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Effects of Long-Term Flutamide Treatment During Development In Zebra Finches

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Abstract

The molecular mechanisms responsible for the sexual differentiation of the zebra finch song system remain mysterious. Androgen receptors are expressed in a sexually dimorphic fashion in the zebra finches song system: males have more cells expressing androgen receptors, and this sex difference appears very early in development (day 9 posthatch). Estrogen administration to hatchling females up-regulates androgen receptor expression in their song system and profoundly masculinizes their song system's morphology. Co-administering flutamide, an androgen-receptor blocker, with estrogen impedes estrogen's masculinizing effects on the song system, suggesting that androgens are required for masculine development.

Accordingly, to investigate further the role of androgens in the sexual differentiation of the zebra finch song system, we sought to block androgen activity in males by administering large, sustained doses of flutamide from just before androgen receptors are expressed in the song system (day 7) through to the day of sacrifice (day 61-63).

Flutamide profoundly reduced the size of the testes, demonstrating that this drug and mode of administration could have a large impact on tissues. In contrast, flutamide had only a minor impact on the song system: the number of RA neurons was slightly reduced, and the corrected HVC volume showed a trend toward demasculinization. Other brain measures (uncorrected HVC, and corrected and uncorrected volumes of Area X, lMAN, RA, and Rotundus; neuron size in lMAN, HVC, and RA; and number of HVC and

LMAN neurons) were not significantly affected. The present results do not support an important role for androgen in masculinizing the song circuit after posthatch day 7.

Keywords: Songbird, antiandrogen, sex differences, flutamide, zebra finch

Introduction

Song behavior in zebra finches is sexually dimorphic: males sing and females do not. This sex difference results from dramatic sex differences in the neural circuit responsible for this behavior. Males have larger song nuclei that have larger neurons and make interconnections that are either absent or vestigial in females [9,19,23,26,31]. Sex differences in androgen accumulation or androgen receptor expression are found in song regions HVC and lMAN [4,7,24] and to a lesser extent in RA and Area X [2,18]; in these areas, males express more androgen receptors than females do. This sex difference is first observed at days 9-11 posthatch in HVC and Area X [7,18].

The sex difference in androgen receptors could play a role in the sexual differentiation of the song system. Administering estradiol to hatchling females masculinizes their song systems [9,11,14] and up-regulates the expression of androgen receptors in HVC, Area X, and lMAN [18,25]. Additionally, co-administering flutamide, an androgen receptor antagonist [22,27], with estradiol markedly blocks estradiol's ability to masculinize the female song system [11], suggesting that activation of androgen receptors is a necessary step for the masculinization of the song system—at least in estrogenized female hatchlings.

Castration and treatment with flutamide at day 20 posthatch results in a demasculinization of Area X and lMAN [6], which is consistent with the interpretation that androgens are involved in the masculinization of the song system of male zebra finches. In contrast, treatment of male hatchings with flutamide on day 1 did not result in a demasculinization of the song system [29]. Day 1 treatment, however, used Silastic "rope" implants that are largely depleted of steroids in less than a week [14] and may also have been depleted of flutamide in a few days. Since androgen receptors are not expressed in the song system until posthatch day 9, the androgen blockade provided by the flutamide implants on Day 1 may have missed the window during which androgens act to masculinize the song system.

We sought to block androgen action via flutamide during the developmental time period when androgen receptors are first expressed in the song system. Thus, we initiated flutamide treatments on day 7. To guard against the possibility of later compensatory androgen action obscuring the effects of treatment, we maintained this androgen blockade throughout subsequent development. Accordingly, the birds remained on a large dose of flutamide until they were sacrificed (day 61-63). Despite flooding their systems with flutamide, we found only a small effect on the number of RA neurons and a trend toward demasculinizing the corrected HVC volume, along with a substantial decrease in testicular weight.

Method

Subjects. 20 male zebra finches (flutamide-treated=9, control=11) hatched and reared at the University of California, Los Angeles (UCLA), were used for this study.

Care in handling and surgical procedures were approved by the UCLA Chancellor's Animal Research Committee.

Procedure. On day 7 posthatch, the finches in the flutamide-treated group were surgically implanted with two "rope" flutamide (Schering) pellets under the skin of the breast. The "rope" pellets were constructed by mixing flutamide with Silastic glue in a ratio of 1:6, extruding this mixture as a thin rope through a syringe without a needle, curing overnight, and then weighing and cutting so that each pellet resulted in a dose of 200 μg of flutamide. Each animal received 2 pellets on day 7, a total of 400 μg flutamide. The control group was similarly implanted on day 7 posthatch with rope pellets composed of pure Silastic glue without flutamide.

On day 14 posthatch, each bird in the flutamide group was anesthetized with 0.01-0.02 cc Equithesin and implanted subcutaneously on the breast with a 5 mm Silastic tube (Dow-Corning 0.76 mm i.d. 1.65 mm o.d.) packed with flutamide and sealed at both ends with Silastic glue. These Silastic tube implants were pierced with a 26-gauge needle, resulting in 10 holes along its length, ensuring that flutamide would leak into the bird once implanted. The finches in the control group were anesthetized and given empty tube implants under the breast skin.

On day 35 posthatch, the flutamide group was again anesthetized with 0.01- 0.02 cc Equithesin, and the flutamide tube implanted on day 14 was removed and replaced with two new flutamide-filled Silastic tubes under the skin of the breast. These new tubes were each identical to the tubes implanted on day 14. The removed implant was checked to ensure that the reservoir of flutamide was nearly but not completely empty, indicating

that the drug was still being delivered at this time. The control males also underwent the same procedure but were implanted with two empty Silastic tubes.

On day 61-63, the birds were sacrificed under deep Equithesin anesthesia. The flutamide tube implants were removed, inspected, and confirmed to be largely but not completely depleted of flutamide. The original flutamide "rope" pellets were also recovered to establish that they had been present during the first week of treatment. Thus, birds in the flutamide group received large doses of flutamide throughout most of their lives, from day 7 to day 61-63.

Once the birds were deeply anesthetized, they were perfused intracardially with 0.75% bird saline followed by 10% buffered formalin. After perfusing thoroughly, the brains were removed and stored in 10% formalin. The brains were embedded in gelatin, soaked overnight in 20% sucrose in 10% formalin solution, and frozen-sectioned at 40 μm. Every third section was mounted on gelatin-coated slides and stained with thionin.

The testes and the syrinx were removed and stored in 10% formalin. Subsequently they were cleaned of extraneous tissues and weighed to the nearest 0.1 mg.

A blind observer measured the cross-sectional areas of right and left Area X, HVC, lMAN, RA, and Rotundus by using NIH image. The volume of the telencephalon was estimated by measuring its cross-sectional area in one hemisphere in all sections containing lMAN (lMAN volumes were not different between groups—see Table 1). Volumes were calculated using the cylindrical method: the sum of the cross-sectional areas was multiplied by the sampling interval of 120 μm (0.12 mm). The volumes calculated for each nucleus were averaged across hemispheres. Due to damage during histology, some animals were excluded for one or more of the dependent measures, so the

size of the groups varies across dependent measures (cf. Table 1). If there was damage in only one hemisphere, the volume obtained from the nucleus in the intact hemisphere was used and not averaged (Area X: two flutamide-treated cases, lMAN: one flutamide-treated case and three control, HVC: two flutamide-treated cases and one control, RA: one flutamide-treated case, Rotundus: three flutamide-treated cases and one control). Analyses were performed on both the original volumes of each nucleus and also on corrected volumes in which average nucleus volume was divided by the volume of the telencephalon for that bird.

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 staining lightly, having little cytoplasm, and often having several nucleoli in each nucleus. The number of neurons was counted in twenty-five frames (each 61760 μm^3) by counting nucleoli and sampling throughout the rostral-caudal extent of lMAN, HVC, and RA across both hemispheres. Counting a smaller profile like nucleoli produces relatively accurate counts and is as reliable as using an optical dissector [33]. The average density of neurons for each animal was calculated and multiplied by the volume of the respective nucleus to determine the number of neurons.

The flutamide-treated group and the control group were compared using t-tests for each dependent measure in the brain, for the combined average weights of the right and left testes, and for syrinx weights. The t-tests provide the least conservative analyses,

bolstering our confidence in the largely negative findings on song system development (see Results below).

Results

Testes weight was significantly reduced by the flutamide treatment, t (16) = 2.396, p = 0.029 (Figure 1 A), but syrinx weight was not (Table 1). There was a modest decrease in the number of RA neurons in the flutamide-treated group, $t(10) = 2.306$, $p < .05$ (Figure 1 B). No other uncorrected brain measures were significantly different between groups, including volumes (all ps > .35, Table 1), cell sizes (all ps $\geq$ .30, Table 1), and counts and densities of neurons in HVC and lMAN ($ps \geq .20$, Table 1). HVC volume corrected by telencepahlic volume showed a trend toward demasculinization in flutamide-treated birds, $t(5) = 2.473$, $p = .0563$, but flutamide did not affect any other corrected volumes (all ps > .37—data not shown).

Discussion

In the present study, we administered the androgen receptor blocker flutamide to male zebra finches from posthatch day 7, before detectable androgen receptor expression in the neural song circuit, until the time of sacrifice just after 60 days of age. Although this treatment is effective at reducing testis size in the same animals, and flutamide treatment blocks estradiol-induced masculinization of the song circuit in females [11], it was largely ineffective in preventing masculinization of the song circuit in males. Flutamide

produced only a modest demasculinization of RA number and a trend toward demasculinization of HVC volume once it had been corrected by telencephalon volume. The results suggest that most of the normal processes of masculinization of the song circuit in males do not require activation of androgen receptors during this period.

The lack of a large effect on the song system could possibly be due to flutamide's failure to act as an antiandrogen in brain tissue or in this species. On the one hand, although flutamide is usually only described as having antiandrogenic properties [22,27], it also has effects similar to dihydrotestosterone (DHT) on hippocampal neurons in mammals [20], suggesting that flutamide can also have androgenic properties in nervous tissue. On the other hand, flutamide's reduction in the number of RA neurons is similar to the effect of inhibiting DHT synthesis in the zebra finch brain [13], suggesting that flutamide has an antiandrogenic effect in the zebra finch brain. Flutamide also has well-established antiandrogenic effects in zebra finches and other passerine species: it demasculinizes the syrinx [35], affects brain measures including morphometric endpoints and androgen receptor regulation [11,21], and has behavioral effects both in zebra finches and other passerines [30,34,36]. Thus, our failure to get a large flutamide effect on the song system with massive, sustained doses is not likely due to its failure to act as an antiandrogen.

Flutamide's reduction of testes size in the present study (Figure 1A) is consistent with its effects in developing rats [5,17]. This reduction in testes size can be explained either by flutamide's direct antiandrogenic effect on the testes or by flutamide's up-regulation of aromatase activity and consequent increased E_2 production [16]. (E_2 can also reduce testes size in developing rats [15] and zebra finches [12]). In the present study, flutamide

treatment did not affect syrinx weight, which is supposedly an androgen-dependent trait and has been affected by flutamide treatment in zebra finches [35]. The failure to find an effect of flutamide on syringeal weight may have occurred because of age differences in the birds: our birds were not yet fully adult and may not have had very high levels of androgens, whereas flutamide did affect syrinx weight when adult birds were used [35]. Notably, our birds had smaller syringes than adult birds [35], suggesting that our birds did not have adult androgen levels. Furthermore, the route of administration may be important: implants of flutamide do not seem to affect the syrinx, whereas daily injections produce a modest effect [35].

We also did not find an effect of flutamide on Rotundus or telencephalic volumes even though castration increases Rotundus volume in zebra finches [1] and exogenous testosterone decreases both Rotundus and telencephalon volume in dark-eyed juncos [32]. Our failure to see an effect of flutamide could be because these traits are not androgen-dependent but are instead affected by other metabolites of testosterone such as estradiol. Also, our birds were not fully adult, but birds in the studies reporting effects on Rotundus and telencephalon size were. Further, the effect size of castration or exogenous testosterone on Rotundus and telencephalic volumes is small, and we used a smaller number of birds than the studies finding effects on these variables.

Although we gave flutamide for a long, sustained time during development, the timing of our flutamide treatment may have been inappropriate to see effects. Androgen receptors have not been detected in the song system before day 9 posthatch [7,18], but they could still be present earlier either at low levels or in cells that will become part of the song system later in development [18]. Notably, androgen receptors appear in other

brain sites *in ovo* [8,28]. Conceivably, androgen-dependent events critical to masculinizing the song system could occur before androgen receptors are expressed at high levels in the song system, perhaps even *in ovo*. Since androgen receptor levels change through life in zebra finches, larger or even more sustained doses of flutamide at different developmental time points could yield larger effects, but the results so far are not encouraging. Neither administration of flutamide on the day of hatching [29] nor *in ovo* (20 μg at day 10 of incubation) [10] resulted in any demasculinization of the song system.

Our failure to find a dramatic effect of flutamide treatment in males contrasts with the effects of combining flutamide treatment with castration during development, which reduced both Area X and lMAN volumes [6]. Since our study's birds were intact, estrogenic metabolites of androgens could have influenced their song system development, possibly accounting for the difference.

The present study also contrasts with the dramatic effect that flutamide has on estrogen-induced masculinization in developing females. E_2 up-regulates androgen receptors in hatchling females [18,25], and co-administering flutamide with E_2 at hatching essentially blocks estrogen-induced masculinization [11]. So, although activation of androgen receptors seems essential for masculinizing the song system in estrogenized females, activation of androgen receptors does not seem to be critical for normal masculine development of this system. Presumably E_2 administration to developing females induces developmental processes that ultimately converge with normal male development of this system. Nonetheless, since the present results do not suggest a large role for androgen in masculinization of the song circuit in males, the

molecular events in the developmental pathway of estrogenized females must not completely parallel those of males. Sex differences in sex chromosome gene expression within brain cells may account for the differential sensitivity to androgens between normal males and estradiol-treated females during song system development [3].

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Figure 1

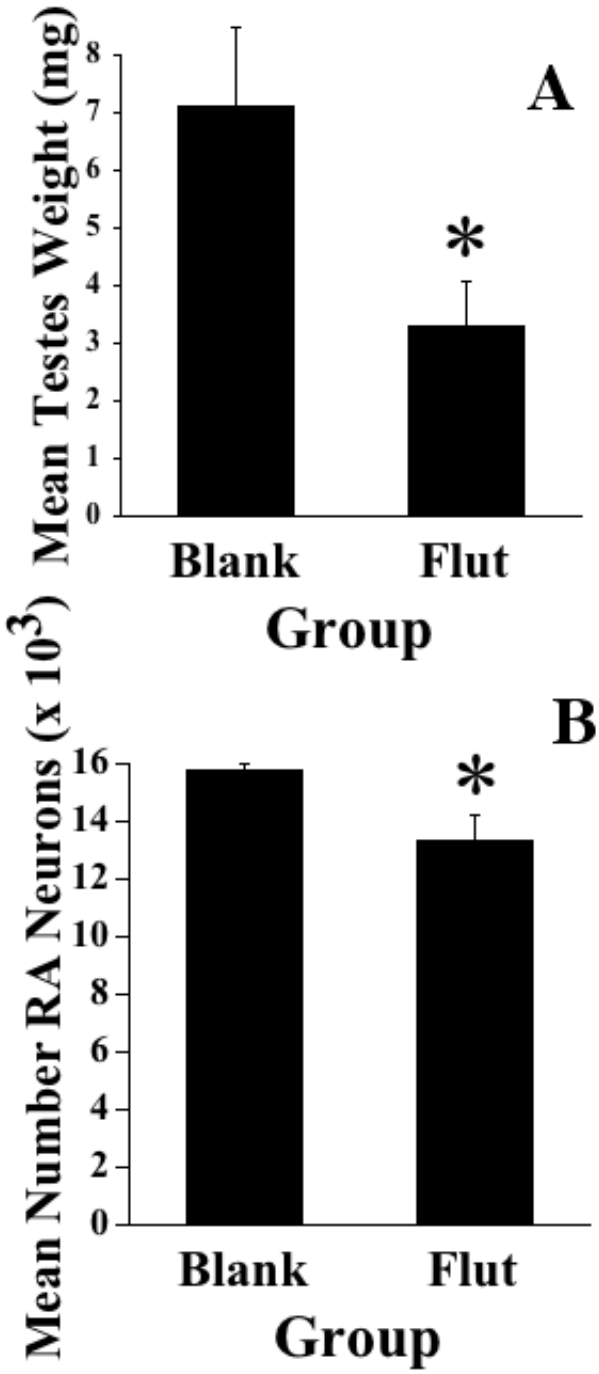

Figure Caption:

Figure 1 A) Mean testes weight as a function of group (Blank n = 9, Flutamide n = 10). B) Mean number of RA neurons as a function of group (Blank n = 5, Flutamide n = 7), asterisk indicates different from blank, $p < 0.05$, in both graphs.

Table 1

Mean ± SEM and probability values based on t-tests of dependent measures that showed no significant difference between control and flutamide-treated males. Volumes are in mm^3, soma sizes are in μm^2, densities are neurons per μm^3, weights are in grams.

Dependent Measure	**Control Mean ± SEM (n size)**	**Flutamide Mean ± SEM (n size)**	**p value**
Area X Volume	1.176 ± .079 (n = 8)	1.155 ± .102 (n = 7)	**p = 0.87**
MAN Volume	0.170 ± .020 (n = 9)	0.170 ± .022 (n = 8)	**p = 0.98**
lMAN Soma Size	199.50 ± 5.54 (n = 11)	208.35 ± 6.06 (n = 8)	**p = 0.30**
lMAN Density	7.23×10^{-5} ± 7.57×10^{-6} (n = 10)	7.78×10^{-5} ± 2.61×10^{-6} (n = 9)	**p = 0.53**
lMAN # neurons	13182 ± 1350 (n = 9)	13708 ± 1813 (n = 8)	**p = 0.96**
HVC Volume	.392 ± .056 (n = 4)	.338 ± .032 (n = 7)	**p = 0.39**
HVC Soma Size	164.68 ± 1.57 (n = 10)	165.32 ± 2.34 (n = 9)	**p = 0.82**
HVC Density	8.41×10^{-5} ± 1.86×10^{-6} (n = 10)	8.53×10^{-5} ± 2.67×10^{-6} (n = 9)	**p = 0.70**
HVC # neurons	36041 ± 3769 (n = 4)	29057 ± 3093 (n = 8)	**p = 0.20**
RA Volume	.227 ± .013 (n =5)	.231 ±.012 (n = 7)	**p = 0.80**
RA Soma Size	194.89 ± 6.98 (n =11)	196.58 ± 5.22 (n =8)	**p = 0.86**
RA Density	6.33×10^{-5} ± 2.79×10^{-6} (n = 11)	5.74×10^{-5} ± 1.78×10^{-6} (n = 9)	**p = 0.11**
Rotundus Volume	.455 ± .032 (n = 4)	.498 ± .031 (n = 6)	**p = 0.95**
Telencephalon Volume	27.05 ± 1.61 (n = 5)	28.73 ± 2.39 (n = 4)	**p = 0.56**
Syrinx weight	0.020 ± .001 (n = 10)	0.020 ± .001 (n = 9)	**p = 0.94**