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A Dose-Response Study of Estradiol's Effects on the Developing Zebra Finch Song System

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Abstract

To gauge the sensitivity of the female zebra finch song system to estradiol (E2), we used subcutaneous implants to administer various doses of E2 to hatchling female zebra finches. Four different doses of E2 were administered: 50, 15, 5 and 0 µg via subcutaneous silicon “ropes” at hatching, and the brains were examined in adulthood. Further, we examined whether masculinization was all-or-none once a threshold was reached or if the morphology of the song system would show a graded response to the various doses of E2. Finally, we asked if the various dependent measures—volume of song nuclei, neuron size, and neuron number—would show differential sensitivity to E2.

Fifteen micrograms was sufficient to masculinize many aspects of the song system and was often as effective as 50 µg, causing a dramatic difference relative to the 0 µg group. Different aspects of the song system seemed differentially sensitive to the effects of E2: volumes of song control nuclei, the size of RA neurons, and the number of HVC neurons were significantly masculinized by 15 µg E2, but the number of RA neurons and HVC and IMAN soma sizes required 50 µg. The results suggest that several developmental processes are influenced by E2, possibly because of multiple sites of action or multiple processes that respond to E2.

Keywords

Estradiol; songbird; dose-response; zebra finch; masculinization; steroid hormone

Song behavior in zebra finches is sexually dimorphic: males sing and females do not. This sex difference in behavior is reflected in an interconnected system of neurons related to song behavior. Nuclei in this system are larger in males and have more and larger neurons than they do in females [10,14,15,24,28].

Estradiol (E2) administration to hatchling females markedly masculinizes the female song system [1,6,10,11,12,14,15,18,20,25,26,28], even to the point where they will produce song as adults [29]. Also, events in the normal masculinization of the song system are directed by

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estrogen [17]. Thus, understanding the mechanisms of estrogen-induced masculinization is required to understand sexual differentiation of this system.

Efforts to disrupt masculinization of the male song system by interfering with estrogen synthesis or blocking receptors has been largely unsuccessful, suggesting that some other agents may contribute to sexual differentiation of the brain (see [13,31] for review). Nonetheless, if the zebra finch song system is exquisitely sensitive to estrogen and interference with estrogen action was less than complete, then such manipulations in males may have had no effect—residual activity may have been sufficient to trigger the masculinizing mechanisms. This idea might hold true especially if the E2 is produced locally at high levels in the brain [27].

The sensitivity of the song system to exogenous E2 has not been systematically studied. Conventional wisdom holds that large doses of E2 are required to induce masculinization of the female song system [4], but this assertion has never been empirically tested. Typically, single implants of fifty to one hundred micrograms E2 have been used to masculinize the song system in females [10,11,14,15,28]. A twenty microgram dose was sufficient to masculinize the brain when given in daily injections [1] and a single two microgram dose was sufficient to produce partial masculinization of HVC soma size when implanted under the skin of the breast [12]. One dose-response study using an oral route of administration gave daily doses of approximately 1.3-3.4 µg/day across an entire week beginning at posthatch day 5 and found only modest masculinization in Area X [26]. Thus, data probing the song system's threshold of sensitivity to estrogen are not available.

We measured the sensitivity of the hatchling female zebra finch song system to various doses of E2 using subcutaneous implants. We asked if masculinization was an all-or-none event once one had reached a threshold or if the morphology of a given aspect of the song system would show increasing masculinization with increasing doses of E2. We also determined if various dependent measures—volume of song nuclei, neuron size, and neuron number—would show differential sensitivity to E2.

Method

Subjects

Twenty-five female zebra finches hatched in the UCLA colony and raised socially were used. Birds had a 12:12 L:D cycle. All birds were treated according to a protocol approved by the Chancellor's Office for the Protection of Research Subjects at UCLA.

Apparatus

The brains were examined using a light microscope connected to computer via a video camera mounted on the microscope. Quantification was done using NIH Image (<http://rsb.info.nih.gov/nih-image/>).

Procedure

E2 was mixed with Silastic in different ratios: 1:6 E2:silastic for a 50 µg dose, 1:20 for a 15 µg dose, 1:60 for a 5 µg dose, and plain Silastic for 0 µg dose control. These mixtures were extruded from a 1 cc syringe without a needle and the resulting “ropes” left overnight to cure. These ropes were then quartered longitudinally, weighed, and cut to the appropriate length for the doses desired so the pellets varied in length. All pellets were within 10% of the desired dose—actual calculated dose for the 50 µg batches were 45-54.6 µg, for 15 µg group the calculated dose was 16.36 µg, and the 5 µg group dose was calculated at 5.04-5.16 µg.

Six birds were assigned to each dose except for 15 μg group, which had seven birds. All birds were implanted on day 1 (day of hatching) under the skin of the breast and were sacrificed on or near day 100 (range 99-103 days). We experienced very little mortality definitively linked to implantation ($n=1$). At sacrifice, the birds were exsanguinated with bird saline (.75%) followed by 10% formalin in saline. Birds were only included if they were female and if the pellets could be found at sacrifice. Sex was confirmed by examination of the gonad. Brains were dissected, stored in 10% formalin, frozen sectioned at 40 μm in the coronal plane, and stained with thionin.

All measurements were made by an observer who was blind to treatment. Cross-sectional areas of song system nuclei and the individual neurons in IMAN, HVC, and RA were measured using NIH Image. The volumes of Area X, IMAN, HVC, and RA were measured by examining the sections at 6.25X, tracing the cross-sectional areas from every third section, adding these areas, and multiplying by the sampling interval (0.12 mm). The volumes of each song system nucleus were averaged across hemispheres for each animal. In cases where the cross-sectional area could be measured only in one hemisphere, only one hemisphere contributed to the average (HVC, one case in the 15 μg group, one case in the 0 μg group). If the cross sectional area in neither hemisphere could be accurately measured, that bird was excluded from the particular volume measure (Area X, one case in the 0 μg group).

The cross-sectional areas of individual neurons were measured at 800X in HVC, RA, and IMAN (25 per hemisphere, 50 total) and their means for each bird were used in the analyses. Neurons were distinguished from glia by their more intense staining, proportionately larger cytoplasm, and presence of one or two nucleoli. Neurons were randomly sampled in no more than five contiguous frames per section that were adjoined in either a dorsal-ventral or medial-lateral direction in both hemispheres along the rostral-caudal extent of a given nucleus. Samples of neurons were also counted in HVC and RA. The number of neurons in HVC and RA was determined by counting the number of nucleoli in neurons in 25 frames (each 45494.8 μm^3). Nucleoli were counted in five contiguous frames either moving in a dorsal-ventral direction or lateral-medial direction in a given section. No more than five frames were sampled in a given section, and care was taken to sample throughout the rostral-caudal extent of both HVC and RA across both hemispheres. The average density of neurons was calculated for each animal and multiplied by the volume of the nucleus (determined by the mean from both hemispheres). Counting a smaller profile like nucleoli produces relatively accurate counts that are as reliable as using an optical dissector [30].

All dependent measures were analyzed via an ANOVA and Fisher's Least Significant Difference test was used for post-hoc comparisons. Because a few samples showed heterogeneity of variance, we also analyzed the data using a statistical method based on resampling of the data (www.resampling.com), which does not depend on assumptions of homogeneity of variance. We used one-tailed tests with the resampling method because the direction of the E2 effect is well known and confirmed by many labs (see above). We only report comparisons as significant if both the result of the ANOVA and resampling test technique agreed. The resampling method confirmed all the significant differences that we found with the parametric tests.

Results

The volumes of RA, HVC, and Area X were all significantly affected by E2 treatments ($F(3,21) = 7.30$, $F(3,21) = 6.127$, and $F(3,20) = 5.10$, respectively, all $p_s < 0.005$ —Figure 1). Post-hoc tests revealed that the 15 and 50 μg groups were significantly different from the 5 and 0 μg groups but that they were not different from each other on these measures (Figure 1). E2 treatments also significantly affected soma size for RA, HVC, and IMAN neurons ($F(3,21)$

= 10.79, $F(3,21) = 6.10$, and $F(3,21) = 4.61$, respectively, all $ps < 0.05$ or less—Figure 2). Post-hoc tests revealed that 15 μg was sufficient to significantly masculinize RA neuron size but only the 50 μg group was significantly different from other groups in the cases of IMAN and HVC neuron size (Figure 2). Moreover, some individual birds showed masculinization of RA soma size with little or no masculinization of HVC or IMAN soma size, suggesting independent mechanisms controlling masculinization of soma size across nuclei (see Figure 2).

E2 treatments affected the number of neurons in RA and HVC ($F(3,21) = 5.08$ and $F(3,21) = 6.76$, respectively, both $p < 0.01$ or less—Figure 3). Fifteen micrograms seemed sufficient to masculinize the number of HVC neurons; 50 μg was necessary to masculinize the number of RA neurons (Figure 3).

Discussion

Clearly, 15 μg of E2 was sufficient to masculinize many aspects of the song system and was often as effective as 50 μg . On the one hand, 15 μg may seem like a large dose of E2; 5 μg E2 is sufficient to masculinize a female rat pup rendering her anovulatory as an adult [9], and a rat pup is much larger than a hatchling zebra finch. On the other hand, anovulation is a physiological endpoint—to our knowledge no dose-response study has ever been undertaken to determine the threshold for an E2-induced change in brain morphology in any other species, so there is no good comparison. Further, we neither know how fast E2 is released from the Silastic implants nor know the rate at which steroids are metabolized by developing zebra finches. Fifty microgram pellets of E2 have a half-life of 80.6 hours and may be thoroughly depleted in a week [14 but see also 28]. If our 15 μg pellets, which masculinized many aspects of the song system, have the same release rate as 50 μg pellets, then they would release less than 7.5 μg E2 across the first three days of implantation.

Different aspects of the song system do not appear to be equally sensitive to E2's effects. We found that the volumes of all the song nuclei, the size of RA neurons, and the number of HVC neurons were all about equally sensitive to the effects E2: fifteen micrograms significantly masculinized these measures. In contrast, 50 μg E2 was necessary to significantly masculinize the size of HVC and IMAN neurons and the number of RA neurons. RA soma size seems more sensitive than other soma sizes: visual inspection of the graphs reveal that RA soma size was masculinized in some individual birds administered 15 μg of E2, yet HVC soma size in some of the same birds was masculinized little if at all (Figure 2). In another study, RA soma size correlated more strongly with proximity to intracranial microimplants of estrogen than did HVC soma size [12], suggesting that RA neuron size is more sensitive to estrogen.

This differential sensitivity to E2 suggests that different mechanisms contribute to masculinization of different dependent variables measured. A given phenotype could be also affected by local factors such as ingrowth of axons [17,22], local vascular changes [21], or local concentrations of trophic factors [3,7,8].

For most dependent measures, individuals seemed either to show a dramatic response to the E2 treatment or to not respond at all, particularly at the 15 μg dose, which seemed to be near threshold for many traits. This individual variability could stem from differences in endogenous steroid background due to laying/hatch order [32] or could be due to variations in individual genotypes, such as variability in individuals' ability to upregulate androgen receptor in response to E2 [19,23]. Also, there seems to be a limit on the response to E2—the masculinization induced by E2 treatment seems to show diminishing returns with the higher doses. Visual inspection of the figures reveal that group mean increases with higher doses of E2 often reflect an increase in the proportion of birds responding more than an overall increase in degree of

masculinization. Further, in other studies, doses greater than 50 μg do not produce markedly more masculinization than does 50 μg [11,20,28].

Clearly, the female song system is not exquisitely sensitive to E2: 5 μg was never sufficient to produce significant masculinization on any measures. Nonetheless, some individuals responded to the 5 μg dose and had Area X volumes above zero (Figure 1), and two birds in this group had bigger volumes than have ever been reported for control females [16]. A previous study also found that Area X volume was more sensitive to the effects of E2 than other measures [26]. Notably, our results do not preclude the possibility that the male song system could still be exquisitely sensitive to E2's influence. Sex differences in sex chromosome gene expression within brain cells could create differential sensitivity to E2 between males and females during song system development [5].

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Figure 1.

Volumes of RA, HVC, and Area X as a function of dose of E2. Each bar represents an individual bird, and the order of birds in each figure is the same so that values for different dependent variables can be compared within and across birds. Asterisks and brackets indicate differences between groups in post-hoc comparisons, $p < .05$ or less.

Figure 2.

Cross sectional area of RA, HVC, and IMAN neurons as a function of dose of E2. All else is as in Figure 1.

Figure 3. Number of RA and HVC neurons as a function of dose of E2. Bars represent individual birds and asterisks and brackets described in Figure 1.