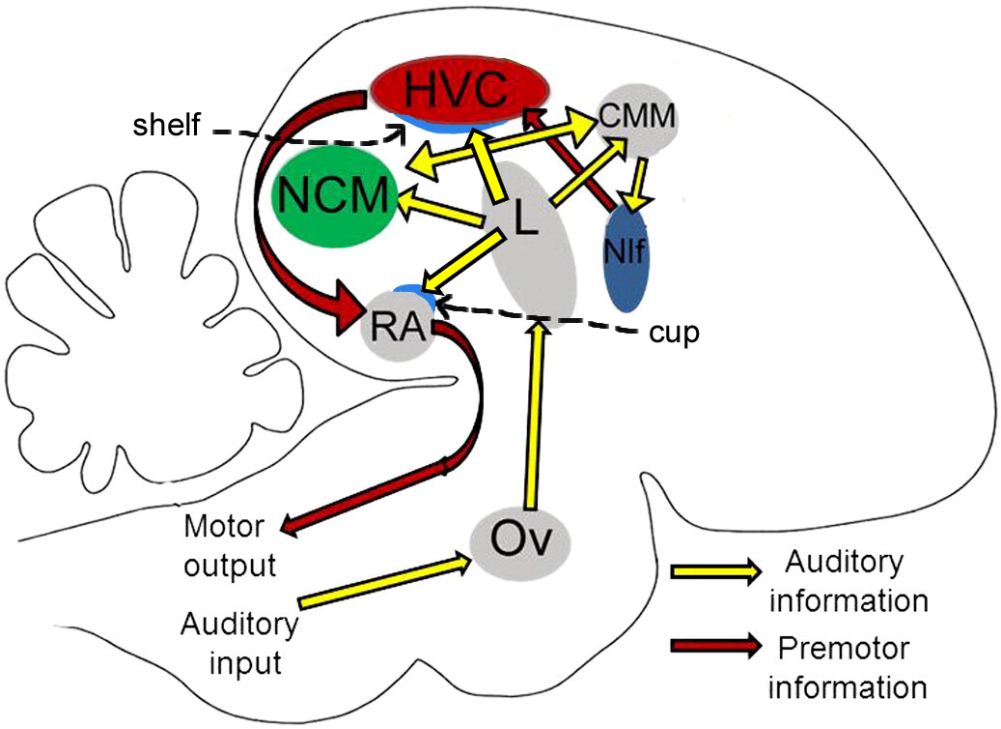

Figure 2.3 Zebra finch auditory network and its connectivity to song control nuclei. The schematic depicts a sagittal view of the major auditory regions and their connections to HVC and RA. Yellow arrows depict auditory pathways. Red arrows outline premotor and sensorimotor connections (figure adapted from Remage-Healey, 2014).

breathing during song production (Bauer et al., 2008) and serves as an interface between auditory and vocal control regions. Specifically, NIf projects directly to HVC (Bottjer et al., 2000) and has been shown to transsynaptically propagate E2-induced auditory neuronal firing from NCM to HVC (Pawlisch and Remage-Healey, 2015). This region is thought to be incapable of synthesizing or responding to E2 due to little expression of aromatase containing cells and lack of nuclear receptors (reviewed in Pawlisch and Remage-Healey, 2015). However, since GPER1 is abundantly expressed within NIf it is possible that E2 directly affects aspects of sensorimotor coordination within this region. We also detected abundant glial labeling within NIf (but it was not evaluated in our analysis). It is not clear whether the GPER1 receptors in glia are directly involved in auditory function within this region, but this may be possible as glia-localized GPER1 receptors can mediate synaptic transmission in rodent brain (Kumar et al., 2015). These ideas warrant further investigation.

In sum, the patterns of GPER1 expression within the major song nuclei suggest that this receptor may influence several aspects of the neural song circuit development in an age- and sex-dependent manner, primarily through neuroprotective and apoptotic functions. The anatomical distribution of GPER1 within auditory regions during the sensitive periods for song learning suggests a role for this receptor in processing of sensory and motor auditory information, and possibly sensorimotor integration. Future studies should confirm these possibilities by examining the effects of pharmacological inhibition of GPER1 throughout early development on song circuit morphology and aspects of auditory function. Furthermore, quantitative analyses should verify possible sex differences in this receptor throughout these developmental time points.

2.5 References

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, 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. Dev Neurobiol 25: 865-877.

Barclay SR, Harding CF. 1990. Differential modulation of monoamine levels and turnover rates by estrogen and/or androgen in hypothalamic and vocal control nuclei of male zebra finches. Brain Res 523:251-62.

Bauer EE, Coleman MJ, Roberts TF, Roy A, Prather JF, Mooney R. 2008. A synaptic basis for auditory–vocal integration in the songbird. J Neurosci 28:1509-22.

Bernard DJ, Bentley GE, Balthazart J, Turek FW, Ball GF. 1999. Androgen Receptor, Estrogen Receptor α, and Estrogen Receptor β Show Distinct Patterns of Expression in Forebrain Song Control Nuclei of European Starlings. Endocrinology 140:4633-4643.

Bottjer SW, Glaessner SL, Arnold AP. 1985. Ontogeny of brain nuclei controlling song learning and behavior in zebra finches. J Neurosci 5:1556-62.

Bottjer SW, Brady JD, Cribbs B. 2000. Connections of a motor cortical region in zebra finches: relation to pathways for vocal learning. J Comp Neurol 420:244-60.

Boumans T, Gobes SM, Poirier C, Theunissen FE, Vandersmissen L, Pintjens W, Verhoye M, Bolhuis JJ, Van der Linden A. 2008. Functional MRI of auditory responses in the zebra finch forebrain reveals a hierarchical organisation based on signal strength but not selectivity. PLoS one 3:e3184.

Brenowitz EA, Larson TA. 2015. Neurogenesis in the adult avian song-control system. Cold Spring Harb Perspect Biol 7:a019000.

Broughton BR, Brait VH, Kim HA, Lee S, Chu HX, Gardiner-Mann CV, Guida E, et al. 2014. Sex-dependent effects of G protein–coupled estrogen receptor activity on outcome after ischemic stroke. Stroke 45:835-841.

Caras ML, Sen K, Rubel EW, Brenowitz EA. 2015. Seasonal plasticity of precise spike timing in the avian auditory system. J Neurosci 35:3431-45.

Chao A, Paon A, Remage-Healey L. 2015. Dynamic variation in forebrain estradiol levels during song learning. Dev Neurobiol 75:271-86.

Chimento A, Sirianni R, Delalande C, Silandre D, Bois C, Andò S, Maggiolini M, et al. 2010. 17β-estradiol activates rapid signaling pathways involved in rat pachytene spermatocytes apoptosis through GPR30 and ERα. Mol Cell Endocrinol 320:136-144.

Chimento A, Sirianni R, Casaburi I, Ruggiero C, Maggiolini M, Andò S, Pezzi V. 2012. 17β-Estradiol activates GPER-and ESR1-dependent pathways inducing apoptosis in GC-2 cells, a mouse spermatocyte-derived cell line. Mol Cell Endocrinol 355:49-59.

Clayton NS. 1988. Song discrimination learning in zebra finches. Animal Behav 36:1016-24.

Ding Q, Gros R, Limbird LE, Chorazyczewski J, Feldman RD. 2009. Estradiol-mediated ERK phosphorylation and apoptosis in vascular smooth muscle cells requires GPR 30. Am J Physiol Cell Physiol 297:C1178-C1187.

Ford J, Hajibeigi A, Long M, Hahner L, Gore C, Hsieh JT, Clegg D, et al. 2011. GPR30 deficiency causes increased bone mass, mineralization, and growth plate proliferative activity in male mice. J Bone Miner Res 26:298-307.

Grisham W, Arnold AP. 1995. A direct comparison of the masculinizing effects of testosterone, androstenedione, estrogen, and progesterone on the development of the zebra finch song system. Dev Neurobiol 26:163-170.

Gurney ME, Konishi M. 1980. Hormone-induced sexual differentiation of brain and behavior in zebra finches. Science 208:1380-1383.

Gurney ME. 1982. Behavioral correlates of sexual differentiation in the zebra finch song system. Brain Res 231:153-172.

Jacobs EC, Grisham W, Arnold, AP. 1995. Lack of a synergistic effect between estradiol and dihydrotestosterone in the masculinization of the zebra finch song system. Dev Neurobiol 27:513-519.

Jacobs EC, Arnold AP, Campagnoni AT. 1999. Developmental regulation of the distribution of aromatase-and estrogen-receptor-mRNA-expressing cells in the zebra finch brain. Dev Neurosci 21:453-72.

Jin H, Clayton DF. 1997. Localized changes in immediate-early gene regulation during sensory and motor learning in zebra finches. Neuron 19:1049-59.

Kirn JR, DeVoogd TJ. 1989. Genesis and death of vocal control neurons during sexual differentiation in the zebra finch. J Neurosci 9:3176-3187.

Konishi M, Akutagawa E. 1985. Neuronal growth, atrophy and death in a sexually dimorphic song nucleus in the zebra finch brain. Nature 315:145-7.

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 *USA* 85:7006-7007.

Krentzel AA, Macedo-Lima M, Ikeda MZ, Remage-Healey L. 2018. A membrane g-protein coupled estrogen receptor is necessary but not sufficient for sex-differences in zebra finch auditory coding. Endocrinology.

Kumar A, Bean LA, Rani A, Jackson T, Foster TC. 2015. Contribution of estrogen receptor subtypes, ERα, ERβ, and GPER1 in rapid estradiol-mediated enhancement of hippocampal synaptic transmission in mice. Hippocampus 25:1556-66.

Maney D, Pinaud R. 2011. Estradiol-dependent modulation of auditory processing and selectivity in songbirds. Front Neuroendocrinol 32:287-302.

Martensson UE, Salehi SA, Windahl S, Gomez MF, Swärd K, Daszkiewicz-Nilsson J, Wendt A, et al. 2009. Deletion of the G protein-coupled receptor 30 impairs glucose tolerance, reduces bone growth, increases blood pressure, and eliminates estradiol-stimulated insulin release in female mice. Endocrinology 1502:687-698.

Mathews GA, Arnold AP. 1991. Tamoxifen's effects on the zebra finch song system are estrogenic, not antiestrogenic. Dev Neurobiol 22:957-969.

Meitzen J, Moore IT, Lent K, Brenowitz EA, Perkel DJ. 2007. Steroid hormones act transsynaptically within the forebrain to regulate neuronal phenotype and song stereotypy. J Neurosci 27:12045-12057.

Miller MW, Potempa G. 1990. Numbers of neurons and glia in mature rat somatosensory cortex: effects of prenatal exposure to ethanol. J Comp Neurol 293:92-102.

Morissette M, Le Saux M, D'Astous M, Jourdain S, Al Sweidi S, Morin N, Estrada-Camarena E, et al. 2008. Contribution of estrogen receptors alpha and beta to the effects of estradiol in the brain. J Steroid Biochem Mol Biol 108:327-338.

Nixdorf-Bergweiler BE. 1996. Divergent and parallel development in volume sizes of telencephalic song nuclei in male and female zebra finches. J Comp Neurol 375:445-456.

Nordeen KW, Nordeen EJ, Arnold AP. 1987. Estrogen accumulation in zebra finch song control nuclei: implications for sexual differentiation and adult activation of song behavior. Dev Neurobiol 18:569-82.

Nordeen EJ, Nordeen KW. 1988. Sex and regional differences in the incorporation of neurons born during song learning in zebra finches. J Neurosci 8:2869-2874.

Pawlisch BA, Remage-Healey L. 2015. Neuroestrogen signaling in the songbird auditory cortex propagates into a sensorimotor network via an 'interface' nucleus. Neurosci 284:522-35.

Prossnitz ER, Barton M. 2011. The G-protein-coupled estrogen receptor GPER in health and disease. Nat Rev Endocrinol 7:715-726.

Remage-Healey L, Maidment NT, Schlinger BA. 2008. Forebrain steroid levels fluctuate rapidly during social interactions. Nat Neurosci 11:1327.

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 107:3852-7.

Remage-Healey L, Dong SM, Chao A, Schlinger BA. 2011. Sex-specific, rapid neuroestrogen fluctuations and neurophysiological actions in the songbird auditory forebrain. J Neurophysiol 107:1621-31.

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. Frank Beach Award Winner: steroids as neuromodulators of brain circuits and behavior. Hormones and behavior. 2014 Aug 31;66(3):552-60.

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.

Srivastava DP, Evans PD. 2013. G-Protein Oestrogen Receptor 1: Trials and Tribulations of a Membrane Oestrogen Receptor. J Neuroendocrinol 25:1219-1230.

Vahaba DM, Macedo-Lima M, Remage-Healey L. 2017. Sensory Coding and Sensitivity to Local Estrogens Shift during Critical Period Milestones in the Auditory Cortex of Male Songbirds. eNeuro 4:ENEURO-0317.

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 366:613-42.

Veney SL, Wade J. 2005. Post-hatching syrinx development in the zebra finch: an analysis of androgen receptor, aromatase, estrogen receptor α and estrogen receptor β mRNAs. J Comp Physiol 191:97-104.

Wade J. 2001. Zebra finch sexual differentiation: the aromatization hypothesis revisited. Microsc Res Tech 54:354-363.

Wade J, Buhlman L, Swender D. 2002. Post-hatching hormonal modulation of a sexually dimorphic neuromuscular system controlling song in zebra finches. Brain Res 929:191-201.

Windahl SH, Andersson N, Chagin AS, Martensson UE, Carlsten H, Olde B, Swanson C, Moverare-Skrtic S, Savendahl L, Lagerquist MK, Leeb-Lundberg LF. 2009. The role of the G protein-coupled receptor GPR30 in the effects of estrogen in ovariectomized mice. Am J Physiol Endocrinol Metab 296:E490-6.

Wnuk A, Rzemieniec J, Lasoń W, Krzeptowski W, Kajta M. 2017. Apoptosis induced by the UV filter benzophenone-3 in mouse neuronal cells is mediated via attenuation of Erα/Pparγ and stimulation of Erβ/Gpr30 signaling. Mol Neurobiol 1-22.

Yoder KM, Phan ML, Lu K, Vicario DS. 2015. He hears, she hears: are there sex differences in auditory processing?. Dev Neurobiol 75:302-14.

Zann RA. 1996. The zebra finch: a synthesis of field and laboratory studies. New York: Oxford University Press. 335 p.

CHAPTER 3

Intracranial Administration of the G-Protein Coupled Estrogen Receptor 1 Antagonist, G-15, Selectively Affects Dimorphic Characteristics of the Song System in Zebra Finches

3.1 Introduction

In zebra finches (*Taeniopygia guttata*), song is controlled by a network of interconnected brain regions High Vocal Center (HVC), robust nucleus of arcopallium (RA), lateral magnocellular nucleus of anterior nidopallium (LMAN), and Area X, as well as the vocal organ (syrinx), which is located at the junction of the trachea and the two bronchi. Within this song circuit there are numerous male-biased dimorphisms, which normally enable only males to sing (reviewed in Arnold, 1992; reviewed in Wade, 2001). However, the exact factor(s) responsible for these sex differences are not clear.

Estradiol (E2) is known to be important by having masculinizing effects on the brain of females. More specifically, systemic E2 administration to hatchlings (via subcutaneous injections or silastic implants) that resulted in supra-physiological plasma hormone levels partially, but significantly, increased neuron number, neuron spacing, and nuclear volumes of RA and HVC (Gurney and Konishi, 1980; Gurney, 1982; Grisham and Arnold, 1995; Jacobs et al., 1995). Even Area X, which is not normally visible in females, was present. These effects enabled E2-treated females to demonstrate male-like singing behavior in adulthood (Gurney and Konishi, 1980;

Simpson and Vicario, 1991; Adkins-Regan et al., 1994).

In males, E2 manipulations have resulted in more varied effects on the brain, with some studies supporting a masculinizing role for this hormone in this sex as well. For example, daily subcutaneous injections of E2 for 20 days starting on the day of hatching increased cell size in HVC and LMAN when brains were examined at post-hatching (P) day 60 (Mathews and Arnold, 1991). Treatment with the estrogen synthesis inhibitor, fadrozole, was effective in preventing the increased level of brain-derived neurotrophic factor (BDNF) mRNA that is normally detectable in male HVC at P30-35 (Dittrich et al., 1999). Moreover, the robust growth of HVC projections into RA was blocked *in vitro* by fadrozole (Holloway and Clayton, 2001).

In contrast to the brain, several studies suggest that the non-neural portion of the song system (i.e., the syrinx) which normally has greater mass and larger muscle fiber sizes in males, is feminized by E2 (reviewed in Wade, 2001). For example, in the mallard duck (*Anas platyrhynchos)*, injections of E2 prior to hatching inhibited growth of the syrinx in males (Noumura et al., 1985) as well as cell proliferation and chondrogenesis in isolated cells of the female syrinx (Takahashi and Noumura, 1987). Treatment with the anti-estrogen (CI-628) prior to hatching masculinized the female syrinx (Noumura et al., 1985). Similarly, in male zebra finches, systemic injections resulting in supra-physiological E2 levels in plasma for the first three weeks post-hatching significantly decreased syrinx mass and muscle fiber sizes (Wade et al., 2002). Inhibiting E2 synthesis in female zebra finches resulted in masculinization of this organ (Gong et al., 1999).

Exactly how and through what receptor(s) E2 is affecting sexual differentiation of the song system is not known. Across species, many effects of E2 are mediated by the nuclear estrogen receptors ERα and ERβ (reviewed in Morissette et al., 2008). In zebra finches, few

neurons expressing ERα mRNA have been detected in the neural song circuit (reviewed in Wade, 2001), and to date, ERβ has not been localized within any song control nuclei (Bernard et al., 1999). In the syrinx ERα mRNA is absent, while ERβ mRNA is present exclusively in the pessulus, a central rigid tissue providing structural support, as well as cartilage (Veney and Wade, 2005). No dimorphisms have been reported in the expression of these receptors within the song system. Since lack of a sex difference in receptor does not eliminate the possibility of dimorphic actions (Duncan et al., 2007), antagonists have been used to determine the influence of these receptors on dimorphisms within the song circuit. Only ICI 182,780 (a high affinity ERα and ERβ antagonist) was effective in selectively demasculinizing the brain (Bender and Veney, 2008) and masculinizing the syrinx (Martin and Veney, 2008). Other antagonists either failed to significantly demasculinize the brain, or in some cases even resulted in masculinization or hypermasculinization (Mathews et al., 1988; Mathews and Arnold, 1990; 1991). To our knowledge (with the exception of ICI) none of these same compounds have ever been tested in the syrinx. While these data suggest that ERα and ERβ by themselves have a limited role in sex differences within the song circuit, the possibility exists that other estrogen receptors are involved.

One candidate is the G-protein-coupled membrane bound estrogen receptor 1 (GPER1). GPER1 is known for mediating rapid cellular responses through regulation of second messengers and kinase pathways, but can also regulate transcription and have long-term cellular effects (reviewed in Prossnitz and Barton, 2011). In mice, this receptor contributes to sexual dimorphisms in cardiovascular function and bone growth (Martensson et al., 2009; Ford et al., 2011; Lenhart et al., 2013). There is also evidence for GPER1-dependent sex differences in brain infarct volume after ischemic stroke (Broughton et al., 2014). In zebra finches, GPER1 is

expressed in the developing and adult syrinx (unpublished observations). In the brain, GPER1 mRNA is present throughout the telencephalon of males and females at select developmental and adult ages (Acharya and Veney, 2012). Immunohistochemical analysis revealed a significant sex difference in GPER1 expressing neurons. At P21, males have more of these neurons in HVC than females, while no sex difference is detected in RA, LMAN, or Area X at either this juvenile age or in adulthood. Dimorphic expression of GPER1 at P21 is significant because the sex difference in HVC volume becomes detectable around this age (Nixdorf-Bergweiler, 1996) and HVC cell number is already dimorphic by this time (Nordeen and Nordeen, 1988). Therefore, GPER1 could be responsible for dimorphic development of this nucleus and perhaps other nuclei within the song system. Mechanistically this could be achieved by direct actions on HVC and indirect actions from HVC on other song nuclei through downstream transynaptic support mechanisms such as those involving growth factors (reviewed in Brenowitz and Larson, 2015).

Given the anatomical and dimorphic distribution of GPER1 in the developing zebra finch song circuit it may significantly contribute to development of this system. In the present study we administered a specific GPER1 antagonist and examined its effects on dimorphic characteristics of the brain and syrinx. We hypothesized that pharmacological blockade of GPER1 would demasculinize the neural song circuit and masculinize the syrinx.

3.2 Methods

3.2.1 Animals

Zebra finches were obtained from our facility at Kent State University. The experimental subjects were collected from nest boxes inside communal aviaries each housing 7-8 adult breeding pairs. The breeders were maintained on a 14:10 light:dark cycle, and had *ad libitum*

access to finch seed and water, along with weekly supplements of hard boiled chicken eggs mixed with bread and fresh spinach or oranges. Adequate measures were taken to minimize pain and discomfort. All procedures were in accordance with Kent State University's Institutional Animal Use and Care Committee and conformed to NIH national guidelines.

3.2.2 Treatments

Following the technique described in Bender and Veney (2008), animals were intracranially injected to increase exposure of the song system to the drug. The injections were made just under the skull, but did not penetrate or otherwise purposefully damage the brain. Males and females received 4 µl of the GPER1 antagonist G-15 (Cayman Chemical; 1.75 µg/µl) or DMSO/propylene glycol vehicle daily for 25 days starting within 24 hours of hatching. This treatment timeframe was selected because E2 is known to have its greatest effects on the song circuit from approximately P1 through P45 (Konishi and Akutagawa, 1988; Adkins-Regan et al., 1994) with many dimorphisms in the song circuit detectable by day 25 (Nordeen and Nordeen, 1988; reviewed in Zann, 1996). The chosen dose of G-15 was within the range of concentrations shown to have potent effects in rodents (Dennis et al., 2009; Hammond and Gibbs, 2011).

3.2.3 Tissue collection and histology

At P25, one hour following their last injection, experimental subjects were deeply anesthetized with Equithesin. They were then transcardially perfused with 0.75% saline followed by 30 ml of 4% phosphate buffered formalin (PBF). The sex of the animals was determined by visual examination of the gonads. The brain and syrinx of each animal was removed, post-fixed overnight in 4% PBF, and cryoprotected in 10% sucrose for 2 hours. This was followed by an

overnight immersion in 20% sucrose and then 30% sucrose the next night. The post-fixation and sucrose immersion steps all occurred at 4˚C. Using a plastic Pasteur pipette the tissues were covered with polyvinylpyrrolidone (0.15 g/ml H_2O) and stored at -80˚C until use. Brains were coronally cryosectioned at 30 μm, syrinxes transversely cryosectioned at 20 μm, and thaw-mounted onto gelatin-coated slides. Brain sections were stained in Thionin, syrinxes in Trichrome.

3.2.4 Histological analysis

Slides were coded to blind the experimenter to the treatment and sex of the subject. CellSens Dimension software was used for analysis. A single brain section in which the cross-sectional area of each song nucleus was the largest was selected as the representative for measurements of neuronal cell size. The area of 50 randomly selected cells (25 per hemisphere) was measured in HVC, RA, LMAN, and Area X (present only in males) under a 40x magnification and averaged. Neurons were distinguished from glia on the basis of their larger size, pale nucleus, and a darkly stained cytoplasm (Kirn and DeVoogd, 1989; Miller and Potempa, 1990). The approximate number of neurons within HVC, RA, and Area X (LMAN was excluded due to difficulty in identifying its boundaries in all sections) was assessed with unilateral counts that alternated left and right hemispheres between animals. For each subject, a single rectangular box of uniform size was placed in three randomly selected, non-overlapping locations in every other adjacent section within each nucleus. The box covered approximately 30-40% of the field of view at 60x magnification and the number of neurons within each of these defined frames was counted. Next, the area of the nucleus in each of these same sections was measured at 4x. This number was then divided by the area of the rectangular box and the

resulting number multiplied by the average number of cells counted within that section. The approximate number of cells within each nucleus was determined by summing the cell number estimates from all sections and multiplying by the sampling interval. No correction was necessary for neuron estimates, as the sampling interval was large enough to eliminate the possibility of double counting. The volumes of HVC, RA, and Area X were also measured (LMAN was excluded for the same reason described previously) by summing the areas of all the sections within the nucleus (measured at 10x) and multiplying by the sampling interval. Volume measurements from the left and right hemispheres were summed and averaged.

For the syrinx, ventralis and dorsalis fiber sizes were measured at 40x by randomly selecting 40 fibers (20 from each side) in each muscle from the section where the pessulus first appeared intact. This represents where the syrinx is fully formed. Measurements of fiber size were summed and averaged across both sides. The effects of G-15 were analyzed (Sigma Stat) using a two-way ANOVA (sex x treatment), followed by Student-Newman-Keuls *post-hoc* analysis when an interaction was detected. A t-test was used for Area X. For all analyses significant effects were considered at a $p < 0.05$.

3.3 Results

3.3.1 Neuron size

In RA **(Figure 3.1A)** there was a male vs. female main effect of sex [$F(1, 46) = 11.24$; $P = 0.002$], no effect of treatment [$F(1, 46) = 2.227$; $P = 0.143$], and no interaction [$F(1, 46) = 0.549$; $P = 0.463$]. While control males had significantly greater neuron size than females, this value approached significance in the G-15 treated group ($P = 0.058$). Within HVC **(Figure 3.1B)** results revealed a main effect of sex [$F(1, 29) = 43.7$; $P < 0.001$], but no treatment effect [F (1,

29) = 0.656; P = 0.425] or an interaction were detected [F (1, 29) = 0.841; P = 0.367]. For region LMAN **(Figure 3.1C)** there was once again a male vs. female main effect of sex [F (1, 29) = 4.539; P = 0.043], but no effect of treatment [F (1, 29) = 0.117; P = 0.735], or an interaction [F (1, 29) = 0.047; P = 0.829]. In Area X **(Figure 3.1D)** neuron size was not affected by G-15 [t = 0.398; P = 0.0695].

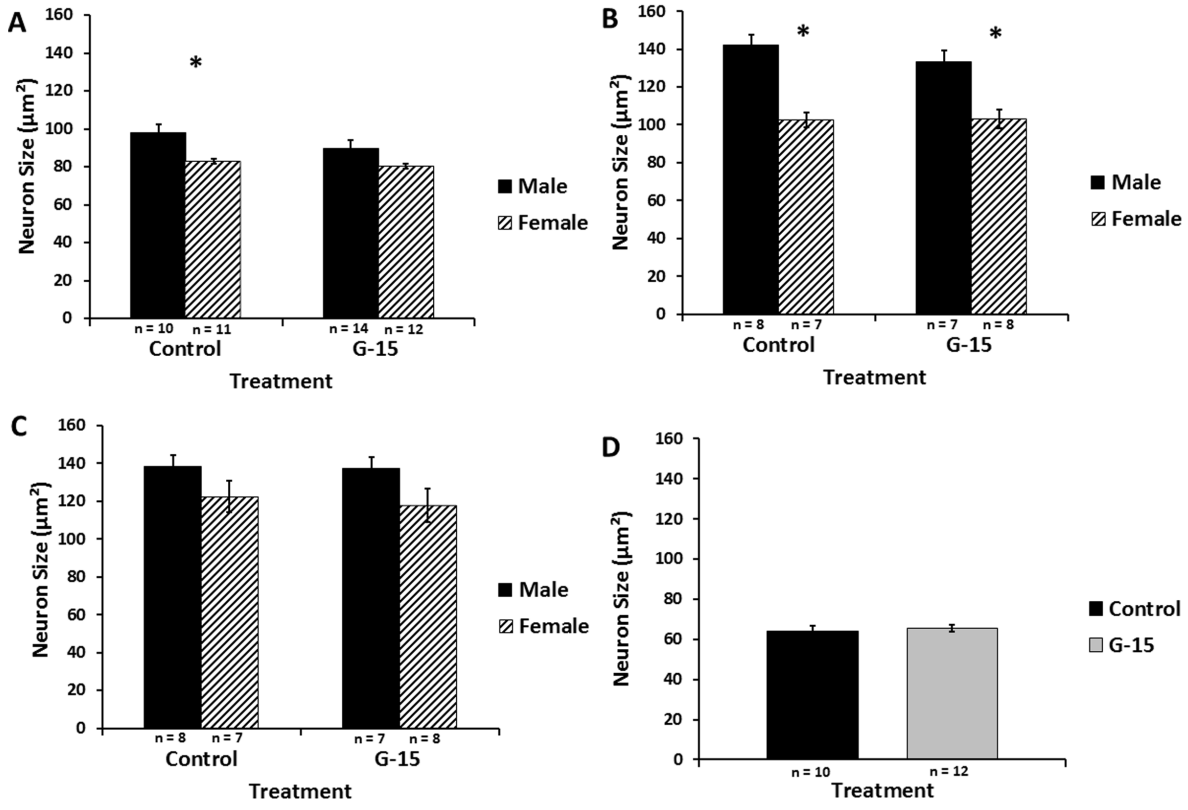

Figure 3.1 The effects of G-15 on neuron size in song nuclei. Treatment with G-15 did not affect the size of neurons in **(A)** RA, **(B)** HVC, **(C)** LMAN, or **(D)** Area X. Bars represent means ± SEM. * denotes a sex difference within each treatment group ($P \leq 0.002$).

3.3.2 Neuron number

A significant main effect of sex was detected in RA **(Figure 3.2A)** [F (1, 41) = 20.92; P < 0.001] but there was no effect of treatment [F (1, 41) = 0.001; P = 0.976], or an interaction [F (1, 41) = 0.226; P = 0.637]. Within HVC **(Figure 3.2B)** results revealed a significant main effect of sex [F (1, 28) = 38.3; P < 0.001], but no main effect of treatment [F (1, 28) = 0.383; P = 0.542], and no interaction [F (1, 28) = 0.999; P = 0.327]. In Area X **(Figure 3.2C)** treatment with G-15 had no effect on neuron number [t = 1.131; P = 0.277].

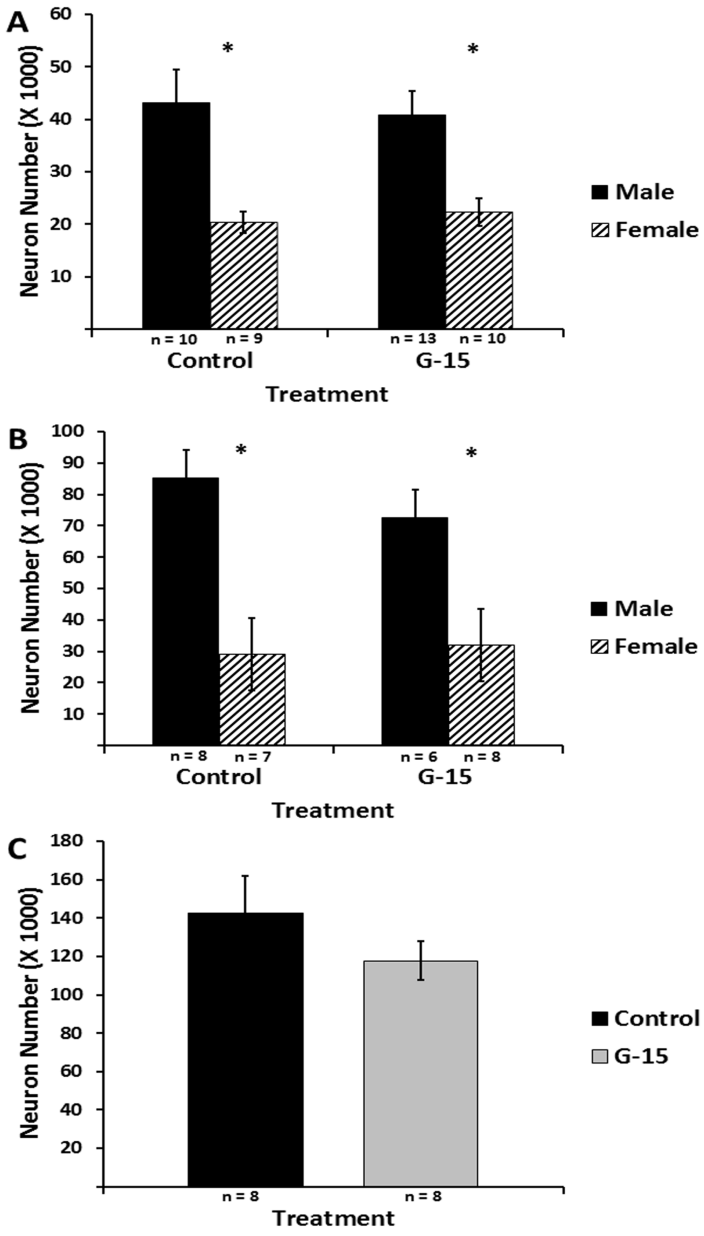

Figure 3.2 The effects of G-15 on neuron number in song nuclei. G-15 did not affect neuron number in **(A)** RA, **(B)** HVC, or **(C)** Area X. Bars represent means ± SEM. * denotes a sex difference within each treatment group ($P \leq 0.004$).

3.3.3 Nuclear volume

Measurements of nuclear volume in RA **(Figure 3.3A)** revealed a significant main effect of sex [$F(1, 45) = 48.29$; $P < 0.001$], but no effect of treatment [$F(1, 45) = 0.059$; $P = 0.809$] and no interaction [$F(1, 45) = 0.1$; $P = 0.753$]. In HVC **(Figure 3.3B)** there was a significant main effect of sex [$F(1, 29) = 57.06$; $P < 0.001$], treatment [$F(1, 29) = 5.502$; $P = 0.027$], and an interaction [$F(1, 29) = 9.893$; $P = 0.004$]. More specifically, exposure to G-15 significantly decreased nuclear volume in males, but had no effect on this measure in females. In Area X **(Figure 3.3C)** there was once again a significant effect of treatment [$t = 3.003$; $P = 0.009$]. The antagonist significantly decreased nuclear volume in this nucleus.

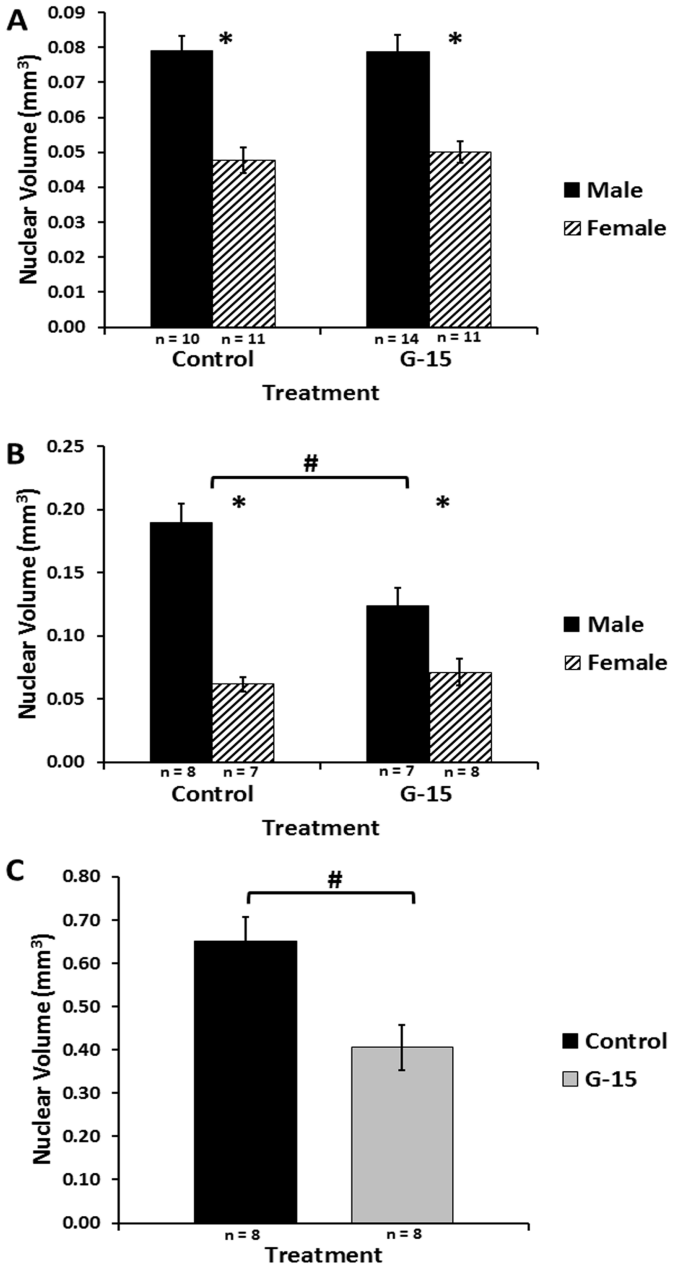

Figure 3.3 The effects of G-15 on nuclear volume in the song system. G-15 had no effect on nuclear volume in **(A)** RA, but it significantly decreased this measure in **(B)** HVC and **(C)** Area X of males. Bars represent means ± SEM. * denotes a sex difference within each treatment group ($P \leq 0.005$), # represents a significant difference across treatment groups ($P \leq 0.009$).

3.3.4 Syrinx muscle fiber sizes

While no main effect of sex was present in ventralis **(Figure 3.4A)** [$F(1, 39) = 0.973$; $P = 0.331$], significant effects of treatment [$F(1, 39) = 4.327$; $P = 0.045$] and an interaction [$F(1, 39) = 30.18$; $P < 0.001$] were detected. More specifically, in response to G-15, muscle fiber sizes decreased in males, but increased in females. In dorsalis **(Figure 3.4B)** measurements yielded no main effects of sex [$F(1, 35) = 0.570$; $P = 0.456$], or treatment [$F(1, 35) = 0.082$; $P = 0.776$] but G-15 did significantly decrease fiber sizes in males, and increased them in females. A significant interaction [$F(1, 35) = 9.150$; $P = 0.005$] was also detected.

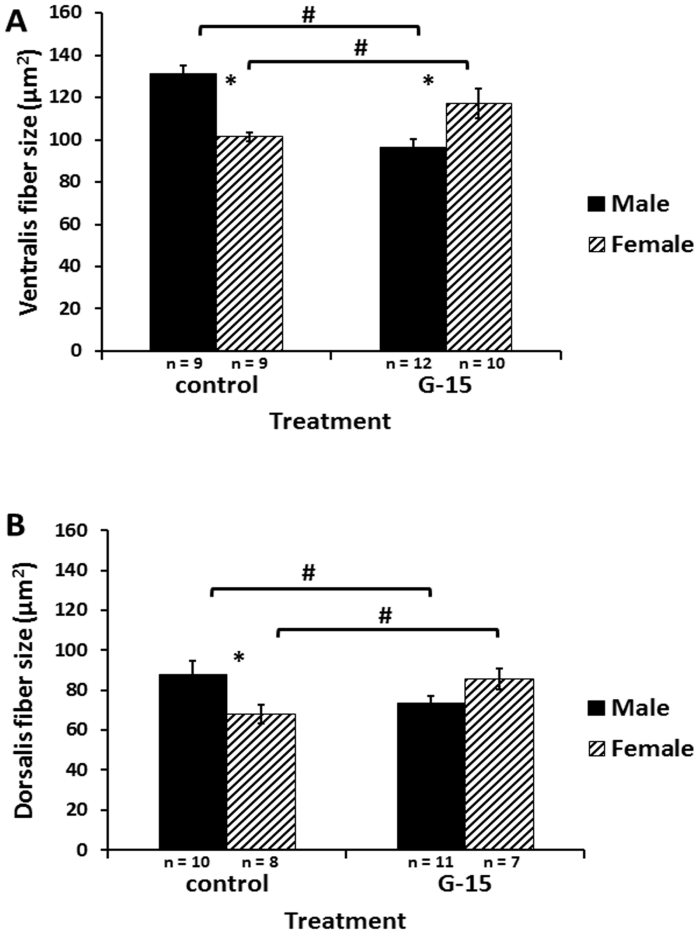

Figure 3.4 The effects of G-15 on syrinx muscle fiber sizes. Daily treatment with G-15 significantly decreased **(A)** ventralis muscle fiber sizes in males, but increased them in females. **(B)** Dorsalis fiber sizes also decreased in males, but increased in females. Bars represent means ± SEM. ***** denotes a sex difference within each treatment group ($P \leq 0.01$). # denotes significant differences across treatment groups ($P \leq 0.04$).

3.4 Discussion

In this study exposure to the GPER1 antagonist G-15 resulted in decreased nuclear volumes of HVC and Area X in males, and increased muscle fiber sizes of ventralis and dorsalis in females. These results partially support the hypothesis that antagonism of the GPER1 receptor demasculinizes the neural song circuit and masculinizes the syrinx in post-hatched developing zebra finches. Interestingly, G-15 had no effect on the examined neural dimorphisms in females. In males, neuron size and number were not affected, nor was nuclear volume of RA. Additionally, the direction of the syrinx effect was opposite that of females with fiber sizes in ventralis and dorsalis decreasing. Taken together, these results suggest that E2 acting through the membrane-bound GPER1 receptor can selectively influence dimorphisms within the song system. However, since not all features were affected, particularly in the brain, other factors (genetic and/or other estrogen receptors) may be involved.

In rodents and birds cell survival and death in central and peripheral tissues significantly contribute to establishing sex differences (Takahashi and Noumura, 1987; Kirn and DeVoogd, 1989, reviewed in Arnold and McCarthy, 2016). These processes are partially regulated by estrogen receptors including GPER1, which can mediate cell survival and death within the same organism (Chimento et al., 2010; 2012; reviewed in Srivastava and Evans, 2013). For example, in rat primary cortical neurons, antagonism of GPER1 blocked neuroprotection by E2 against oxygen-glucose deprivation through signaling pathways involving PI3K, Src, and ERK (Abdelhamid et al., 2011). In a mouse model of Parkinson's disease, inhibition of GPER1 reversed the protective effect of raloxifene on dopaminergic striatal neurons by inhibiting the upregulation of the antiapoptotic protein Bcl-2 and BDNF (Bourque et al., 2014). In addition, treatment with the specific GPER1 agonist G-1 promoted cell survival in the heart by reducing

infarct size and post-ischemic cardiac dysfunction in rats (Deschamps and Murphy, 2009). In contrast to the above examples, which describe protective functions of GPER1, this receptor is also known to promote apoptosis. For example, in mice, combined activation of GPER1 and ERα resulted in increased ERK and c-Jun phosphorylation leading to upregulation of the proapoptotic Bax protein and death of spermatocytes (Chimento et al., 2012). In rats, treatment with G-1 mediated apoptosis in vascular smooth muscle cells (Ding et al., 2009).

GPER1 can also have cell death and survival functions across sexes but on the same tissue. For instance, knockout of the GPER1 gene inhibited bone growth in female mice (Martensson et al., 2009), but enhanced it in males (Ford et al., 2011). In mouse brain, treatment with G-1 resulted in an increase in infarct volume following induction of stroke in males, while it decreased neuronal damage and apoptosis in females (Broughton et al., 2014). It is not exactly clear what factor(s) determine whether GPER1 is protective or apoptotic but we propose that similar dual functions of this receptor contribute to sexual dimorphisms within the zebra finch song system.

Our data suggest that in the male zebra finch brain GPER1 has protective actions. Treatment with G-15 significantly decreased nuclear volumes of HVC and Area X. The developing HVC has GPER1 receptors, thus it is likely that G-15 acted directly on this nucleus to affect volume. In contrast, Area X has no GPER1 receptor expression (unpublished observation), but does receive neuronal connections from HVC, which are known to significantly contribute to its development through downstream transsynaptic mechanisms (reviewed in Brenowitz and Larson, 2015). Thus, the significant effect on nuclear volume in this region was possibly due to GPER1's actions on the signaling stemming from HVC.

HVC also has neuronal connections with RA, which does contain GPER1 receptors, yet treatment with G-15 was ineffective in altering dimorphisms in this region. This suggests that GPER1 in RA may not be the primary facilitator of development in this nucleus. Instead, transsynaptic support from HVC likely contributes, just as it does for Area X, with GPER1 in RA possibly playing a modulatory role. This idea warrants further investigation but is supported by studies in which implants containing androgens near HVC significantly increased RA nuclear volume and neuron size (Brenowitz and Lent, 2002; Meitzen et al., 2007). Implants near RA had no effect despite an abundance of androgen receptors in this nucleus that were hypothesized to amplify the trophic signals from HVC (Meitzen et al., 2007). It is not clear why the afferent input to RA was not affected by G-15 (similar to what we hypothesized occurred to Area X) but HVC is known to provide different trophic factor support to RA and Area X (Denisenko-Nehrbass et al., 2000; reviewed in Brenowitz and Larson, 2015). This involves different signaling mechanisms, which we propose G-15 may selectively act on.

In the male zebra finch brain, cell number and size were also not affected by G-15 suggesting that the significant effect on nuclear volume was possibly due to a shift in cell spacing and/or a modest decrease in cell size or number. The reasons for a lack of effect are not clear but there are two possible explanations. One, these measures may be regulated by other factors/estrogen receptor(s). Secondly, this result could be due to differential sensitivities of these attributes to the antagonist. From at least one study it is proposed that dimorphic features of the song system such as cell number, size, and nuclear volume are not equally sensitive to E2 (Grisham et al., 2008). Therefore, it is reasonable to conclude that these measures may also not be similarly affected by an antagonist. Although we cannot explain the lack of a neural response

in females to G-15, differences in sensitivity to the drug, such as with the male data, may partially explain the results.

In the syrinx, fiber sizes of ventralis and dorsalis significantly decreased with G-15 treatment in males, but increased in females. This suggests that in the syrinx GPER1 promotes cell survival in males and is apoptotic in females. Again, the reason for these effects is unknown, but is consistent with the reported opposite actions of GPER1. Because this study employed intracranial injections, there are some possibilities of how the antagonist could have impacted the syrinx. First, it is possible that E2 acted on the tracheosyringeal portion of the hypoglossal nucleus (nXIIts), which receives afferent projections from RA and whose motor neurons innervate the syrinx (Nottebohm et al., 1976). Though rich in androgen receptors, this region also has GPER1 receptor expression (unpublished observation). Although we do not know if the two are co-localized in zebra finches, GPER1 receptors are expressed in spinal cord motoneurons in rats (Hu et al., 2012). Therefore, it is possible that in zebra finches G-15 may have affected the function of nXIIts motoneurons, which in turn altered muscle fiber sizes in the syrinx. Secondly, G-15 injections may have unintentionally resulted in some leakage into the periphery such that GPER1 receptors in the syrinx were directly activated.

In sum, our study provides evidence that GPER1 has limited influences on sexual differentiation of the song system (particularly in the brain) during early zebra finch development. To determine if more robust effects can be achieved, particularly by focusing on estrogens, it may be necessary in future studies to target multiple receptors for this hormone. One idea is that GPER1 works in conjunction with ERα and/or ERβ (reviewed in Hadjimarkou and Vasudevan, 2017) to influence select dimorphisms of this circuit. These receptors have overlapping distributions within the song circuit, and since antagonism of the nuclear receptors

(Bender and Veney, 2008; Martin and Veney, 2008) and GPER1 result in minimal effects, perhaps a combinational inhibition would be more effective.

In addition to contributing to development of the song circuit, GPER1 may function in song learning (in both sexes) and/or production (in males) by modulating auditory stimuli. HVC receives input from auditory areas Nif, Field L, and NCM (reviewed in Theunissen et al., 2004). In addition to HVC at least two of these areas (NCM and Field L) contain GPER1 receptors (unpublished observation). There is a suggestion that E2 acting through GPER1 can alter the firing rates of neurons (Remage-Healey and Joshi, 2012). We propose that this modulation occurs in these auditory regions and HVC, thus affecting the output from this nucleus and impacting song learning and/or production. Future studies should additionally examine the role of GPER1 in auditory function.

3.5 References

Abdelhamid R, Luo J, VandeVrede L, Kundu I, Michalsen B, Litosh VA, Schiefer IT, et al. 2011. Benzothiophene selective estrogen receptor modulators provide neuroprotection by a novel GPR30-dependent mechanism. ACS Chem Neurosci 2:256-268.

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, 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. Dev Neurobiol 25: 865-877.

Arnold AP. 1992. Developmental plasticity in neural circuits controlling birdsong: sexual differentiation and the neural basis of learning. Dev Neurobiol 23:1506-1528.

Arnold AP, McCarthy MM. 2016. Sexual differentiation of the brain and behavior: a primer. In: Pfaff DW, Volkow ND, editors. Neuroscience in the 21st century: from basic to clinical. New York: Springer, p 2139–2168.

Bender AT, Veney SL. 2008. Treatment with the specific estrogen receptor antagonist ICI 182,780 demasculinizes neuron soma size in the developing zebra finch brain. Brain Res 1246:47-53.

Bernard DJ, Bentley GE, Balthazart J, Turek FW, Ball GF. 1999. Androgen Receptor, Estrogen Receptor α, and Estrogen Receptor β Show Distinct Patterns of Expression in Forebrain Song Control Nuclei of European Starlings. Endocrinology 140:4633-4643.

Bourque M, Morissette M, Di Paolo, T. 2014. Raloxifene activates G protein-coupled estrogen receptor 1/Akt signaling to protect dopamine neurons in 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine mice. Neurobiol Aging 35:2347-2356.

Brenowitz EA, Lent K. 2002. Act locally and think globally: intracerebral testosterone implants induce seasonal-like growth of adult avian song control circuits. Proc Natl Acad Sci *USA* 99:12421-12426.

Brenowitz EA, Larson TA. 2015. Neurogenesis in the adult avian song-control system. Cold Spring Harb Perspect Biol 7:a019000.

Broughton BR, Brait VH, Kim HA, Lee S, Chu HX, Gardiner-Mann CV, Guida E, et al. 2014. Sex-dependent effects of G protein–coupled estrogen receptor activity on outcome after ischemic stroke. Stroke 45:835-841.

Chimento A, Sirianni R, Delalande C, Silandre D, Bois C, Andò S, Maggiolini M, et al. 2010. 17β-estradiol activates rapid signaling pathways involved in rat pachytene spermatocytes apoptosis through GPR30 and ERα. Mol Cell Endocrinol 320:136-144.

Chimento A, Sirianni R, Casaburi I, Ruggiero C, Maggiolini M, Andò S, Pezzi V. 2012. 17β-Estradiol activates GPER-and ESR1-dependent pathways inducing apoptosis in GC-2 cells, a mouse spermatocyte-derived cell line. Mol Cell Endocrinol 355:49-59.

Denisenko-Nehrbass NI, Jarvis E, Scharff C, Nottebohm F, Mello CV. 2000. Site-specific retinoic acid production in the brain of adult songbirds. Neuron 27:359-370.

Dennis MK, Burai R, Ramesh C, Petrie WK, Alcon SN, Nayak TK, Bologa CG, et al. 2009. In vivo effects of a GPR30 antagonist. Nat Chem Biol 5:421-427.

Deschamps AM, Murphy E. 2009. Activation of a novel estrogen receptor, GPER, is cardioprotective in male and female rats. Am J Physiol Heart Circ Physiol 297:H1806-H1813.

Ding Q, Gros R, Limbird LE, Chorazyczewski J, Feldman RD. 2009. Estradiol-mediated ERK phosphorylation and apoptosis in vascular smooth muscle cells requires GPR 30. Am J Physiol Cell Physiol 297:C1178-C1187.

Dittrich F, Feng Y, Metzdorf R, Gahr M. 1999. Estrogen-inducible, sex-specific expression of brain-derived neurotrophic factor mRNA in a forebrain song control nucleus of the juvenile zebra finch. Proc Natl Acad Sci *USA* 96:8241-8246.

Duncan KA, Carruth LL. 2007. The sexually dimorphic expression of L7/SPA, an estrogen receptor coactivator, in zebra finch telencephalon. Dev Neurobiol 67:1852-1866.

Ford J, Hajibeigi A, Long M, Hahner L, Gore C, Hsieh JT, Clegg D, et al. 2011. GPR30 deficiency causes increased bone mass, mineralization, and growth plate proliferative activity in male mice. J Bone Miner Res 26:298-307.

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.

Grisham W, Arnold AP. 1995. A direct comparison of the masculinizing effects of testosterone, androstenedione, estrogen, and progesterone on the development of the zebra finch song system. Dev Neurobiol 26:163-170.

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.

Gurney ME, Konishi M. 1980. Hormone-induced sexual differentiation of brain and behavior in zebra finches. Science 208:1380-1383.

Gurney ME. 1982. Behavioral correlates of sexual differentiation in the zebra finch song system. Brain Res 231:153-172.

Hadjimarkou MM, Vasudevan N. 2017. GPER1/GPR30 in the brain: Crosstalk with classical estrogen receptors and implications for behavior. J Steroid Biochem Mol Biol 10:1016.

Hammond R, Gibbs RB. 2011. GPR30 is positioned to mediate estrogen effects on basal forebrain cholinergic neurons and cognitive performance. Brain Res 1379:53-60.

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.

Hu R, Sun H, Zhang Q, Chen J, Wu N, Meng H, Cui G, et al. 2012. G-protein coupled estrogen receptor 1 mediated estrogenic neuroprotection against spinal cord injury. Crit Care Med 40:3230-3237.

Jacobs EC, Grisham W, Arnold, AP. 1995. Lack of a synergistic effect between estradiol and dihydrotestosterone in the masculinization of the zebra finch song system. Dev Neurobiol 27:513-519.

Kirn JR, DeVoogd TJ. 1989. Genesis and death of vocal control neurons during sexual differentiation in the zebra finch. J Neurosci 9:3176-3187.

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 *USA* 85:7006-7007.

Lenhart PM, Broselid S, Barrick CJ, Leeb-Lundberg LF, Caron KM. 2013. G-protein-coupled receptor 30 interacts with receptor activity-modifying protein 3 and confers sex-dependent cardioprotection. J Mol Endocrinol 51:191-202.

Mårtensson UE, Salehi SA, Windahl S, Gomez MF, Swärd K, Daszkiewicz-Nilsson J, Wendt A, et al. 2009. Deletion of the G protein-coupled receptor 30 impairs glucose tolerance, reduces bone growth, increases blood pressure, and eliminates estradiol-stimulated insulin release in female mice. Endocrinology 1502:687-698.

Martin LC, Veney SL. 2008. The specific estrogen receptor antagonist ICI 182,780 masculinizes development of the zebra finch syrinx. Gen Comp Endocrinol 156:434-439.

Mathews GA, Brenowitz EA, Arnold AP. 1988. Paradoxical hypermasculinization of the zebra finch song system by an antiestrogen. Horm Behav 22:540-551.

Mathews GA, Arnold AP. 1990. Antiestrogens fail to prevent the masculine ontogeny of the zebra finch song system. Gen Comp Endocrinol 80:48-58.

Mathews GA, Arnold AP. 1991. Tamoxifen's effects on the zebra finch song system are estrogenic, not antiestrogenic. Dev Neurobiol 22:957-969.

Meitzen J, Moore IT, Lent K, Brenowitz EA, Perkel DJ. 2007. Steroid hormones act transsynaptically within the forebrain to regulate neuronal phenotype and song stereotypy. J Neurosci 27:12045-12057.

Miller MW, Potempa G. 1990. Numbers of neurons and glia in mature rat somatosensory cortex: effects of prenatal exposure to ethanol. J Comp Neurol 293:92-102.

Morissette M, Le Saux M, D'Astous M, Jourdain S, Al Sweidi S, Morin N, Estrada-Camarena E, et al. 2008. Contribution of estrogen receptors alpha and beta to the effects of estradiol in the brain. J Steroid Biochem Mol Biol 108:327-338.

Nixdorf-Bergweiler BE. 1996. Divergent and parallel development in volume sizes of telencephalic song nuclei in male and female zebra finches. J Comp Neurol 375:445-456.

Nordeen EJ, Nordeen KW. 1988. Sex and regional differences in the incorporation of neurons born during song learning in zebra finches. J Neurosci 8:2869-2874.

Nottebohm F, Stokes TM, Leonard CM. 1976. Central control of song in the canary, *Serinus canarius*. J Comp Neurol 165:457-486.

Noumura T, Matsumoto E, Takahashi M. 1985. On the development of sexual dimorphism in the duck syrinx and estradiol binding. Current trends in comparative endocrinology. Hong Kong: Hong Kong University Press, p 601-602.

Prossnitz ER, Barton M. 2011. The G-protein-coupled estrogen receptor GPER in health and disease. Nat Rev Endocrinol 7:715-726.

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.

Simpson HB, Vicario DS. 1991. Early estrogen treatment alone causes female zebra finches to produce learned, male-like vocalizations. Dev Neurobiol 22:755-776.

Srivastava DP, Evans PD. 2013. G-Protein Oestrogen Receptor 1: Trials and Tribulations of a Membrane Oestrogen Receptor. J Neuroendocrinol 25:1219-1230.

Takahashi MM, Noumura T. 1987. Sexually dimorphic and laterally asymmetric development of the embryonic duck syrinx: effect of estrogen on in vitro cell proliferation and chondrogenesis. Dev Biol 121:417-422.

Theunissen FE, Amin N, Shaevitz SS, Woolley S, Fremouw T, Hauber ME. 2004. Song selectivity in the song system and in the auditory forebrain. Ann N Y Acad Sci 1016:222-245.

Veney SL, Wade J. 2005. Post-hatching syrinx development in the zebra finch: an analysis of androgen receptor, aromatase, estrogen receptor α and estrogen receptor β mRNAs. J Comp Physiol 191:97-104.

Wade J. 2001. Zebra finch sexual differentiation: the aromatization hypothesis revisited. Microsc Res Tech 54:354-363.

Wade J, Buhlman L, Swender D. 2002. Post-hatching hormonal modulation of a sexually dimorphic neuromuscular system controlling song in zebra finches. Brain Res 929:191-201.

Zann RA. 1996. The zebra finch: a synthesis of field and laboratory studies. New York: Oxford University Press. 335 p.

CHAPTER 4

Intramuscular Antagonism of the G-Protein Coupled Estrogen Receptor 1 Partially Affects Dimorphic Characteristics of the Syrinx, but is Ineffective in the Neural Song Circuit in Zebra Finches

4.1 Introduction

In zebra finches song is controlled by interconnected brain nuclei High Vocal Center (HVC), robust nucleus of arcopallium (RA), lateral magnocellular nucleus of the anterior nidopallium (LMAN), and Area X, which project to the syrinx (vocal organ) (reviewed in Arnold, 1992; reviewed in Wade, 2001). Throughout this song system, several morphological attributes are more robust in males as compared to females, permitting only males to normally sing (Nottebohm and Arnold, 1976; Gurney, 1981; reviewed in Wade and Arnold, 2004).

Estradiol (E2) is among the major contributors to sexually dimorphic differentiation of the song system. In the male and female neural song circuit this hormone tends to have partial masculinizing effects, whereas in the syrinx, E2 has feminizing actions (Gurney and Konishi, 1980; Gurney, 1982; Mathews and Arnold, 1991; Grisham and Arnold, 1995; Jacobs et al., 1995; reviewed in Wade, 2001; Wade et al., 2002). Despite its potent effects the mechanisms underlying E2 action have remained largely unclear.

The nuclear receptors (ERα and ERβ) have limited and non-dimorphic expression within the song system, suggesting that other estrogen receptor(s) may be involved. The G-protein-

coupled membrane bound estrogen receptor 1 (GPER1) is a possible candidate. Sexually dimorphic function has been reported for this receptor in rodents (Martensson et al., 2009; Ford et al., 2011; Lenhart et al., 2013; Broughton et al., 2014). In zebra finches, GPER1 mRNA and (less extensively) protein localizations have been described in the developing and adult brain, with protein levels being dimorphic at least during one developmental age (Acharya and Veney, 2012). GPER1 protein is also expressed within the syrinx during development and in adulthood (unpublished observations).

In addition to the important receptor(s) for E2 action not being known in this system, curiously, an analysis of prior studies has suggested that the method of drug delivery may influence the outcome of E2 manipulation. More specifically, studies that have used the aromatase inhibitor fadrozole (FAD) have been particularly intriguing. When primary dissociated cell cultures from telencephalon of hatchling zebra finches were treated with FAD, aromatase activity was significantly reduced (Wade et al., 1994). Elsewhere, FAD significantly inhibited the normal masculine pattern of axonal projections from HVC into RA in male and E2-exposed female cultured brain slices (Holloway and Clayton, 2001). Whereas the results of *in vitro* manipulations demonstrate efficacy of FAD in blocking E2 synthesis and altering dimorphic characteristics of the neural song system, peripheral administration of this drug to whole organism has generally not been as successful. For instance, treatment of male zebra finches with FAD *in ovo* only partially demasculinized RA volume but the volumes of HVC and Area X, as well as neuron soma size in RA were not affected when these measures were examined in adulthood (Wade and Arnold, 1996). In another report, daily intramuscular injections of FAD to male and female zebra finches from post-hatching (P) day 1 through 30 did not affect RA and Area X volume, or soma sizes within RA and HVC (Wade and Arnold, 1994)

but in a similar study the same treatment from P3 through P27 did increase the volume of LMAN, the size of RA neurons, and the projections from LMAN to RA in males (Beach and Wade, 2015). The reason(s) for these hypermasculinizing effects are not clear but highlight the complexity of sexual differentiation. The fact that FAD largely failed to effectively demasculinize the neural song circuit when administered peripherally, but did so when delivered in culture, suggests that method of drug delivery may have influenced the experimental outcome. This idea is further supported by studies examining the effects of E2 on auditory function. Specifically, local injection of FAD into the caudomedial nidopallium during playback of song significantly decreased E2-induced neuronal firing activity (Remage-Healey et al., 2010) but intramuscular injections had no effect (Yoder et al., 2012).

Overall, the concept that method of drug delivery is a crucial factor affecting experimental outcome is not just limited to FAD or zebra finches. In overiectomized rats the capacity of two different nuclear estrogen receptor antagonists (CI628 and CI680) to prevent uptake of radiolabeled E2 was explored within brain, pituitary, and uterine tissues (Chazal et al., 1975). Compared to the uterus and pituitary where the greatest degree of inhibition was achieved, intravenous injections of these compounds were only minimally effective within the hypothalamus and had no effect in the cerebral cortex. Similarly, a dopamine receptor agonist (SK&F 82526) was successful in inducing contralateral muscle rotation in rats when administered intracerebrally but not intraperitoneally (reviewed in Hahn et al., 1982). Also in rats, intracerebroventricular but not intravenous administration of the selective aminopepdidase A inhibitor EC33 significantly decreased blood pressure by blocking synthesis of brain angiotensin III (Reaux et al., 1999).

Method of drug administration having an effect on experimental outcome is not novel, but we argue that it is often overlooked, and in zebra finches, may be a significant factor accounting for discrepancies in experimental manipulations. More specifically, inability to cross the blood-brain barrier (BBB) from the periphery due to size or tertiary structure are all possible reasons for why compounds may not enter the brain. Furthermore, enzymatic cleavage of drugs into inactive metabolites or non-specific binding to peripheral ligands can also hinder a drug from reaching the brain or being delivered in effective doses (reviewed in Upadhyay, 2014).

The goal of this study is two-fold. The first is to further investigate the role of GPER1 in sexually dimorphic development of the song system. Secondly, I will confirm that peripherally administered compounds are likely to be less effective in altering dimorphisms within the neural portion of the song circuit. In a prior experiment, central (intracranial) injections of an antagonist to GPER1 significantly affected select dimorphic features in the brain and the syrinx. In the current study, G-15 will be administered peripherally. The hypothesis is that G-15 will demasculinize the brain and masculinize the syrinx, but the neural effects will be less robust as compared to those from the intracranial manipulations.

4.2 Methods

4.2.1 Animals

All procedures were approved by the Institutional Animal Care and Use Committee at Kent State University and were in agreement with NIH national guidelines. Hatchling zebra finches were obtained from nest boxes within aviaries containing 7-8 breeding pairs of adults housed in our animal facility. The birds were maintained on a 14:10 light:dark cycle. Finch seed and water were provided *ad libitum*. This diet was supplemented with hard-boiled chicken eggs,

bread, and fresh spinach or oranges once a week. For all procedures, care was taken to minimize animal pain and discomfort.

4.2.2 Treatments

To block the GPER1 pathway during a hypothesized sensitive period for influencing dimorphisms within the song system (Konishi and Akutagawa, 1988; Adkins-Regan et al., 1994) the specific GPER1 antagonist G-15 (Cayman Chemical; 1.75 μg/μl) or DMSO/propylene glycol vehicle was administered to males and females. Starting within 24 hours of hatching, a 4 μl injection was given in the breast muscle and repeated daily for 25 days. This dose of G-15 has been demonstrated to be effective in and was derived based on rodent studies (Dennis et al., 2009; Hammond and Gibbs, 2011).

4.2.3 Tissue collection and processing

Within one hour after receiving the last injection at P25, animals were deeply anesthetized with Equithesin and transcardially perfused with 0.75% saline and 30 ml of 4% phosphate buffered formalin (PBF). The gonads were visually examined to determine the sex of the bird. Both the brain and syrinx were collected and post-fixed overnight in 4% PBF at 4˚C. The tissues were then cryoprotected in 10% sucrose for 2 hours followed by an overnight incubation each in 20% and 30% sucrose. Next they were covered with Polyvinylpyrrolidone (0.15 g/ml H_2O) and stored at -80˚C until use. Brains were cryosectioned coronally at 30 μm, syrinxes transversely sectioned at 20 μm, thaw-mounted onto gelatin-coated slides, and stained with Thionin (brains) or Trichrome (syrinxes).

4.2.4 Histological measurements

To prevent experimental bias, slides were coded to mask the treatment and sex of each animal. CellSens Dimension software was used for histological analyses. Neuron soma size was measured by selecting a single section in which the cross-sectional area of the song nucleus appeared largest. Within this representative section 25 cells were selected per hemisphere at random (50 total) in HVC, RA, LMAN, and Area X (present only in males). Soma sizes were measured under a 4x magnification and averaged across hemispheres. Neurons typically have larger sizes, pale nuclei, and darkly stained cytoplasm (Kirn and DeVoogd, 1989; Miller and Potempa, 1990) and these criteria were used to distinguish this cell type from glia. Neuron number was measured in the same brain regions with the exception of LMAN as it was difficult to identify the borders of this nucleus in all sections. This value was approximated by unilaterally sampling from sections within each animal, but between animals sampling alternated left and right hemispheres. For each nucleus, neurons were counted within a single rectangular box (counting frame) placed in three non-overlapping, random locations in every other adjacent section. The box covered approximately 30-40% of the field of view at 60x magnification. The size of the box was then divided by the area of each corresponding cross-sectional song nucleus measured at 4x. Next, this value was multiplied by the number of neurons averaged across the three counting frames in the same section. The cell number estimates from all sections were then summed and multiplied by the sampling interval to obtain the approximate neuron number in each nucleus. As the sampling interval was large (240 μm) double-counting cells was highly unlikely. Therefore, no correction was made for neuron estimates. Nuclear volumes were measured in HVC, RA, and Area X (LMAN was not included for the reason stated previously). Cross-sectional areas of all the sections within each nucleus were measured in each hemisphere

at 10x, summed and then multiplied by the sampling interval. Values from the left and right hemispheres were averaged together. In the syrinx the single section in which the pessulus first appeared intact (fully formed) was selected for analysis. 20 randomly selected fibers in ventralis and dorsalis were measured in both left and right sides (40 total) at 40x. These values were then summed and averaged.

4.2.5 Data analysis

Sigma Stat was used to analyze the effects. A two-way ANOVA (sex x treatment) was conducted followed by Student-Newman-Keuls *post-hoc* analyses whenever an interaction was present. A t-test was used for Area X. For all analyses significant effects were considered at a $p < 0.05$.

4.3 Results

4.3.1 Neuron size

A main effect of sex was detected in RA **(Figure 4.1A)** [$F(1, 38) = 8.178$; $P = 0.007$] but there was no effect of treatment [$F(1, 38) = 0.790$; $P = 0.380$] or an interaction [$F(1, 38) = 0.558$; $P = 0.460$]. Whereas G-15 treated males had significantly larger neuron soma sizes as compared to females, for unknown reason(s) we did not detect the expected sex effect in the control group. In HVC **(Figure 4.1B)** there was once again a main effect of sex [$F(1, 38) = 54.18$; $P < 0.001$], but no main effect of treatment [$F(1, 38) = 0.572$; $P = 0.454$] or an interaction [$F(1, 38) = 0.755$; $P = 0.391$] was detected. Results revealed a male vs. female main effect of sex within LMAN **(Figure 4.1C)** [$F(1, 34) = 6.862$; $P = 0.014$] but no main effect of treatment

[F (1, 34) = 1.087; P = 0.305] or an interaction [F (1, 34) = 171; P = 0.682] **(Figure 4.1D)** [t = 1.788; P = 0.089].

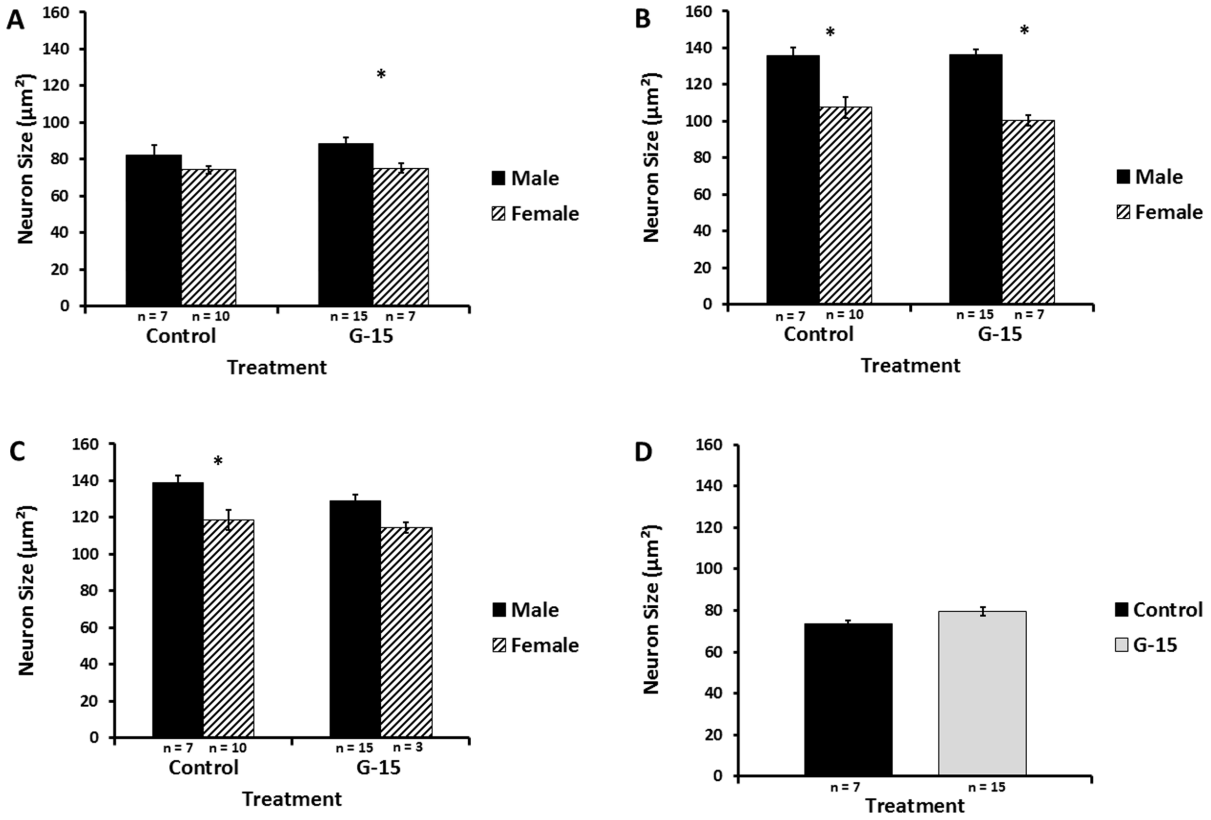

Figure 4.1 The effects of G-15 on neuron soma size in song nuclei. Treatment with G-15 did not affect the size of neurons in **(A)** RA, **(B)** HVC, **(C)** LMAN, or **(D)** Area X. Bars represent means ± SEM. * denotes a sex difference within each treatment group ($P \leq 0.02$).

4.3.2 Neuron number

In RA **(Figure 4.2A)** there was a main effect of sex [$F(1, 38) = 55.40$; $P < 0.001$] but no effect of treatment [$F(1, 38) = 0.569$; $P = 0.456$] or an interaction [$F(1, 38) = 0.942$; $P = 0.339$]. A main male vs. female effect of sex was revealed within HVC **(Figure 4.2B)** [$F(1, 38) = 64.10$; $P < 0.001$] but no effect of treatment [$F(1, 38) = 0.048$; $P = 0.827$] or an interaction [$F(1, 38) = 1.162$; $P = 0.289$] was detected. Lastly, there was no effect of treatment in Area X **(Figure 4.2C)** [$t = 1.063$; $P = 0.301$].

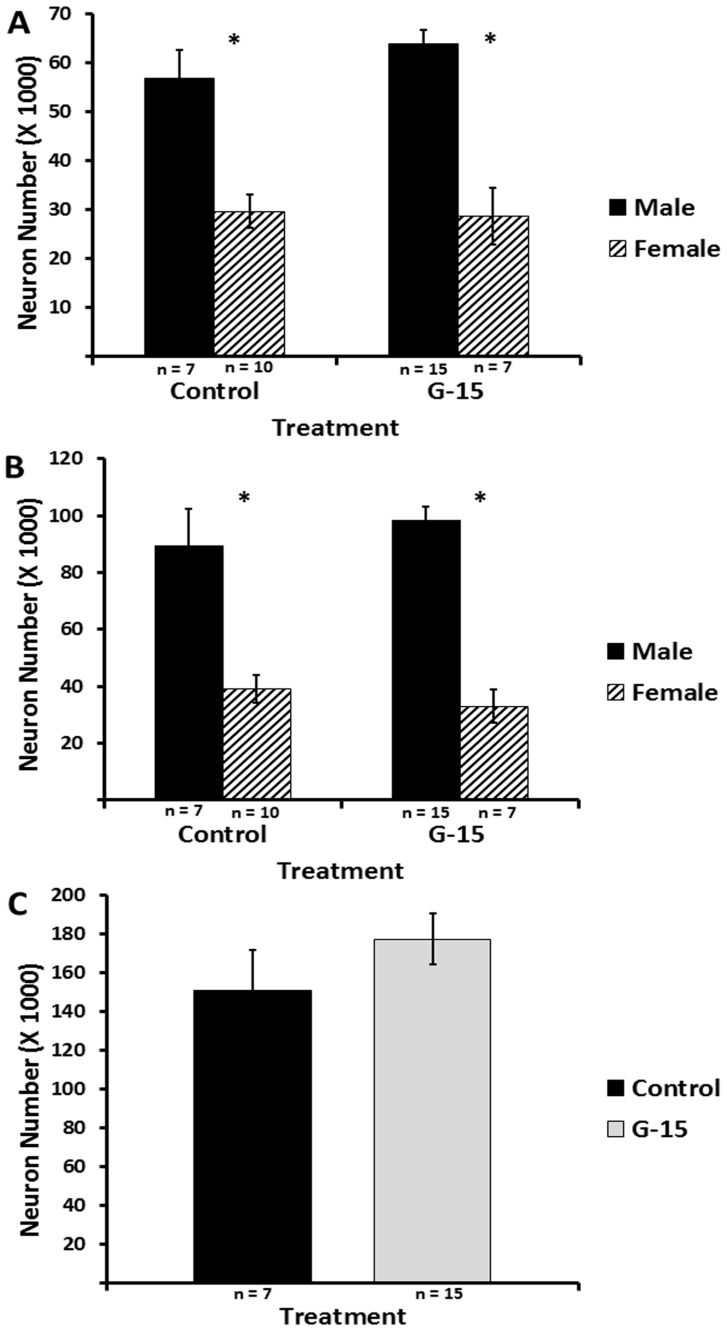

Figure 4.2 The effects of G-15 on neuron number in song nuclei. G-15 did not affect neuron number in **(A)** RA, **(B)** HVC, or **(C)** Area X. Bars represent means ± SEM. * denotes a sex difference within each treatment group ($P < 0.001$).

4.3.3 Nuclear volume

In RA **(Figure 4.3A)** there was a significant main effect of sex [$F(1, 38) = 40.05$; $P < 0.001$] but measurements of nuclear volume did not reveal an effect of treatment [$F(1, 38) = 0.360$; $P = 0.553$] or an interaction [$F(1, 38) = 0.607$; $P = 0.441$]. A similar pattern was observed within HVC **(Figure 4.3B)** where a main effect of sex existed [$F(1, 38) = 70.35$; $P < 0.001$] but no effect of treatment [$F(1, 38) = 0.008$; $P = 0.929$] or an interaction [$F(1, 38) = 0.202$; $P = 0.656$] was detected. Treatment with G-15 did not affect nuclear volume in Area X **(Figure 4.3C)** [$t = 0.483$; $P = 0.634$].

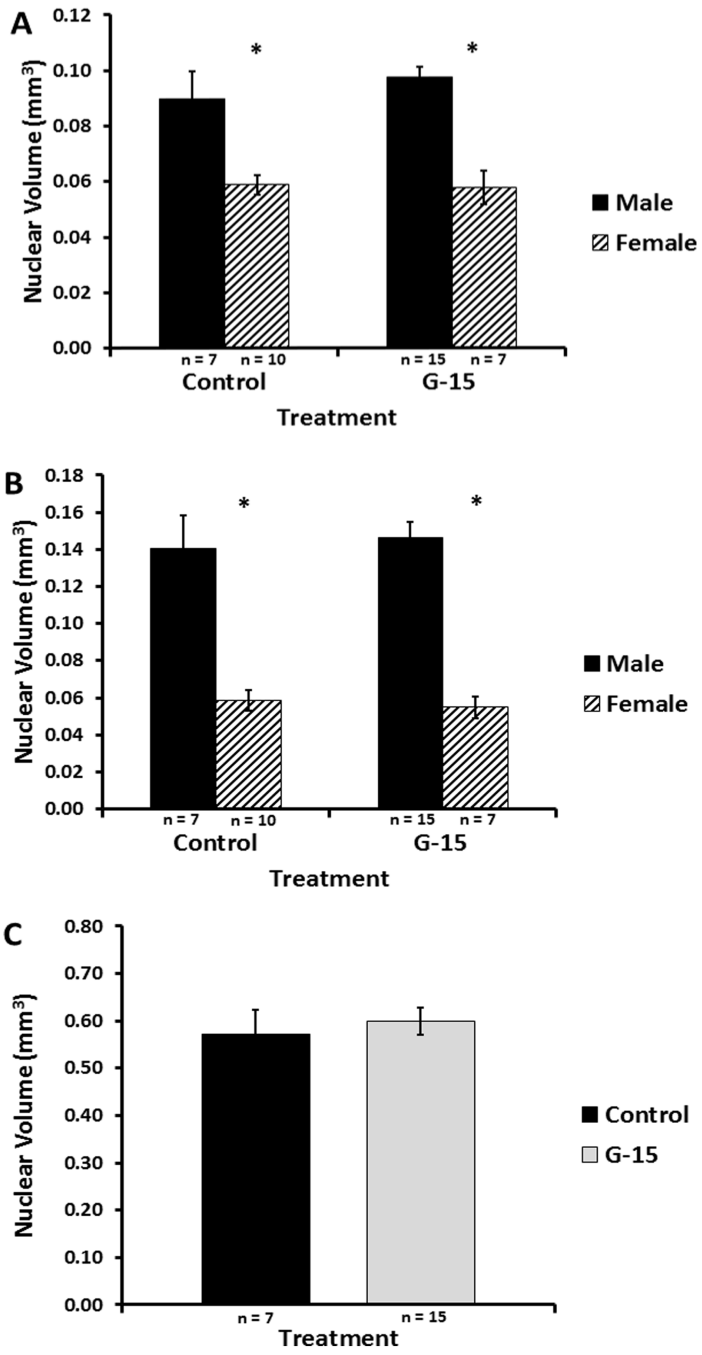

Figure 4.3 The effects of G-15 on nuclear volume in the song system. G-15 had no effect on nuclear volume in **(A)** RA, **(B)** HVC or **(C)** Area X. Bars represent means ± SEM. * denotes a sex difference within each treatment group ($P < 0.001$).

4.3.4 Syrinx muscle fiber sizes

Measurements of muscle fiber size within ventralis **(Figure 4.4A)** revealed a significant main effect of sex [F (1, 38) = 6.989; P = 0.012]. While no main effect of treatment [F (1, 38) = 0.196; P = 0.661] was detected, G-15 did significantly decrease muscle fiber sizes in males only. A significant interaction [F (1, 38) = 5.422; P = 0.026] was also detected. In dorsalis **(Figure 4.4B)** there was a significant main effect of sex [F (1, 44) = 8.421; P = 0.006], no main effect of treatment (though it approached significance) [F (1, 44) = 3.785; P = 0.059], and a significant interaction [F (1, 44) = 11.80; P = 0.001]. More specifically, dorsalis fiber sizes significantly decreased in males in response to treatment with G-15.

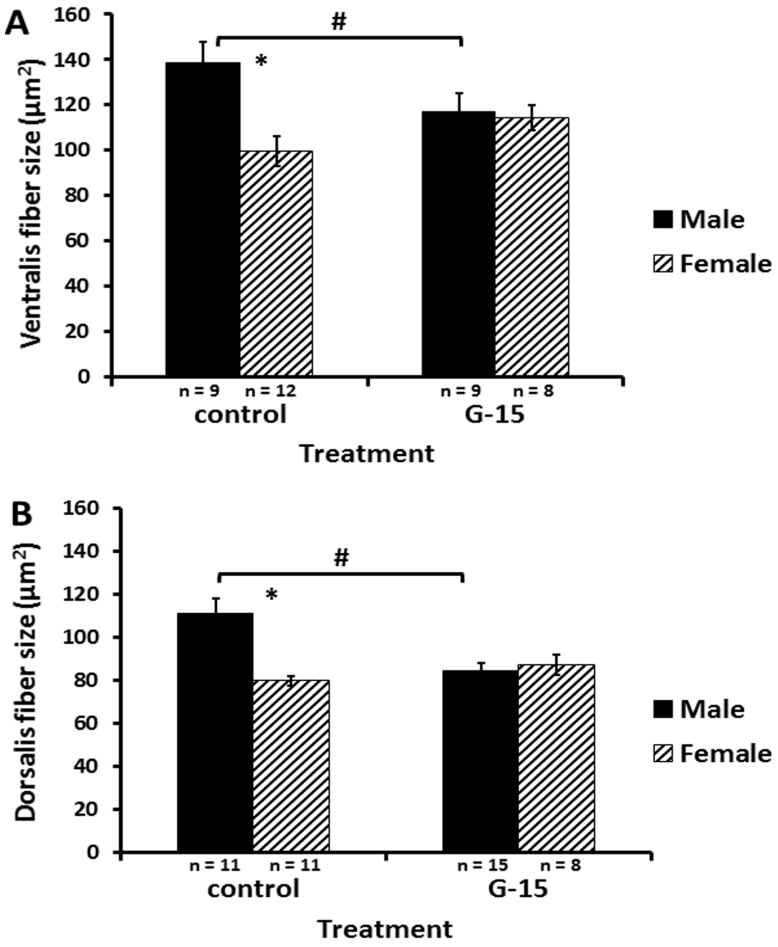

Figure 4.4 The effects of G-15 on syrinx muscle fiber sizes. G-15 significantly decreased **(A)** ventralis muscle fiber sizes in males, but had no effect in females. **(B)** Dorsalis fiber sizes also significantly decreased in males, but females were not affected by this treatment. Bars represent means ± SEM. ***** denotes a sex difference within each treatment group ($P \leq 0.001$). # denotes significant differences across treatment groups ($P \leq 0.05$).

4.4 Discussion

In this study, systemic treatment with G-15 significantly decreased muscle fiber sizes of ventralis and dorsalis in males, but had no effect in the female syrinx. It also had no effect in either sex within the neural song circuit on some of the most common dimorphisms that are altered by E2 administration or by exposure to centrally infused antagonists. Whereas results did not support the hypothesis that inhibition of GPER1 will demasculinize the brain and masculinize the syrinx, they did demonstrate that peripherally administered compounds may not reach the brain as efficiently as centrally administered agents. Thus, route of drug delivery is an important factor that should receive more attention when designing studies in zebra finches.

Lack of effects in the brain suggests that G-15 may have failed to reach the CNS from the periphery or did not enter the brain in sufficient concentrations to affect song nuclei. The BBB, the brain's protective capillary endothelium, has been described as a major obstacle for drug delivery into the CNS by being highly restrictive (reviewed in Banks, 2009; reviewed in Upadhyay, 2014). In order to freely diffuse through this barrier, at a minimum a compound must be lipid soluble and have a molecular weight around a maximum of 400 Da. Still, a majority of compounds that do meet these criteria are unable to permeate the brain (reviewed in Pardridge, 2012) since other factors such as charge and tertiary structure also play a role (Upadhyay, 2014). G-15 is a quinoline (oily) compound with a molecular weight of 370.2 Da (https://www.caymanchem.com/ pdfs/14673.pdf) but our results suggest that it may not have successfully reached the brain possibly due to not satisfying the remaining criteria for BBB permeability. Alternatively, even when a drug does have the ability to cross the BBB, it may not do so in therapeutically effective concentrations due to its association with non-specific ligands outside the brain, or its conversion into inactive metabolites by catalytic enzymes (Upadhyay,

2014). Therefore, another possible interpretation of the results is that G-15 was similarly affected in the periphery and did not reach the brain in sufficient concentrations.

Given that E2 is feminizing in the syrinx, G-15 was hypothesized to masculinize this organ. Surprisingly, and contrary to this prediction, inhibition of GPER1 demasculinized the male syrinx. These results suggest that GPER1 may not underlie the full scope of E2 action in development of this organ. For example, it is possible that ERβ is involved, as its mRNA is expressed in juvenile male and female syrinx (Veney and Wade, 2005). Blockade of this receptor with the specific antagonist ICI 182,780 for the first 25 days of development significantly increased muscle fiber sizes of ventralis and dorsalis in both sexes (Martin and Veney, 2008). This suggests that ERβ has feminizing effects on the syrinx. In contrast, we discovered that antagonism of GPER1 led to a feminization of the male syrinx, which is consistent with this receptor not feminizing, but rather, having a masculinizing role in this organ! We hypothesize that these two receptors work in parallel to influence syrinx dimorphisms. A proposed model is presented in **Figure 4.5**.

In the male syrinx, under physiological conditions **(Figure 4.5A)**, E2 acts on ERβ and GPER1, but because of sex differences in receptor number, and/or differences in intracellular signaling mechanisms, the response via GPER1 is greater resulting in masculinization. When males are exposed to supraphysiological doses of E2 **(Figure 4.5B)** we propose that both receptors are initially activated, but due to unidentified compensatory/feedback effects the activation of ERβ is up-regulated leading to feminization. Thus in summary, treatment with ICI 182,780 masculinizes, and G-15 feminizes the syrinx in males **(Figure 4.5C)**. Interestingly, GPER1 protein is not expressed in muscle fibers of the syrinx but it is localized to the syringeal pessulus (central boney tissue providing structural support) and chondrocytes (unpublished

observation) **(Figure 4.6)**. E2 influences differentiation of chondrocytes and bone formation in a number of species (Corvol et al., 1987; Nasatzky et al., 1993; Schwartz et al., 1995). Therefore, it could act within these tissues to indirectly alter muscle morphology. This may be achieved by inducing differentiation of myogenic bone marrow progenitor cells in the pessulus of the syrinx into muscle fibers (Ferrari et al., 1998; Hamada et al., 2006), or indirectly influencing muscle morphology by promoting chondrocyte and bone growth.

Similar to males, ICI 182,780 masculinizes the female syrinx further supporting the feminizing role of ERβ in this organ (Martin and Veney, 2008). However, GPER1 may have a different function. More specifically, we recently demonstrated that intracranial administration of G-15 resulted in a masculinization of the female syrinx (unpublished study). Thus, unlike in males where GPER 1 has masculinizing effects on the syrinx, in females, it appears that this receptor is feminizing! This is not a baseless conclusion, as dual functions for GPER1 within the same tissue and/or organism are not unique and have been described in rodent studies (Martensson et al., 2009; Chimento et al., 2010; Ford et al., Chimento et al., 2011; 2012; reviewed in Srivastava and Evans, 2013; Broughton et al., 2014).

In our model we propose that under endogenous E2 exposure, feminization occurs as a result of binding to both ERβ and GPER 1 **(Figure 4.7A)**. Supraphysiological concentrations of E2 do not have additional feminizing effects on the syrinx beyond those occurring normally (Wade et al., 2002) **(Figure 4.7B)**. In summary, treatment with ICI 182,780 masculinizes the syrinx, and theoretically G-15 should have also resulted in a masculinization, but curiously there was no effect **(Figure 4.7C)**.

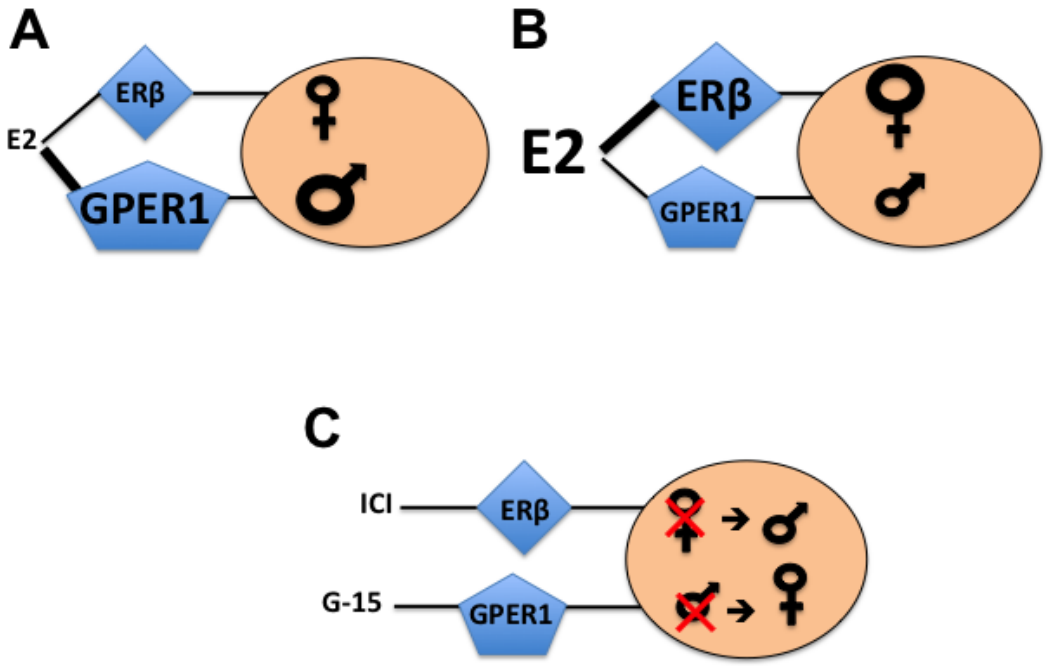

Figure 4.5 The proposed model for effects of ERβ and GPER1 in the syrinx of males. (A) Endogenous E2 masculinizes the syrinx via preferential GPER1 activation (depicted by bolded pathway lines and enlarged male symbol). **(B)** Supraphysiological E2 levels induce enhanced activation of ERβ receptors resulting in an overall feminization. **(C)** Inhibition of ERβ with ICI 182,780 masculinizes, and treatment with G-15 feminizes the syrinx.

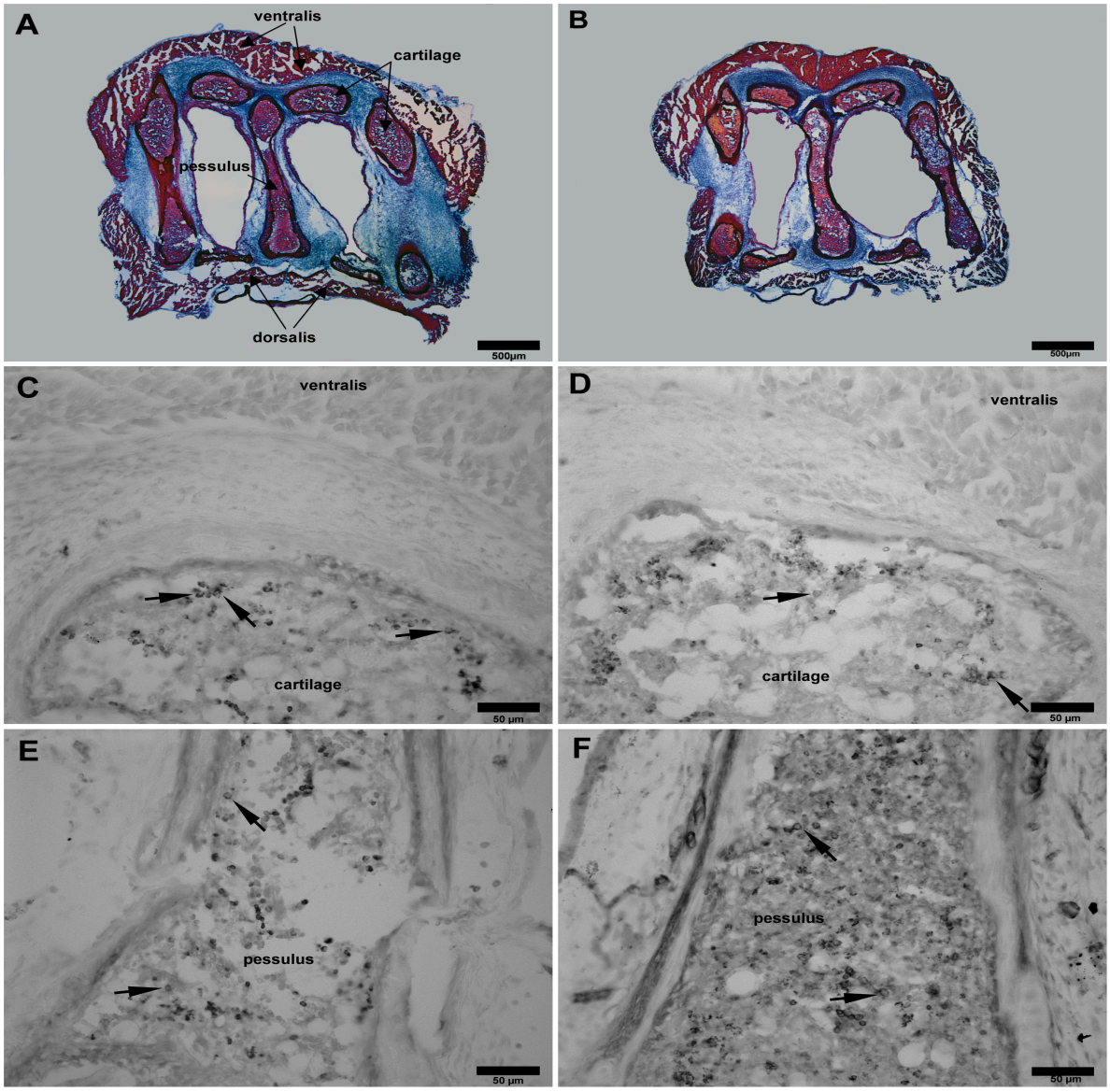

Figure 4.6 Representative photomicrographs of syrinx anatomy and GPER1 immunoreactivity in the syrinx of P25 zebra finch. Transverse sections stained with Trichrome are depicted in a **(A)** male and a **(B)** female. **(C)** and **(D)** represent magnified views of synringeal cartilage showing GPER1+ chondrocytes (black arrows) in a male and a female, respectively. Note lack of immunoreactivity in ventralis muscle fibers. Black arrows depict GPER1 labeling in the pessulus of a **(E)** male and a **(F)** female. Scale bars, 500 µm (A, B), 50 µm (C, D, E, F).

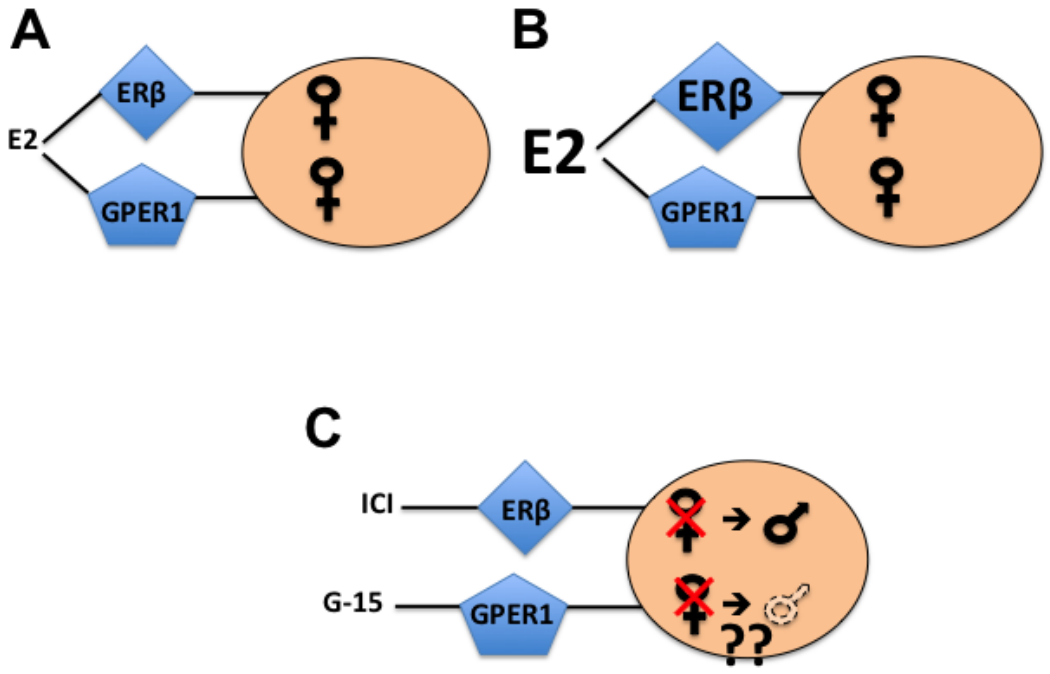

Figure 4.7 The proposed model for effects of ERβ and GPER1 in the syrinx of females. (A) physiological E2 feminizes the syrinx by acting on both ERβ and GPER1 receptors. **(B)** Supraphysiological doses of this hormone do not have added feminizing effects. **(C)** Feminization is blocked by ICI 182,78 leading to a masculine development. G-15 did not alter the female syrinx in this study, but it has been shown to masculinize this organ when administered centrally.

While unexpected, lack of effects on characteristics of the female syrinx in this study could be explained by sex differences in drug metabolism, a phenomenon that has primarily been reported in mammals (reviewed in Waxman and Chang, 1995; reviewed in Franconi et al., 2007). Dimorphisms in drug metabolism mainly involve differences in hepatic catalytic enzyme expression that lead to differences in rates of drug activity (reviewed in Waxman and Holloway, 2009). For instance, in rats, many drugs are metabolized 3-5 times more quickly in males as compared to females resulting in their shorter duration of action (reviewed in Kato, 1975), whereas this direction is reversed in mice (Vesell, 1968). In addition, at least one study in white Leghorn chickens reported that activities of several hepatic microsomal monooxygenases were significantly greater in roosters as compared to hens (Pampori and Shapiro, 1993). Although, to our knowledge, this concept has not been directly examined in zebra finches, it is possible that they exhibit a similar sex difference in drug metabolism, which would explain the effects of G-15 on the syrinx. This antagonist may have been metabolized more quickly in females as compared to males, thus resulting in no effects on the syrinx.

In summary, our results demonstrate a limited role for GPER1 in development of the syrinx. Future studies should investigate combinational effects of GPER1 and ERβ on syrinx development through co-administration of G-15 and ICI 182,78. Our study also demonstrates that in zebra finches, a peripheral method for drug delivery may not be as effective as central administration in altering aspects of brain development, possibly due to limitations imposed by the BBB, enzymatic breakdown of drugs in the periphery, and/or nonspecific binding outside the brain. Sex differences in drug metabolism may also account for some of the discrepancies observed in studies involving E2 manipulation in zebra finches. Therefore, future studies should

consider the influence of the method of drug delivery when designing experiments in this model organism.

4.5 References

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, 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. Dev Neurobiol 25: 865-877.

Arnold AP. 1992. Developmental plasticity in neural circuits controlling birdsong: sexual differentiation and the neural basis of learning. Dev Neurobiol 23:1506-1528.

Banks WA. 2009. Characteristics of compounds that cross the blood-brain barrier. In: BMC Neurol 9 (Suppl 1), p. S3.

Beach LQ, Wade J. 2015. Masculinisation of the Zebra Finch Song System: Roles of Oestradiol and the Z-chromosome Gene Tubulin-Specific Chaperone Protein A. J Neuroendocrinol 27:324-34.

Broughton BR, Brait VH, Kim HA, Lee S, Chu HX, Gardiner-Mann CV, Guida E, et al. 2014. Sex-dependent effects of G protein–coupled estrogen receptor activity on outcome after ischemic stroke. Stroke 45:835-841.

Chazal G, Faudon M, Gogan F, Rotsztejn W. 1975. Effects of two estradiol antagonists upon the estradiol uptake in the rat brain and peripheral tissues. Brain Res 89:245-54.

Chimento A, Sirianni R, Delalande C, Silandre D, Bois C, Andò S, Maggiolini M, et al. 2010. 17β-estradiol activates rapid signaling pathways involved in rat pachytene spermatocytes apoptosis through GPR30 and ERα. Mol Cell Endocrinol 320:136-144.

Chimento A, Sirianni R, Zolea F, Bois C, Delalande C, Ando S, Maggiolini M, Aquila S, Carreau S, Pezzi V. 2011. Gper and ESRs are expressed in rat round spermatids and mediate oestrogen-dependent rapid pathways modulating expression of cyclin B1 and Bax. Int J Androl 34:420-9.

Chimento A, Sirianni R, Casaburi I, Ruggiero C, Maggiolini M, Andò S, Pezzi V. 2012. 17β-Estradiol activates GPER-and ESR1-dependent pathways inducing apoptosis in GC-2 cells, a mouse spermatocyte-derived cell line. Mol Cell Endocrinol 355:49-59.

Corvol MT, Carrascosa A, Tsagris L, Blanchard O, Rappaport R. 1987. Evidence for a direct in vitro action of sex steroids on rabbit cartilage cells during skeletal growth: influence of age and sex. Endocrinology 120:1422-9.

Dennis MK, Burai R, Ramesh C, Petrie WK, Alcon SN, Nayak TK, Bologa CG, et al. 2009. In vivo effects of a GPR30 antagonist. Nat Chem Biol 5:421-427.

Ferrari G, Angelis D, Coletta M, Paolucci E, Stornaiuolo A, Cossu G, Mavilio F. 1998. Muscle regeneration by bone marrow-derived myogenic progenitors. Science. 279:1528-30.

Ford J, Hajibeigi A, Long M, Hahner L, Gore C, Hsieh JT, Clegg D, et al. 2011. GPR30 deficiency causes increased bone mass, mineralization, and growth plate proliferative activity in male mice. J Bone Miner Res 26:298-307.

Franconi F, Brunelleschi S, Steardo L, Cuomo V. Gender differences in drug responses. 2007. Pharmacol Res 55:81-95.

Grisham W, Arnold AP. 1995. A direct comparison of the masculinizing effects of testosterone, androstenedione, estrogen, and progesterone on the development of the zebra finch song system. Dev Neurobiol 26:163-170.

Gurney ME, Konishi M. 1980. Hormone-induced sexual differentiation of brain and behavior in zebra finches. Science 208:1380-1383.

Gurney ME. 1981. Hormonal control of cell form and number in the zebra finch song system. J Neurosci 1:658-73.

Gurney ME. 1982. Behavioral correlates of sexual differentiation in the zebra finch song system. Brain Res 231:153-172.

Hahn RA, Wardell JR, Sarau HM, Ridley PT. 1982. Characterization of the peripheral and central effects of SK&F 82526, a novel dopamine receptor agonist. J Pharmacol Exp Ther 223:305-13.

Hamada H, Kim MK, Iwakura A, Ii M, Thorne T, Qin G, Asai J, Tsutsumi Y, Sekiguchi H, Silver M, Wecker A. 2006. Estrogen receptors α and β mediate contribution of bone marrow–derived endothelial progenitor cells to functional recovery after myocardial infarction. Circulation. 114:2261-70.

Hammond R, Gibbs RB. 2011. GPR30 is positioned to mediate estrogen effects on basal forebrain cholinergic neurons and cognitive performance. Brain Res 1379:53-60.

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.

Jacobs EC, Grisham W, Arnold, AP. 1995. Lack of a synergistic effect between estradiol and dihydrotestosterone in the masculinization of the zebra finch song system. Dev Neurobiol 27:513-519.

Kato R. 1975. Sex-related differences in drug metabolism. Drug Metab. Rev 3:1-32.

Kirn JR, DeVoogd TJ. 1989. Genesis and death of vocal control neurons during sexual differentiation in the zebra finch. J Neurosci 9:3176-3187.

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 *USA* 85:7006-7007.

Lenhart PM, Broselid S, Barrick CJ, Leeb-Lundberg LF, Caron KM. 2013. G-protein-coupled receptor 30 interacts with receptor activity-modifying protein 3 and confers sex-dependent cardioprotection. J Mol Endocrinol 51:191-202.

Martensson UE, Salehi SA, Windahl S, Gomez MF, Swärd K, Daszkiewicz-Nilsson J, Wendt A, et al. 2009. Deletion of the G protein-coupled receptor 30 impairs glucose tolerance, reduces bone growth, increases blood pressure, and eliminates estradiol-stimulated insulin release in female mice. Endocrinology 1502:687-698.

Martin LC, Veney SL. 2008. The specific estrogen receptor antagonist ICI 182,780 masculinizes development of the zebra finch syrinx. Gen Comp Endocrinol 156:434-439.

Mathews GA, Arnold AP. 1991. Tamoxifen's effects on the zebra finch song system are estrogenic, not antiestrogenic. Dev Neurobiol 22:957-969.

Miller MW, Potempa G. 1990. Numbers of neurons and glia in mature rat somatosensory cortex: effects of prenatal exposure to ethanol. J Comp Neurol 293:92-102.

Nasatzky E, Schwartz Z, Boyan BD, Soskolne WA, Ornoy A. 1993. Sex-dependent effects of 17-beta-estradiol on chondrocyte differentiation in culture. J Cell Physiol 154:359-67.

Nottebohm F, Arnold AP. 1976. Sexual dimorphism in vocal control areas of the songbird brain. Science 194:211-3.

Pampori NA, Shapiro BH. 1993. Sexual dimorphism in avian hepatic monooxygenases. Biochem pharmacol 46:885-90.

Pardridge WM. 2012. Drug transport across the blood–brain barrier. J Cereb Blood Flow Metab 32:1959-72.

Reaux A, Fournie-Zaluski MC, Llorens-Cortes C. 2001. Angiotensin III: a central regulator of vasopressin release and blood pressure. Trends Endocrinol Metab 12:157-62.

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 107:3852-7.

Schwartz Z, Gates PA, Nasatzky E, Sylvia VL, Mendez J, Dean DD, Boyan BD. 1996. Effect of 17β-estradiol on chondrocyte membrane fluidity and phospholipid metabolism is membrane-specific, sex-specific, and cell maturation-dependent. Biochim Biophys Acta Biomembr 1282:1-0.

Srivastava DP, Evans PD. 2013. G-Protein Oestrogen Receptor 1: Trials and Tribulations of a Membrane Oestrogen Receptor. J Neuroendocrinol 25:1219-1230.

Upadhyay RK. 2014. Drug delivery systems, CNS protection, and the blood brain barrier. BioMed Res Int.

Veney SL, Wade J. 2005. Post-hatching syrinx development in the zebra finch: an analysis of androgen receptor, aromatase, estrogen receptor α and estrogen receptor β mRNAs. J Comp Physiol 191:97-104.

Vesell ES. 1968.Genetic and environmental factors affecting hexobarbital metabolism in mice. Ann N Y Acad Sci 151:900-12.

Wade J, Arnold AP. 1994. Post-hatching inhibition of aromatase activity does not alter sexual differentiation of the zebra finch song system. Brain Res 639:347-50.

Wade J, Schlinger BA, Hodges L, Arnold AP. 1994. Fadrozole: a potent and specific inhibitor of aromatase in the zebra finch brain. Gen Comp Endocrinol 941:53-61.

Wade J, Arnold AP. 1996. Functional testicular tissue does not masculinize development of the zebra finch song system. Proc Natl Acad Sci 93:5264-8.

Wade J. 2001. Zebra finch sexual differentiation: the aromatization hypothesis revisited. Microsc Res Tech 54:354-363.

Wade J, Buhlman L, Swender D. 2002. Post-hatching hormonal modulation of a sexually dimorphic neuromuscular system controlling song in zebra finches. Brain Res 929:191-201.

Wade J, Arnold AP. 2004. Sexual differentiation of the zebra finch song system. Annals of the New York Academy of Sciences. 1016:540-59.

Waxman DJ, Chang TK. 1995. Hormonal regulation of liver cytochrome P450 enzymes. In: Cytochrome P450. Boston: Springer. pp. 391-417.

Waxman DJ, Holloway MG. 2009. Sex differences in the expression of hepatic drug metabolizing enzymes. Mol Pharmacol 76:215-28.

Yoder KM, Lu K, Vicario DS. 2012. Blocking estradiol synthesis affects memory for songs in auditory forebrain of male zebra finches. Neuroreport 23:922.

CHAPTER 5

General Discussion

5.1 Implications of Research

Studies of mammals have established a central role for estradiol (E2) in sexual differentiation of the brain. However, non-traditional animal models such as the zebra finch have challenged and expanded our understanding of how these E2-mediated processes take place. One active area of research has been to identify the receptor mechanism(s) through which E2 influences brain dimorphisms. In the zebra finch song system, expression of the nuclear estrogen receptors (ERα and ERβ) is limited and their manipulations have largely failed to reverse the sex differences (reviewed in Wade, 2001). Historically, efforts to elucidate the role of E2 in brain sexual differentiation in rodents and birds have focused on ERα and ERβ as these receptors are known to have long-term, transcriptional function that can last several hours to days consistent with the time frame of developmental processes (reviewed in Morissette et al., 2008). But contribution to longer-term processes has also been proposed for membrane-bound forms of ER that typically act within seconds to minutes (Toran-Allerand et al., 2002). This raises the possibility that a membrane-bound receptor could be important for effects of E2 in the song system.

In zebra finches, E2 has also been studied in the context of auditory processing demonstrating enhancing effects on neuronal auditory function within a rapid time scale as birds hear songs of conspecifics (Remage-Healey et al., 2010). Whereas these modulatory effects have

been well demonstrated in adults, our understanding of how E2 contributes to juvenile auditory processing is less clear. Similar to the song system, in the auditory forebrain, ERα and ERβ have minimal expression and their blockade does not inhibit E2's effects (reviewed in Remage-Healey et al., 2013). These results suggest that an alternate form of ER may be important for these functions.

Among the few identified membrane receptors, GPER1 is a candidate for influencing song system dimorphisms and auditory function in zebra finches. This receptor has widespread distribution within the rodent brain (Hazell et al., 2009), regulates rapid as well as transcriptional activity (reviewed in Prossnitz and Barton, 2011), and is involved in a wide range of neural processes such as learning, memory acquisition, synaptic transmission and dendritic spine remodeling (reviewed in Alexander et al., 2017). While typically known for its rapid effects, chronic activation of GPER1 enhances (Hammond et al., 2009) and its long-term inhibition impairs spatial memory formation (Hammond et al., 2012) suggesting sustained effects for this receptor.

As an alternate approach, in this dissertation I studied the contribution of GPER1 to dimorphic development of the song system. In addition, I characterized its protein distribution within the major song nuclei and select auditory regions throughout a range of juvenile ages which would help shed light on its functional significance in these areas during development. In my first study, I semi-quantitatively described GPER1 protein expression in zebra finches within the song system and select auditory regions at several post-hatching (P) ages representing the hypothesized sensitive period for E2 action and when birds extensively hear and process song (reviewed in Adkins-Regan et al., 1994). My analysis revealed robust distribution of the receptor in HVC, RA, and LMAN in both males and females. Additionally, three major auditory regions

NCM, Field L and NIf had widespread expression in both sexes. I found age- and sex- specific variation in GPER1 immunoreactivity within song nuclei that coincides with some of the major cytostructural changes occurring in these regions during this period. GPER1 expression also varied within select auditory regions across the sensory and sensorimotor phases of song learning and between the sexes, consistent with the phase-specific effects of E2 on auditory response (Vahaba et al., 2017). Taken together, my data suggest that in zebra finches GPER1 may play an active role in sexual differentiation of the song system, and regulation of and possible sex differences in auditory processing. To my knowledge, this is the first study to comprehensively describe GPER1 protein distribution in the juvenile zebra finch brain.

My second study examined the effects of GPER1 inhibition on the dimorphisms within the song system by administering a specific GPER1 antagonist, G-15, directly in the brain. Daily treatment of zebra finches for the first 25 days after hatching significantly decreased nuclear volumes of HVC and Area X in males, but not females. G-15 also decreased muscle fiber sizes of ventralis and dorsalis in the vocal organ syrinx of males, but increased these measures in females. These results suggest that GPER1 does play a role in sexually dimorphic development of the song nuclei and the syrinx, but these effects are limited suggesting that other receptors, and even non-hormonal factors such as genetic influences, are likely also important (McCarthy and Arnold, 2011). Furthermore, the opposing effects of G-15 on the syrinx and lack of effects within the female brain were interesting as they suggest that similar to rodents GPER1 has dual and sex-specific functions in the zebra finch song system possibly by influencing processes such as cell survival and apoptosis (reviewed in Srivastava and Evans, 2013). Overall, my study demonstrates for the first time that a G-protein coupled estrogen receptor can also contribute to sexually dimorphic and long-term developmental functions within the zebra finch song system.

A central theme in my dissertation was to explore whether the route by which drugs are administered influence experimental results. The overwhelming majority of the past studies of song system development have attempted to block E2 synthesis and/or action peripherally, neglecting the possible limitations imposed by factors such as the blood-brain barrier on drug efficacy or sex differences in drug metabolism. Using the same experimental paradigm as my second study, in my third experiment I put this hypothesis to the test by administering G-15 intramuscularly and investigated the influence of GPER1 on song system dimorphisms. My data revealed that as compared to the intracranial administration, peripheral delivery of G-15 did not affect any of the dimorphisms within the neural song circuit. The effects in the syrinx partially agreed with those obtained from the intracranial method in that both ventralis and dorsalis muscle fiber sizes were demasculinized only in males, but they were unaffected in females, overall supporting a limited role for GPER1 in organizing the song system dimorphisms. Collectively, the results from my second and third experiments suggest that in zebra finches route of drug delivery does influence the results and must be given attention when designing experiments.

5.2 Future Directions

In order to gain a more complete understanding of how E2 affects song system dimorphisms expression of GPER1 should be quantitatively analyzed to confirm possible sex differences. This will be necessary specifically at ages and within regions for which the semi-quantitative analysis revealed divergent patterns of distribution between the sexes. Another future direction is to address the possible role of transsynaptic support. G-15 significantly decreased nuclear volumes of HVC and Area X in males, but interestingly, GPER1 appears to be

expressed only within HVC, whereas Area X appears almost entirely devoid of labeling. Area X does receive trophic support involving growth factors such as BDNF from HVC (reviewed in Brenowitz and Lent, 2001). Therefore, E2 may be indirectly influencing growth of Area X through growth factor release by acting on GPER1 receptors within HVC. This is likely as GPER1 has been shown to mediate neuroprotection via upregulation of BDNF in mice (Bourque et al., 2014). Future studies should investigate this possibility by analyzing BDNF levels in Area X following blockade of GPER1 receptors in HVC.

Since G-15 did not reverse all of the dimorphisms examined in the brain even when administered centrally, other membrane ERs may also be important. Future studies should investigate candidates such as ER-X (Toran-Allerand et al., 2002) and ER-γ (Hawkins et al., 2000) whose expression has been reported in mice and fish, respectively, but never explored in birds. Additionally, although antagonism of ERα and ERβ have not completely reversed sex differences within the song system, some limited effects have been reported. For instance, intracranial treatment with ICI 182,780, a specific nuclear ER blocker, demasculinized neuron soma sizes in select song nuclei of both sexes (Bender and Veney, 2008). These results suggest that GPER1 may work in combination with the nuclear ERs. Future studies could investigate this possibility by centrally co-administrating G-15 and ICI 182,780 during the first 25 days post-hatching as potency of these antagonists have now been verified in zebra finches. Various modes of functional cross-talk between GPER1 and the nuclear ERs have been described in rodents (reviewed in Hadjimarkou and Vasudevan, 2017) suggesting similar interactions may also be occurring in the zebra finch song system.

One other interesting future direction for this research is to explore the behavioral consequence of early GPER1 inhibition on behavior in adulthood. Will the effects on the song

circuit early post-hatching influence aspects of song production later in life? Additionally, it would be interesting to determine if GPER1 inhibition has consequences for auditory processing. Does not having functional GPER1 receptors affect a male's ability to learn song, or a female's ability to choose a high quality male mate?

Although the results of my study provided new insight into the zebra finch model, one limitation that was faced (particularly in experiment 2) was ensuring effective delivery of G-15 to all target areas within the brain. While the intracranial injections were administered just under the skull we did not verify whether G-15 had in fact reached all target areas in the brain. One way to address this limitation would be to co-inject a lipid soluble dye with G-15 in order to track the extent of its diffusion throughout the brain. Alternatively, site-specific injections of G-15 would have been desirable, however, this is not a viable option in early post-hatching zebra finches as their brain is too small for surgical cannula implantations. To bypass this limitation, future experiments could make use of compounds such as lipid nanoparticles designed for targeted brain delivery (Blasi et al., 2007) to ensure maximal and more sustained delivery of G-15 which would also possibly eliminate the need for daily injections.

5.3 References

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. Dev Neurobiol 25: 865-877.

Alexander A, Irving AJ, Harvey J. 2017. Emerging roles for the novel estrogen-sensing receptor GPER1 in the CNS. Neuropharmacology 113:652-60.

Bender AT, Veney SL. 2008. Treatment with the specific estrogen receptor antagonist ICI 182,780 demasculinizes neuron soma size in the developing zebra finch brain. Brain Res 1246:47-53.

Blasi P, Giovagnoli S, Schoubben A, Ricci M, Rossi C. 2007. Solid lipid nanoparticles for targeted brain drug delivery. Adv Drug Deliv Rev 59:454-77.

Bourque M, Morissette M, Di Paolo, T. 2014. Raloxifene activates G protein-coupled estrogen receptor 1/Akt signaling to protect dopamine neurons in 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine mice. Neurobiol Aging 35:2347-2356.

Brenowitz EA, Lent K. 2001. Afferent input is necessary for seasonal growth and maintenance of adult avian song control circuits. J Neurosci 21:2320-9.

Hadjimarkou MM, Vasudevan N. 2017. GPER1/GPR30 in the brain: Crosstalk with classical estrogen receptors and implications for behavior. J Steroid Biochem Mol Biol 10:1016.

Hammond R, Mauk R, Ninaci D, Nelson D, Gibbs RB. 2009. Chronic treatment with estrogen receptor agonists restores acquisition of a spatial learning task in young ovariectomized rats. Horm Behav 56:309-14.

Hammond R, Nelson D, Kline E, Gibbs RB. 2012. Chronic treatment with a GPR30 antagonist impairs acquisition of a spatial learning task in young female rats. Horm Behav 62:367-74.

Hawkins MB, Thornton JW, Crews D, Skipper JK, Dotte A, Thomas P. 2000. Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts. Proc Natl Acad Sci 97:10751-6.

Hazell GG, Yao ST, Roper JA, Prossnitz ER, O'Carroll AM, Lolait SJ. Localisation of GPR30, a novel G protein-coupled oestrogen receptor, suggests multiple functions in rodent brain and peripheral tissues. 2009. J Endocrinol 202:223-36.

McCarthy MM, Arnold AP. 2011. Reframing sexual differentiation of the brain. Nat Neurosci 14:677.

Morissette M, Le Saux M, D'Astous M, Jourdain S, Al Sweidi S, Morin N, Estrada-Camarena E, et al. 2008. Contribution of estrogen receptors alpha and beta to the effects of estradiol in the brain. J Steroid Biochem Mol Biol 108:327-338.

Prossnitz ER, Barton M. 2011. The G-protein-coupled estrogen receptor GPER in health and disease. Nat Rev Endocrinol 7:715-726.

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 107:3852-7.

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 25:1024-31.

Srivastava DP, Evans PD. 2013. G-Protein Oestrogen Receptor 1: Trials and Tribulations of a Membrane Oestrogen Receptor. J Neuroendocrinol 25:1219-1230.

Toran-Allerand CD, Guan X, MacLusky NJ, Horvath TL, Diano S, Singh M, Connolly ES, 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 22:8391-401.

Vahaba DM, Macedo-Lima M, Remage-Healey L. 2017. Sensory Coding and Sensitivity to Local Estrogens Shift during Critical Period Milestones in the Auditory Cortex of Male Songbirds. eNeuro 4:ENEURO-0317.

Wade J. 2001. Zebra finch sexual differentiation: the aromatization hypothesis revisited. Microsc Res Tech 54:354-363.