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THE EFFECTS OF ESTRADIOL ON 17 β -HYDROXYSTEROID DEHYDROGENASE TYPE IV AND ANDROGEN RECEPTOR EXPRESSION IN THE DEVELOPING ZEBRA FINCH SONG SYSTEM

J. Bayley Thompson^a, Eldin Dzubur^a, Juli Wade^b, and Michelle Tomaszycski^{a,CA}

^a Wayne State University, Department of Psychology, Detroit MI 48202, USA

^b Michigan State University, Department of Psychology and Neuroscience Program, East Lansing, MI 48824, USA

Abstract

Recent work in zebra finches suggests that genes and hormones may act together to masculinize the brain. This study tested the effects of exogenous estradiol (E2) on 17 β -Hydroxysteroid Dehydrogenase type IV (HSD17B4) and the co-localization of HSD17B4 and androgen receptor (AR) mRNA. We asked three primary questions: First, how does post-hatching E2 treatment affect HSD17B4 mRNA expression in males and females? Second, is this gene expressed in the same cells as AR, and, third, if so does E2 modulate co-expression? Female finches implanted with 50 μ g of E2 on the third day post-hatching showed a significant increase in the density of cells expressing HSD17B4 and AR in HVC at day 25. Co-localization of AR cells that also expressed HSD17B4 was high across groups (>81%). We found significant sex differences in co-localization in both the HVC and Area X of control animals, with males showing a higher percentage of cells expressing AR mRNA that also expressed HSD17B4 in comparison to females. However, although E2 treatments significantly increased the number of cells expressing HSD17B4 mRNA and AR mRNA in the HVC of females, the percentage of HSD17B4 cells co-expressing AR was reduced in HVC and Area X in E2-treated animals. These results lend support to the hypothesis that genes and hormones may act in concert to modulate the sexually differentiation of the zebra finch song system. Further, the data suggest that a single hormonal mechanism cannot mimic the complex development of male singing behavior and associated song nuclei.

Keywords

Zebra finch; masculinization; estradiol; song nuclei; genes; hormones; 17 β hydroxysteroid dehydrogenase type IV; *in situ* hybridization

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Corresponding author: Michelle L. Tomaszycski, PhD, Department of Psychology, Wayne State University, 5057 Woodward, Detroit MI, 48202, Phone: (313)577-0341, michelletomas@wayne.edu.

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1. Introduction

Sex differences in behavior are widespread across species and can be linked to corresponding sex differences in the brain. For example, zebra finches exhibit striking sexual dimorphisms in singing behavior that parallel differences in brain morphology. Male finches sing and females do not, and the brain areas controlling song are substantially larger in males. For example, Area X, a region important for song learning, is present in adult males, but does not develop in females (Nottebohm & Arnold, 1976). The lateral magnocellular nucleus of the anterior nidopallium (LMAN) is also important for song learning. Although LMAN is sexually monomorphic in volume, the LMAN of males has larger somas and nucleoli than that of females (Nixdorf-Bergweiler, 2001). HVC (proper name) and the robust nucleus of the arcopallium (RA) are responsible for the motor aspects of song and these areas are larger in volume, have more cells, and larger somas in males than in females (Wade, 2001).

Research has focused primarily on hormonal control of song system masculinization. In females, E2 administered early in development can partially masculinize song nuclei and can even result in the production of male-typical song, although results vary greatly between individual females (Holloway & Clayton, 2001; Simpson & Vicario, 1991a, 1991b). However, no hormonal treatments have reliably masculinized females, and few studies have demasculinized males by limiting hormone exposure. For example, treatments with anti-estrogens (Mathews & Arnold, 1990; Mathews & Arnold, 1991; Mathews et al., 1988) or estrogen-synthesis inhibitors (Balthazart et al., 1995; Wade & Arnold, 1994) do not inhibit masculinization (Mathews & Arnold, 1990). Castration concurrent with flutamide treatment (an androgen antagonist) does not completely prevent masculinization (Bottjer & Hewer, 1992). However, treating slices from male song nuclei with estrogen antagonists inhibits male-typical connections between HVC and RA (Holloway & Clayton, 2001), suggesting some role for hormones, albeit in vitro. More recent research has revealed that male-typical cell sizes in HVC and RA can be demasculinized by direct intracranial injections of an estrogen receptor antagonist (Bender & Veney, 2008). Thus, the role for hormones in the sexual differentiation of the song system remains unclear.

One might expect that E2 masculinizes females by acting at estrogen receptors (ERs) in song nuclei. However, adult zebra finches have relatively few ERs in the song system; these are detectable only in HVC and at low levels (Metzdorf et al., 1999). In contrast, androgen receptors (ARs) are abundant in Area X, LMAN, RA and HVC (Gahr & Metzdorf, 1997; Kim et al., 2004). AR expression is sexually dimorphic in HVC as early as P9 (Gahr & Metzdorf, 1999a) and parallels the increase in volume of HVC in male zebra finches (Kim, et al., 2004). Despite the abundance of ARs in song nuclei, androgen treatments in female zebra finches have little effect on masculinization, and treatments with an androgen receptor antagonist, flutamide only partially demasculinize song regions in males (Grisham et al., 2007). However, flutamide prevents the masculinizing effects of E2 in females, suggesting that ARs are an important component of the process through which masculinization occurs (Grisham et al., 2002). Additionally, E2 up-regulates AR mRNA expression in Area X and HVC (Kim, et al., 2004; Nordeen et al., 1986), and treatments of fadrozole (an aromatase inhibitor) decrease AR mRNA expression in these same regions (Kim, et al., 2004).

In addition to effects of steroid hormones, it is likely that the masculinization of the song system involves direct genetic effects. This idea was first illustrated by a gynadromorphic zebra finch. The right side of this spontaneously occurring bird had a male gonad, masculine plumage, and male-biased expression of sex chromosome genes (males birds are ZZ; females ZW), while the right was genotypically and phenotypically female (Agate et al., 2003). Morphology of the forebrain song control system indicated roles for both direct

genetic effects and one or more diffusible factors on sexual differentiation. Studies have identified specific genes that are both sex-linked and expressed at increased levels in the male than the female song system (Chen et al., 2005; Duncan & Carruth, 2007; Tang et al., 2007; Tang & Wade, 2006; Tomaszycski et al., 2009). Recent research suggests that some of these genes may interact with AR to facilitate masculinization (Tang & Wade, 2010; Wu, et al., 2010).

The present study investigated the Z-linked gene encoding HSD17B4, which converts E2 into a less active estrogen, estrone (de Launoit & Adamski, 1999). Genomic analysis of HSD17B4 confirms a role of this gene in steroid synthesis and, indeed, ER α response elements have been predicted from this analysis (London & Clayton, 2010). HSD17B4 mRNA is sexually dimorphic in the song system across a range of ages. At day 25 post-hatching, this gene is expressed in 3 song regions: LMAN, Area X and HVC (Tomaszycski, et al., 2009). Expression is higher in the male zebra finch LMAN as early as day 5 (London et al., 2010) and is significantly increased in males than in females in HVC at post hatch day 25 (Tomaszycski, et al., 2009). This study asks three primary questions: First, how does E2 treatment affect HSD17B4 mRNA expression in day 25 males and females? This age is particularly relevant to development of male brain morphology and behavior. The forebrain song circuit is rapidly differentiating, and it is early in the period when males learn song from their fathers. We hypothesize that E2 will increase HSD17B4 expression in the HVC of females, since E2 can partially masculinize females and one plausible mechanism is an increase in male-biased gene expression. Second, is this gene located in the same cells as AR? If cells express both AR and HSD17B4, it will suggest that they may act in concert to produce masculinization. Third, does E2 treatment also affect co-localization of AR and HSD17B4? If so, E2 may influence masculinization by modulating the interaction between these two genes.

2. Results

2.1 HSD17B4

Two-way (sex by treatment) ANOVAs were conducted for each brain region. We examined three song nuclei: HVC, LMAN and Area X. RA was not analyzed because it did not show specific staining for HSD17B4 in this or a previous study (Tomaszycski, et al., 2009). In HVC, there was no significant main effect of sex ($F=2.550$, $p=0.127$) or treatment ($F<1$) on HSD17B4 mRNA expression. However, a significant sex by treatment interaction for HSD17B4 was detected ($F=7.213$, $p=0.015$, fig 1). Control males had a greater number of cells expressing HSD17B4 than did control females ($t=4.188$, $p=0.002$) and treatment with E2 increased the expression of HSD17B4 mRNA expression in females but decreased it in males, such that E2-treated animals did not significantly differ from each other ($t<1$).

In Area X, no significant main effects were detected for [sex: ($F<1$), treatment: ($F=1.961$, $p=0.178$)], nor was there a significant interaction ($F<1$, fig 2). The same was true for HSD17B4 mRNA expression in LMAN [sex: ($F<1$), treatment: ($F=1.964$, $p=0.177$), interaction: ($F<1$), fig 3].

2.2 Androgen Receptor

For HVC, no main effect of either sex ($F<1$) or treatment ($F<1$) existed. However, a significant sex by treatment interaction was found for AR mRNA expression in this brain region ($F=17.780$, $p=0.001$, fig 1). E2 increased the number of cells expressing AR in the HVC of females and decreased expression in the same region in males, such that E2 males had lower levels of expression than did E2 females ($t=3.318$, $p=0.008$). Also, control males

had a significantly higher number of cells expressing AR than did control females ($t = 2.990$, $p = 0.014$).

In Area X, a main effect of treatment was observed for cells expressing AR mRNA ($F = 11.348$, $p = 0.003$, fig 2). Estrogen-treated animals had significantly fewer cells expressing AR mRNA than did controls. However, there was no significant effect of sex, ($F < 1$), nor was there a significant sex by treatment interaction, ($F < 1$).

In LMAN, AR mRNA expression did not differ overall between the sexes, ($F < 1$), or across treatments, ($F = 2.756$, $p = 0.113$), nor was there a significant interaction between the two, ($F = 0.428$, $p = 0.521$, fig 3).

2.3 Co-localization of HSD17B4 and AR mRNA

ANOVAs were run on arcsine transformed percentages to test the effects of sex and E2 treatment on the co-localization of HSD17B4 and AR mRNA. Co-localization was examined in two ways: the percentage of AR mRNA cells expressing HSD17B4 and the percentage of HSD17B4 mRNA cells expressing AR, to obtain a more complete picture of co-localization patterns.

The percentage of AR mRNA cells expressing HSD17B4 was high across groups and brain regions (>81%). However, males (regardless of treatment) had a greater percentage of AR mRNA expressing cells that also expressed HSD17B4 than did females in HVC ($F = 6.482$, $p = 0.020$), as well as Area X ($F = 4.653$, $p = 0.044$, see Table 1). This effect did not occur in LMAN ($F < 1$). Significant effects of treatment were not detected in any of the regions examined [HVC: ($F < 1$); Area X: ($F = 2.76$, $p = 0.113$); LMAN: ($F < 1$)]. Also, there were no significant sex by treatment interactions in HVC ($F = 1.83$, $p = 0.192$), Area X ($F = 2.22$, $p = 0.153$), or LMAN ($F < 1$).

The percentage of HSD17B4 expressing cells that co-expressed AR mRNA was also high in HVC across groups, but lower and more variable within Area X and LMAN. While an effect of sex was not detected [HVC: ($F < 1$); Area X: ($F = 1.313$, $p = 0.266$); LMAN: ($F = 1.008$, $p = 0.328$), see Table 1], E2 significantly decreased co-localization in Area X (E2: $F = 6.602$, $p = 0.019$) and LMAN ($F = 5.094$, $p = 0.036$), but not in HVC ($F < 1$). Finally, no significant sex by treatment interactions were seen in any of the song regions [HVC: ($F = 2.240$, $p = 0.151$); Area X: ($F < 1$); LMAN: ($F < 1$)].

3. Discussion

Overall, we confirmed findings that sexual dimorphisms in HSD17B4 mRNA expression at post hatch day 25 are confined to HVC (Tomaszycki et al., 2009). We further demonstrated that E2 treatments beginning at post hatch day 3 increased expression of HSD17B4 mRNA in the HVC of females and confirmed E2-induced increases in AR mRNA expression in the female HVC as well (Kim, et al., 2004). These same treatments decreased HSD17B4 and AR mRNA expression in the HVC of males. Co-localization of HSD17B4 and AR was high across sexes and treatment groups, although males had a significantly greater degree of co-localization than did females in HVC and Area X, but not in LMAN. Taken together, these results suggest that E2 may partially masculinize females by separately increasing HSD17B4 and AR in the song system, but the inability of E2 to completely masculinize females may relate to the failure to increase the co-localization of these two factors.

3.1 Effects of E2 on HSD17B4 and AR mRNA expression

3.1.1 HVC—The higher number of cells expressing HSD17B4 mRNA in the HVC of males compared to females is consistent with the hypothesis that this gene is involved in

masculinization. Since HSD17B4 converts E2 into estrone, for which estrogen receptors have a lower affinity (de Launoit & Adamski, 1999), HSD17B4 may effectively decrease activity at ERs in HVC. In parallel, ER-immunoreactivity decreases in males in this region at post hatch day 30 (Schlinger, 1997). Further research should evaluate this relationship.

Sex differences in HSD17B4 mRNA expression, however, may not lead to sexually differentiated downstream responses. Recently, London et al. (2010) showed that adult sex differences in HSD17B4 mRNA expression do not translate into a sex difference in enzyme activity. Further research should determine whether or not this is the case during development.

Given that E2 can partially masculinize females, we predicted that E2 might accomplish this at least in part by increasing expression of male-biased genes, such as HSD17B4. Our hypothesis was supported, suggesting that E2 may partially masculinize females by increasing the activity of Z-linked genes. Surprisingly, E2 treatments decreased HSD17B4 expression in males. At least two explanations are plausible, the first of which fits with the hypothesis above: (1) too much E2 may demasculinize males, or (2) E2 may not masculinize males at all. One should also consider, however, that group differences in the number of cells within a specified area (as measured here) may not reflect relationships in the total number of cells expressing HSD17B4 or the level of its activity. These patterns should be investigated before firm conclusions can be drawn.

We also replicated previous findings on sex differences in AR mRNA expression in the HVC of males compared to females (Gahr & Metzdorf, 1999a) and an increase in AR mRNA expression in females as the result of E2 treatments (Kim, et al., 2004), using a lower dosage of E2 (50 µg vs. 83 µg).

We predicted that HSD17B4 would be located in the same cells as androgen receptors. This co-localization might play a part in the masculinization process, since estrogen receptors are low in the song system (Gahr & Metzdorf, 1999b). Males had a significantly higher degree of co-localization (AR cells expressing HSD17B4) in HVC in comparison to females. These results mirror those found with sorting nexin 2 and ribosomal proteins L17 and L37 (Tang & Wade, 2010; Wu et al., 2010), and collectively point to the possibility that the co-localization of a variety of Z-chromosome genes and AR may be important for the masculinization of the song system. However, E2 treatments had no effect on the extent of the co-localization in the present study. This failure of E2 to increase co-localization along with the up-regulation of AR and HSD17B4 may explain why E2 only partially masculinizes the female zebra finch. Simply mimicking male levels of expression is not enough; male-typical patterns of co-localization may be necessary for complete masculinization. Previous research has shown that E2 treatments do not reliably masculinize singing behavior in females (Arnold, 1997; Simpson & Vicario, 1991a), and we may now have at least one underlying mechanism for this result. It is possible that there are other mechanisms by which male-typical levels of co-localization are achieved. Perhaps females have a complementary system by which genes on the W chromosome may act to inhibit masculine patterns of co-localization (Arnold, 1996).

Thus, in HVC at least, we have some novel information that might elucidate the mechanisms by which E2 masculinizes females, via HSD17B4 and AR. However, the processes do not account for normal masculinization, which suggests that separate mechanisms are likely involved.

3.1.2 Area X—Lesions of Area X during development lead to deficits in song production in males (Doupe & Solis, 1997), suggesting that sex differences in this region at day 25 may

be important for song learning. Area X is detectable only in male finches, the most striking sexual dimorphism among the song nuclei. Nonetheless, males and females had similar numbers of cells expressing HSD17B4 mRNA in this region, confirming previously reported findings (Tomaszycki, et al., 2009). This result suggests that HSD17B4 may not mediate differentiation of the structure or function of Area X and that other mechanisms are likely involved.

Furthermore, there was no sex difference in the number of cells expressing AR mRNA. This is surprising, since earlier work documented a sex difference in AR protein expression in Area X at this same age (Wu, et al., 2010). However, mismatches between mRNA and protein expression do occur (Chen et al., 2002; Greenbaum et al., 2003), and Wu and colleagues (2010) quantified the total number of cells expressing AR-ir rather than the number within a specified area as we did. Another study (Kim, et al., 2004) measured mRNA and also found a sex difference. However, that study focused on an earlier developmental time point, and differences may be attributable to the rapidly changing environment within song nuclei at these points. Further, radio labeled *in situ* hybridization was used to estimate the total volume of Area X that expressed AR mRNA in contrast to the number of cells within a portion of Area X in the present study. It is possible that these differences in measurements might contribute to differences in our results, although our previous study also used radio labeled *in situ* hybridization to study HSD17B4 and found results comparable to this study (Tomaszycki, et al., 2009).

Despite the lack of a sex difference in cells within Area X expressing HSD17B4 or AR, we found that males had a higher degree of AR cells co-expressing HSD17B4 than did females in this region. This result lends some support to the idea that AR and HSD17B4 may act in concert to masculinize Area X. Further, E2 may not be necessary for this relationship, since E2 treatments in females did not increase expression of either gene, nor did E2 alter co-localization of AR and HSD17B4 in Area X of either sex. This suggests that E2 likely exerts its masculinizing effects on Area X using different mechanisms.

E2 treatment did not increase HSD17B4 mRNA in the Area X of females or males. However, we found a dramatic reduction of the number of cells expressing AR mRNA, and the percentage of HSD17B4 cells co-expressing AR, in E2 treated animals of both sexes. These results contradict previous work on the effects of E2 on AR mRNA (Kim et al., 2004), which showed that E2 increases AR mRNA in Area X. In addition to considering different developmental time points (day 11 vs. day 25 in the present study), our study also used a lower dose of E2 (50 µg) than did the earlier study (83 µg), which may account for this difference. Nonetheless, another study found that our dosage was sufficient to masculinize many morphological measures in the song regions of females (Grisham et al., 2008), and increased AR mRNA expression in HVC in the present study. AR mRNA in Area X may therefore be less responsive to E2.

3.1.3 LMAN—No sex differences were found in expression of HSD17B4 mRNA in LMAN, confirming previous results at this same age (Tomaszycki et al., 2009). There were also no sex differences in AR mRNA expression or co-localization of AR and HSD17B4. London et. al. (2010) found increases in the expression of HSD17B4 in males compared to females at day 5 in this region, suggesting that HSD17B4 might be more important earlier in the development of LMAN. Finally, E2 treatments did not affect the expression of HSD17B4 or AR in males or females, but decreased the percentage of HSD17B4 cells that also expressed AR. Earlier work also did not find an effect of E2 administration on AR mRNA in LMAN (Kim, et al., 2004).

3.2 Summary

In conclusion, we found that males had higher numbers of cells expressing HSD17B4 and AR mRNA, as well as a greater degree of co-localization of HSD17B4 and AR, in HVC than did females. In Area X, males had a higher degree of cells expressing AR that also expressed HSD17B4 than did females, but there were no other significant sex differences in Area X or LMAN. In females, E2 independently increased HSD17B4 and AR mRNA expression in HVC to male-typical levels, but had no effect on the co-localization of HSD17B4 and AR. Additionally, E2 treatments reduced AR mRNA in Area X and LMAN in both males and females. Thus, a single hormonal mechanism can masculinize aspects of the song system, but cannot completely mimic the complex development of male singing behavior and associated song nuclei.

4. Methods

4.1 Animals and Tissue Preparation

Tissues were collected from animals living in colony cages containing multiple males and females, as well as their offspring. Birds were implanted with either a 1mm silastic glue pellet containing 50µg of E2 or a blank pellet on the third day post hatching. At post hatching day 25, the brains were collected following rapid decapitation, frozen in cold methyl-butane and stored at -80°C. Sex was determined by examining the gonads post-mortem under a dissecting microscope. The presence of the pellet and sex of the animal was confirmed in all subjects. Final sample sizes included six animals in each of the four groups (females and males treated with E2 or blank capsules). Thus, a total of 24 animals were included in the study.

Brains were sectioned coronally (20µm) and mounted onto SuperFrost Plus slides (Fisher Scientific, Hampton, NH). Six series of sections representing the whole brain were collected and stored at -80°C with dessicant.

4.2 Probe Preparation

Colonies used to generate probes were obtained from glycerol stocks (HSD17B4: Tomaszycski et al., 2009; AR: Kim et al., 2004), and DNA was isolated and confirmed through sequencing. We then used a Qiagen Maxi Prep kit (Valencia, CA), and the templates were linearized using the restriction enzymes XhoI (T3) and NotI (T7). For HSD17B4 mRNA, T3 was the anti-sense strand and T7 was the sense strand. For AR the reverse was true. HSD17B4 was labeled using a digoxigenin kit per manufacturer's instructions (Cat # 11 175 025 910, Roche Applied Science, Indianapolis, IN). AR was labeled using a Biotin kit also per manufacturer's instructions (Cat # 11 685 597 910, Roche Applied Science, Indianapolis, IN).

4.3 Double-label Fluorescence In Situ Hybridization

The *in situ* hybridization protocol was adapted from (Pinaud et al., 2004), and optimized to provide minimal staining in AR and HSD17B4 sense slides. Briefly, all slides were brought to room temperature, fixed in 3% paraformaldehyde and rinsed in phosphate buffered saline (PBS). Slides were incubated for 10 minutes in 0.1M triethanolamine hydrochloride with 0.25% acetic anhydride then rinsed three times in 0.2M sodium phosphate, sodium chloride and EDTA (SSPE), dehydrated in ethanols and air dried for 10 minutes. Tissue was incubated overnight at 55°C in 200µl of hybridization buffer, which included 10µl of HSD17B4 probe and 8µl of AR probe.

Parafilm coverslips were removed by rinsing in 2X SSPE, then the slides were washed in 2X SSPE at room temperature for 30 minutes on a shaker. This was followed by a wash in 2X

SSPE/50% formamide for 1 hour and 65°C, and two washes in 0.1X SSPE for 30 min at 65°C. FITC signal detection was accomplished by incubating slides in 0.3% hydrogen peroxide in TNT buffer for 10 minutes followed by 3 rinses in TNT buffer (5 minutes each) on a shaker. Slides were then washed in TNB buffer (TNT buffer with 2mg/BSA) for 30 minutes, then incubated in TNB buffer containing Anti-DIG-POD antibody (1:100; 10 µg/ml, Cat # 11 207 733 910, Roche Diagnostics, Indianapolis, IN) for 2 hours, followed by further washes. This procedure was followed by a 30 minute incubation in a 1:100 tyramide-conjugated TRITC fluorophore in the manufacturer's buffer (Alexa 594, Molecular Probes, Carlsbad, CA). Slides were then incubated in 0.3% Hydrogen peroxide in TNT buffer for 10 minutes and washed for 5 minutes in TNT buffer. For biotin detection, slides were incubated for one hour in TNT buffer containing anti-biotin antibody (1:500; 10µg/ml, Cat # SP-3010, Vector Labs, Burlingame, CA). After a final series of washes, the slides were coverslipped with Slow Fade (Molecular Probes, Carlsbad, CA), dried in a light proof box overnight, and the edges were sealed with clear nail polish the following day.

4.4 Analysis

Images were analyzed using a Nikon (Eclipse 80i) microscope with Nikon Elements (AR 3.0) software. Each brain area of interest (HVC, LMAN, and Area X) was first located in adjacent sections stained with thionin using brightfield microscopy. In females, we measured the area of the medial striatum in which Area X is located in males, based on surrounding landmarks. For each section, three separate images were analyzed in fluorescently labeled slices, FITC illuminating cells expressing HSD17B4 mRNA, TRITC illuminating cells expressing AR, and a merged image showing the co-localization of HSD17B4 mRNA and AR mRNA expression. Since all slides were processed in one *in situ* hybridization run, we used a standard threshold across subjects and brain regions: only cells above this threshold were considered stained. Cells were counted in six slices per animal (3 in each hemisphere; slices were chosen from the center of each nucleus in the anterior-posterior plane), using a $256 \times 196 \mu\text{m}^2$ box placed in the center of each area (in the medial-lateral plane) for each section, by an observer blind to sex and treatment. This box covered approximately 50% of LMAN in both sexes, 50% of Area X and HVC in males, and 95% of HVC in females. The average number of each type of cell was calculated for each individual within each brain region. These values were analyzed using sex by treatment ANOVAs with SPSS (18.0, Chicago, IL), with follow-up t-tests. To determine the degree of co-localization, we examined the proportion of merged cells to cells expressing AR and the proportion of merged cells to cells expressing HSD17B4, and analyzed these data using sex by treatment ANOVAs on arcsine transformed data to compensate for a non-normal distribution.

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Abbreviations

E2	estradiol
HSD17B4	17β hydroxysteroid dehydrogenase type IV
LMAN	lateral magnocellular nidopallium
ER	estrogen receptor
AR	androgen receptor

mRNA**messenger RNA****References**

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Fig. 1. Sex by treatment interaction for 17 β hydroxysteroid dehydrogenase type IV (HSD17B4) and androgen receptor (AR) mRNA expression in male and female zebra finches at day 25 in HVC

A) Bars represent mean number of cells expressing HSD17B4 or AR mRNA in birds treated with 50 μ g of estradiol (E2) or blank capsules (average counts within a $256 \times 196 \mu\text{m}^2$ box). Error bars indicate standard errors. Different lower case letters denote significant differences ($p < 0.05$) within each gene. **B)** First column: *in situ* hybridization using DIG-labeled probes for HSD17B4. Second column: *in situ* hybridization using Biotin-labeled probes for AR. Third column: co-localization of HSD17B4 and AR.

Fig. 2. Effect of E2 on expression of 17 β hydroxysteroid dehydrogenase type IV (HSD17B4) and androgen receptor (AR) mRNA in male and female zebra finches at day 25 in Area X

A) Bars represent mean densities of cells expressing HSD17B4 or AR mRNA in birds treated with 50 μ g of estradiol (E2) or blank capsules (average counts within a 256 $\times$ 196 μ m² box). Error bars indicate standard errors. Different lower case letters denote significant differences ($p < 0.05$) within each gene. **B)** First column: *in situ* hybridization using DIG-labeled probes for HSD17B4. Second column: *in situ* hybridization using Biotin-labeled probes for AR. Third column: co-localization of HSD17B4 and androgen receptors.

Fig 3. No effects of sex or E2- treatment on 17β hydroxysteroid dehydrogenase type IV

(HSD17B4) or androgen receptor (AR) mRNA expression in LMAN

A) Mean counts per $256 \times 196 \mu\text{m}^2$ and standard errors in E2-treated and control birds are indicated. **B)** First column: *in situ* hybridization using DIG-labeled probes for HSD17B4. Second column: *in situ* hybridization using Biotin-labeled probes for AR. Third column: co-localization of HSD17B4 and androgen receptors.

Percentage of HSD17B4 and AR Co-localization in HVC, LMAN and Area X in Estradiol (E2) treated and Control (Blank) Male and Female Zebra Finches

Table 1

	% of AR mRNA cells expressing HSD17B4			% of HSD17B4 mRNA cells expressing AR		
	HVC*	Area X*	LMAN	HVC	Area X*	LMAN*
M+Blank	91.6 ± 0.03 ^a	95.5 ± 0.02 ^a	87.4 ± 0.06	84.2 ± 0.04	73.0 ± 0.13 ^a	59.8 ± 0.16 ^a
M+E2	94.7 ± 0.03 ^a	94.5 ± 0.03 ^a	91.7 ± 0.05	71.0 ± 0.06	36.28 ± 0.13 ^b	34.6 ± 0.10 ^b
F+Blank	88.1 ± 0.04 ^b	81.2 ± 0.04 ^b	88.8 ± 0.05	76.5 ± 0.05	53.1 ± 0.10 ^a	38.2 ± 0.09 ^a
F + E2	81.1 ± 0.03 ^b	92.1 ± 0.04 ^b	89.6 ± 0.10	83.3 ± 0.06	34.68 ± 0.07 ^b	38.7 ± 0.06 ^b

* Different letters denote significantly different from each other.
The left panel indicates sex differences in HVC and Area X.
The right panel indicates an effect of treatment in Area X and LMAN.