Estradiol and Flutamide Effects on the Song System of Developing Male Zebra Finches

Informative

Notable sex differences exist in the neuronal system underlying song in songbirds; males' song systems are more elaborated than females'. Estradiol administration to hatchling males largely *de-masculinized* aspects of song system development, which is quite opposite of its effects on females in which it masculinizes their song system. Although there are many studies describing the effects of early steroids on song system development in females, very few describe steroid influences on male song system development. The present study remedies this situation.

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Acknowledgements

Thanks to Natalie Schottler who drew the figures and made the final edits and Kay Yang-Stayner and Janet Lee who helped with the execution of the study.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Abstract

Estradiol (E_2) masculinizes the developing song system of female zebra finches (Taeniopygia castanotis) if it is administered in early life, but the effects of E_2 on the developing song system in males have not been thoroughly explored. Also, reports of early antiandrogen administration in developing male zebra finches differ in their findings. Therefore. this study was conducted to further explore the effects of administering E_2 alone, flutamide (Flut) alone, or the two in combination during early development. Brains and testes were examined after day 100. The results showed definite demasculinizing effects of early E_2 on the song nucleus HVC (proper name)—its volume and neuron number were markedly reduced. Nonetheless, early E_2 hypermasculinized HVC neuronal size. Flut slightly hypermasculinized RA volume (Robust nucleus of the Arcopallium), which replicates a previous study, but the absence of any additional effects is at odds with other studies. Arguments are put forward suggesting that early E_2 action on HVC development could be a consequence of differential steroid action on the male versus female genotype and/or a disruption of the temporal sequence of gene regulation in developing males. Early E_2 resulted in markedly reducing testes size, which is likely to be a consequence of hijacking endogenous endocrine feedback mechanisms. The reduction in testes size suggests low steroid secretion, which also could contribute to the demasculinization of HVC.

Keywords: song bird, steroid, estradiol, endocrine system, sex differences

Introduction

Song behavior in zebra finches (Taeniopygia castanotis) is sexually dimorphic; males sing and females do not. This sex difference in behavior is reflected by dramatic sex differences in the neural underpinnings of this behavior. Males have larger song nuclei such as HVC, RA—robust nucleus of the arcopallium, and Area X (Grisham & Arnold, 1995), with more neurons in HVC and RA (Nordeen & Nordeen, 1988; Schlinger & Arnold, 1991), and larger neurons in HVC, lMAN—Lateral Magnocellular nucleus of the Anterior Nidopallium, and RA (Nottebohm & Arnold, 1976; Grisham & Arnold, 1995; Arnold & Saltiel, 1979; Nordeen et al., 1992).

The female's song system can be masculinized by administering estradiol (E_2) during development (Adkins-Regan et al., 1994; Gurney & Konishi, 1980; Gurney, 1981, 1982; Jacobs et al., 1995; Simpson & Vicario, 1991). The influence of early E_2 on song system development in males is less clear, however.

Although two studies report that E_2 has no effect on the song system of developing male songbirds either when administered at hatching (Gurney & Konishi, 1980) or *in ovo* (Wade et al., 1997), other studies have noted mixed effects. Tang and Wade (2009) found early E_2 in males increased Area X volume when examined at day 25 posthatch but had no impact on HVC. Conversely, when males were treated for the first 25 days posthatch with estradiol benzoate (EB) and examined at day 25, the volume of Area X and HVC

were demasculinized (Mathews & Arnold, 1991). But, this effect on volumes was not found when birds were treated until day 20 and then examined at day 60. Rather, when examined at Day 60, early EB treatment hypermasculinized male neuronal sizes in lMAN and HVC (Mathews & Arnold, 1991). Hypermasculinization was observed in Area X and HVC volume when male starlings were implanted with E_2 as hatchlings and examined at day 210 but not day 345 (Casto & Ball, 1996).

Mixed effects of androgenic manipulations in development also have been reported. Administration of antiandrogens in early development have been reported to hypermasculinize RA volume and number of its neurons (Schlinger & Arnold, 1991), to demasculinize RA neuron number and slightly decrease HVC volume (Grisham et al., 2007), and demasculinize lMAN and Area X but not influence other song regions (Bottjer & Hewer, 1992). Blocking synthesis of an androgen—dihydrotestosterone—reduced the number and density of RA neurons (Grisham et al., 1997).

Given the mixed results of both estrogen and androgenic manipulation of males in early life, we decided to re-examine the effects of these treatments. We examined the effects of both E_2 and Flut alone and in combination on hatchling males, particularly since we had found such dramatic effects on the combined treatment in hatchling females (Grisham et al., 2002).

Methods

All aspects of this study were approved by the Animal Research Committee of the University of California, Los Angeles. Further, the ARRIVE guidelines 2.0 were followed.

Estradiol (E_2) and flutamide (Flut) were administered in a 2x2 design. Randomly assigned males were implanted within 1-3 days of hatching under the skin of the breast with E_2 pellets, Flut pellets, both, or neither. The E_2 implants were made by mixing finely ground E_2 with Medical grade Silastic glue in a ratio of 1:6 and extruding this mixture into polyethylene tubing (Clay Adams no. 7411, i.d. 0.58 mm, o.d. 0.965 mm). These implants were cut into 2 mm sections, weighed, and their average dose calculated to be 83 µg (batches of pellets ranged from 73 µg to 87 µg E_2). The Flut implants were made in a manner described by Schlinger and Arnold (1991). Briefly, Flut was mixed with Silastic glue and then extruded through a 1cc syringe without a needle. The resulting strips were left to cure overnight, quartered, weighed, and cut into lengths that would result in a dose of 200 µg. Each bird received two pellets (E_2 + blank, E_2 + Flut, Flut + blank, or two blanks) except for unimplanted controls.

All dependent measures were made blind to the birds' treatment group. At sacrifice (101-136 days of age), the birds were deeply anesthetized and then perfused with 0.75% saline followed by 10% formalin in saline. Birds were only included if the pellets could be found at sacrifice. (Birds in the blank+blank group were included regardless.) Even after having been implanted for 101 days or more, the E_2 pellets all appeared to contain E_2

crystals (they had white opacities). The Flut pellets were clear and appeared depleted. The testes were dissected and stored in 10% formalin until they were weighed; at which time they were cleaned of extraneous tissue, dabbed dry, and weighed to the nearest 0.1 mg (if testes weighed less than .01 mg, they were assigned a 0.1 mg weight). Testes weights were taken from 26 adult males (n = 5 E_2 treated; n = 4 E_2+Flut treated; n=8 Flut treated; and 9 untreated/blanks).

The brains were dissected, stored in 10% formalin, and frozen-sectioned at 40 µm in the coronal plane. Brain measures were taken from 28 individuals (n = 5 E_2-treated; n = 4 E_2+Flut treated; n = 8 Flut treated; n = 11 untreated/blanks), but due to histological problems HVC data was n = 26. Data and records can be accessed via UCLA Dataverse at https://doi.org/10.25346/S6/VNSKRP.

The brains were examined with a light microscope connected to a computer via a videocamera. Cross-sectional areas of song system nuclei and their individual neurons were measured using NIH Image (http://rsb.info.nih.gov/nih-image/).

The volumes of Area X, lMAN, HVC, and RA were calculated by means of the cylindrical method: tracing the cross-sectional areas on every third section magnified at 6.25X, adding these areas, and multiplying by the sampling interval (120 µm). The cross-sectional volumes of each song system nucleus were averaged across hemispheres for each animal unless one hemisphere was damaged.

The area of individual neurons was measured at 800X. Twenty-five neurons were sampled through the rostral-caudal extent of the nucleus in each hemisphere for a total of fifty neurons in each nucleus of each animal. Neurons were distinguished from glia by their dark staining, ample cytoplasm, and nuclei containing only one or two nucleoli. Glia were distinguished by light staining, little cytoplasm, often with several nucleoli in each nucleus. The number of neurons in HVC was determined by counting the number of nucleoli in twenty-five frames (each 45,350 μm^3) that were sampled throughout its rostral-caudal extent in both hemispheres. Counting a small profile like nucleoli produces counts as reliable as using an optical dissector (Tramontin et al., 1998). The average density of neurons was calculated for each animal and multiplied by the mean volume of the given song system nucleus.

Dependent variables were analyzed via JASP https://jasp-stats.org/ and using a 2x2 ANOVA using estradiol treatment vs. none as one factor and Flut treatment vs. none as the other factor. Testes weights were analyzed by an ANOVA with 2x2 between (E_2 vs. none and flut vs. none) and one within variable (side).

Results

HVC volume was significantly demasculinized (reduced) by E_2 treatment, $F(1,22) = 8.38$, $p < 0.01$, $\eta^2 = 0.272$ (Figure 1A), as was the number of HVC neurons, $F(1,22) = 4.51$, $p < 0.05$, $\eta^2 = 0.168$ (Figure 1B). Despite E_2's demasculinizing effect on the volume of HVC and on the number of its neurons, E_2 hypermasculinized the size of HVC neurons, $F(1,24) = 6.00$, $p < 0.05$, $\eta^2 = 0.195$ (Figure 1C). There was a weak effect/strong trend for Flut hypermasculinizing RA volume, $F(1,24) = 4.133$, $p = 0.053$, $\eta^2 = 0.131$ (Figure 1D). No other brain measures were affected by either E_2 or Flut alone or in combination (all p values > 0.09).

Testes weight was markedly reduced by E_2 treatment, $F(1,69) = 74.63$, $p < 0.0001$, $\eta^2 = 0.475$ (Figure 2A). There was no effect of Flut alone or an E_2 x Flut interaction on testes size, all ps > 0.50 (Figure 2A). A significant difference due to side was found: testes on the left side were significantly heavier, $F(1,20) = 26.845$, $p < 0.001$, $\eta^2 = 0.063$, and there was a interaction between side and E_2 treatment $F(1,20) = 9.449$, $p < 0.01$, $\eta^2 = 0.022$ (data not shown). Both HVC volume and the number of HVC neurons significantly correlated with mean testicular weight, $r(22) = .576$, $p < 0.01$ and $r(22) = .487$, $p < 0.02$, respectively (Figure 2B & 2C).

Discussion

Administration of E_2 to male hatchlings dramatically reduced HVC volume and the number of its neurons when they were examined in adulthood (Fig. 1A, 1B). This demasculinizing effect of early E_2 on male HVC contrasts sharply with the effects of early E_2 in females, which masculinizes HVC by increasing its volume and size of its neurons (Adkins-Regan et al., 1994; Gurney & Konishi, 1980; Gurney, 1981, 1982; Jacobs et al., 1995, Grisham & Arnold, 1995; Simpson & Vicario, 1991). We posit two conceivable mechanisms to explain the marked difference of early E_2 effects between males and females. Early E_2 administration could 1) differentially affect the male and female genome, possibly triggering temporally inappropriate genetic cascades, or 2) indirectly by interfering with hormonal feedback mechanisms and gonadal function.

Clearly, early E_2 differentially interacts with the male versus female genome (Choe et al., 2021). Early E_2 tends to upregulate genes in females whereas it down regulates genes in males, particularly in Area X (Choe et al., 2021), which is a target of HVC projections (Benezra et al., 2018). Early E_2 administration also has opposite effects on certain gene expression in females versus males; the number of HVC neurons expressing 17β-hydroxysteroid dehydrogenase and androgen receptor mRNA decreased in males but increased in females when birds were examined at day 25 (Thompson et al., 2011). Further, early E_2 alters BDNF gene expression in male but not female HVC (Dittrich et al., 1999), suggesting early E_2 disrupts the temporal sequence of male gene expression, which could alter the adult phenotype.

Temporal differences in early E_2 administration effects may explain some discrepancies in the literature. For example, Choe et al. (2021) did not detect any effect of early E_2 on male HVC volume, but we did. Nonetheless, they used a different dose regimen; they defined the HVC with CADPS2, and they examined the song nuclei at day 30; whereas we used a Nissl stain and examined birds at 101-136 days of age. HVC neurons are added well after day 30 (Walton et al., 2012; Diez et al., 2021), which could explain the discrepancy between their findings and ours. In agreement with our findings, however, Choe et al. (2021) also found early E_2 increased HVC cell size in males.

Similarly, discrepancies in Flut's effects can be due to differences in the onset and duration of treatment as well as the time when birds are examined. Our early Flut treatment showed a strong trend toward hypermasculinizing RA volume (Figure 1D) similar to Schlinger and Arnold (1991), who also treated birds at hatching. In contrast, castration and Flut treatment in adolescence (Day 20) had a different pattern of effects: lMAN and Area X volumes were demasculinized but not RA or HVC (Bottjer & Hewer, 1992). Lastly, when Flut was chronically administered from hatching until day 61-63, Flut only decreased the number of RA neurons (Grisham et al., 2007). Thus, blocking androgen action could also affect the usual temporal pattern of gene activation in development, which could cause differential phenotypic changes depending on timing.

We did not find an effect of early Flut on testicular size (Fig. 2A), which contrasts with a finding in this lab when we used chronic flut treatments (Grisham et al., 2007). The temporal efficacy of our Flut implants is debatable (Fusani et al., 2007). Our Flut pellets were likely to be depleted before the birds were examined, which could explain the difference in outcomes.

The dramatic decrease in testes size induced by early E_2 administration (Fig. 2A) suggests that there are estrogen-mediated endocrine feedback mechanisms in zebra finches. This finding is paralleled in other studies on zebra finches (Mathews & Arnold, 1991), starlings (Casto & Ball, 1996), and other bird species (Lorenz, 1954; Soma et al., 2000). Notably this effect of early E_2 exposure is also found in rats (Hendricks & Gerall, 1970; Zadina et al., 1979). So, our early E_2 exposure could have permanently altered hormonal feedback in a sensitive period that resulted in small testicular size.

Indeed, the testes in early E_2-treated birds were more like that of babies than adults. Our E_2 treated birds likely did not undergo puberty. Notably, HVC volume and the number of its neurons were significantly correlated with testes size (Fig 2B, 2C). Blocking androgen's action pre-pubertally had no impact on HVC (Grisham et al., 2007). But, if the markedly reduced testes size in our birds resulted in reduced testicular secretions not only before but also after puberty, then demasculinizing effects could be a consequence. The best estimate of the onset of puberty in zebra finches is about Day 60-70 posthatch (Bölting & von Englehardt, 2017; Pröve, 1983). HVC neurons are still being added in adulthood—even into the crystalized song period and beyond (Walton et al., 2012; Diez et al., 2021). Although a direct link between adult HVC neuron numbers and androgen levels in zebra finches has yet to be firmly established, other work strongly suggests this connection exists (Williams et al., 2003). The number of androgen target cells in HVC

increases during adolescence in male zebra finches (Bottjer, 1987; Tang & Wade, 2010). Also, testosterone increases the recruitment and/or survival of HVC neurons in adult female canaries (Rasika et al., 1994), an effect that is dependent upon the androgenic rather than estrogenic metabolites of testosterone (Fusani et al., 2002). Early E_2 treatment decreases androgen receptor levels in HVC (Thompson et al., 2011), so there may have been not only low androgen levels, but also fewer receptors upon which to act. So, endocrine effects in adulthood could also explain some of the effects observed in the present study.

In conclusion, the same steroid manipulations have markedly different effects on developing males and females. Discrepancies in the literature can be understood by noting the different onset and duration of treatment of such manipulations as well as the time point when the birds were examined. These temporal discrepancies in steroid action also could uncover not only what gene(s) can be activated to produce differential effects on the male and female phenotype but also the timing of genetic and hormonal feedback mechanisms.

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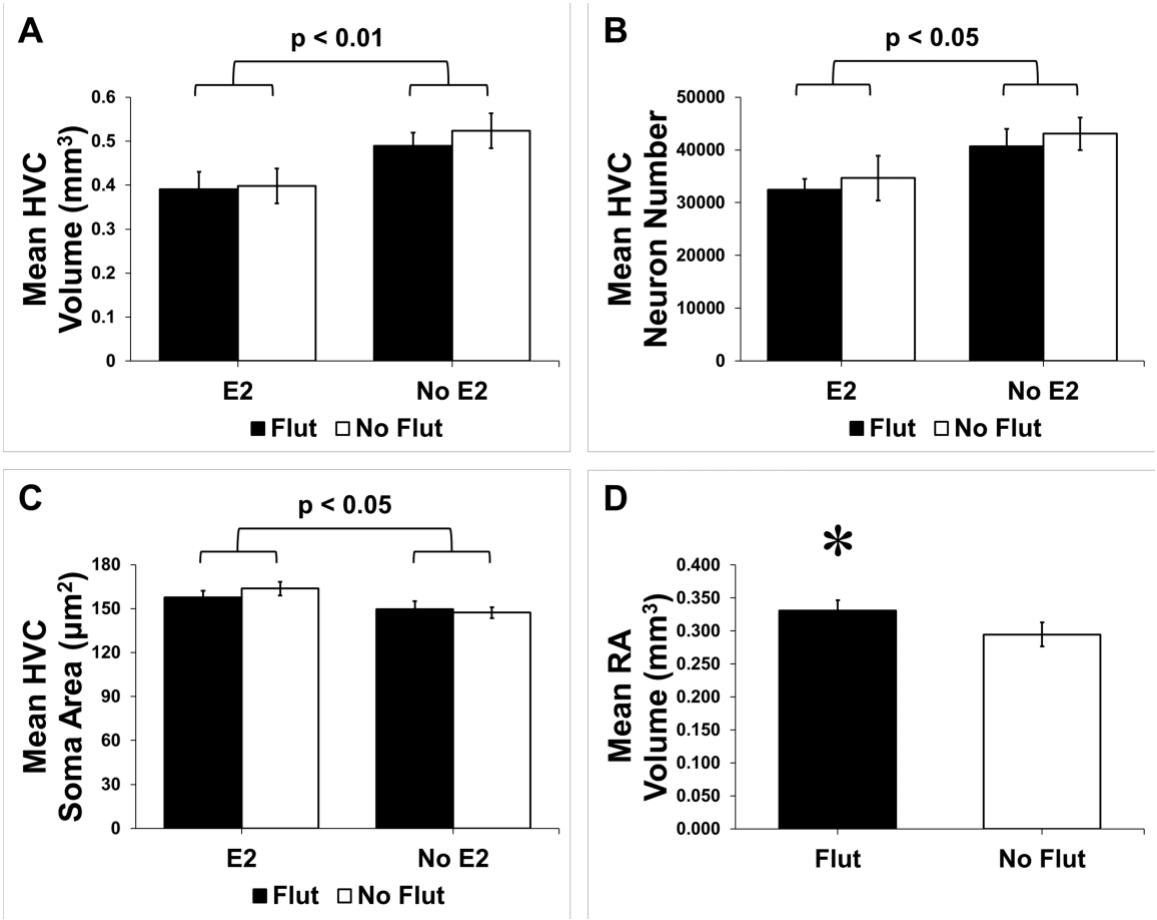

Figure 1 A) Mean HVC volume and B) number of HVC neurons as a function of treatment group. Males receiving E_2 or E_2 +Flut were demasculized. C) Mean HVC neuron size—males in E_2-treated groups had significantly larger neurons. D) RA volume--males receiving Flut were hypermasculinized. Error bars = SEM.

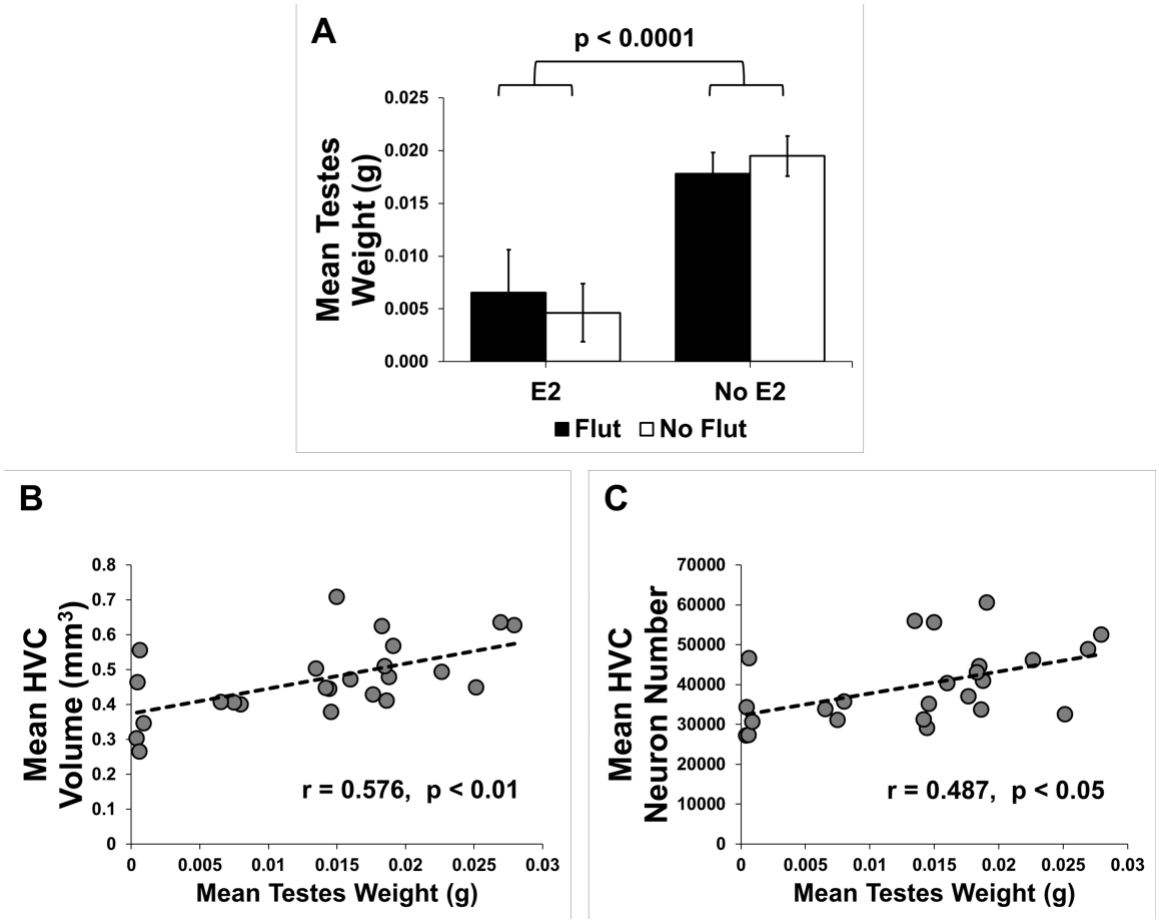

Figure 2 A) Mean testes weight as a function of treatment--E_2 treated birds had markedly reduced testes weights. Error bars = SEM. B) Scatterplot of HVC volume and C) HVC neuron numbers as a function of testes size.