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Testosterone selectively affects aromatase and 5 α -reductase activities in the green anole lizard brain

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Abstract

Testosterone (T) and its metabolites are important in the regulation of reproductive behavior in males of a variety of vertebrate species. Aromatase converts T to estradiol and 5 α -reductase converts T to 5 α -dihydrotestosterone (DHT). Male green anole reproduction depends on androgens, yet 5 α -reductase in the brain is not sexually dimorphic and does not vary with season. In contrast, aromatase activity in the male brain is increased during the breeding compared to non-breeding season, and males have higher levels than females during the breeding season. Aromatase is important for female, but not male, sexual behaviors. The present experiment was conducted to determine whether 5 α -reductase and aromatase are regulated by T. Enzyme activity was quantified in whole brain homogenates in both the breeding and non-breeding seasons in males and females that had been treated with either a T or blank implant. In males only, T increased 5 α -reductase activity regardless of season and up-regulated aromatase during the breeding season specifically. Thus, regulation of both enzymes occurs in males, whereas females do not show parallel sensitivity to T. When considered with previous results, the data suggest that aromatase might influence a male function associated with the breeding season other than sexual behavior. 5 α -Reductase can be mediated by T availability, but this regulation may not serve a sex- or season-specific purpose.

Keywords

Steroid hormone; Steroid metabolism; Sexual dimorphism; Seasonal difference

1. Introduction

Steroid hormones regulate reproductive behavior in many vertebrate species. They are synthesized from cholesterol, which, through a series of reactions, forms a variety of hormones including testosterone (T). T is further metabolized into other steroids, including estradiol (E2) and 5 α -dihydrotestosterone (DHT, reviewed in Lephart et al., 2001).

The aromatase enzyme converts T to E2 and is found in the brains of most male and female vertebrates (Balthazart and Foidart, 1993). This enzyme is important for male sexual behavior in a variety of species, including rodents and songbirds (Timonin and Wynne-Edwards, 2008; Bakker et al., 2004; Schlinger, 1997a). Aromatase levels in the brain are often sexually dimorphic, with male individuals having increased activity compared to

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females in species including rats, Japanese quail, gray short-tailed opossums, zebrafish, and European sea bass (Forlano et al., 2006; Roselli and Resko, 1997; Fadem et al., 1993; Balthazart, 1991). T-treatment increases aromatase activity in male golden hamsters, male and female rats, and female canaries (Fusani et al., 2001; Romeo et al., 1999; Roselli et al., 1996). Thus, T commonly regulates activity of this enzyme.

The 5 α -reductase enzyme converts T to DHT, which is a non-aromatizable androgen and has a higher affinity for androgen receptors than T (Negri-Cesi et al., 1996). 5 α -Reductase is widely distributed in the brain, and many species have no observable sex differences in activity, including zebra finches, Japanese quail, and a variety of mammals (reviewed in Celotti et al., 1997; Balthazart, 1991; Balthazart et al., 1990). However, DHT appears necessary for the full display of male sex behavior in rodents, including rats and adult hamsters (Baum and Vreeburg, 1973; Romeo et al., 2001). Unlike aromatase, 5 α -reductase is generally not affected by T administration (reviewed in Negri-Cesi et al., 1996; Balthazart et al., 1990). However, one of the two isozymes of 5 α -reductase (type II) present in the brain shows an increase in mRNA with T-treatment (Negri-Cesi et al., 2008; Torres and Ortega, 2003).

Aromatase and 5 α -reductase activities have been examined in some seasonally breeding animals, and the regulation tends to mirror what is observed following T administration. For example, in song sparrows neural aromatase mRNA decreases outside of the breeding season (BS), and activity increases during the BS in seasonally breeding teleost fishes (Forlano et al., 2006; Soma et al., 2003). In contrast, 5 α -reductase does not differ between the BS and non-breeding season (NBS) in song sparrows (Soma et al., 2003).

Green anole lizards (*Anolis carolinensis*) are seasonally breeding animals and, similar to others, show a decrease in circulating steroid hormones during the NBS (Lovern et al., 2001; Crews, 1980). Anoles have distinct male courtship displays consisting of head-bobbing, pushups, and repeated extension of a red throat fan, or dewlap (Orrell and Jenssen, 2003). Unlike some vertebrates, male green anole reproductive behavior depends primarily on T, although DHT is necessary for maximal behavioral expression (Mason and Adkins, 1976; Rosen and Wade, 2000). Despite the role of DHT in facilitating male reproductive behavior, 5 α -reductase activity in whole brain samples of anoles is not sexually dimorphic and appears not to vary with season (Rosen and Wade, 2001). Aromatase activity, on the other hand, is increased during the BS in male green anoles, and they have higher levels of activity than females in this season (Rosen and Wade, 2001). In female green anoles, aromatization of T facilitates receptivity (Winkler and Wade, 1998), similar to what is seen in musk shrews (Rissman, 1991).

The factors regulating aromatase and 5 α -reductase in the green anole brain are unclear. The goal of this experiment was to determine whether activity of these enzymes is regulated by the amount of T available, and whether effects differ between the BS and NBS. These issues were addressed in both males and females.

2. Methods

2.1. Animals

Green anole lizards were obtained from Charles Sullivan Co. (Nashville, TN) in April (BS) and October (NBS). The animals were housed individually in 10-gal aquaria with peat moss, sticks, rocks, and water dishes. They were misted daily with water and fed crickets or mealworms 3 times per week (BS) or 2 times per week (NBS). In addition to the fluorescent lighting in the room, full spectrum and heat lamps were provided directly above each cage, which allowed for basking temperatures that were 10 °C warmer than ambient. During the

BS, the animals were kept on a 14:10 light/ dark cycle with temperatures ranging from 28 °C during the day to 19 °C at night. During the NBS the animals were kept on a 10:14 light/ dark cycle with room temperatures ranging from 24 °C during the day to 15 °C at night. Relative humidity was kept at approximately 70% during both the BS and NBS.

All procedures adhered to the Michigan State University Institutional Animal Care and Use Committee and NIH guidelines.

2.2. Surgeries

One week after arrival in the lab, animals were deeply anesthetized by hypothermia and gonadectomized. Using a dissecting microscope, a small incision was made on each side of the animal. The gonad was gently pulled out of the body cavity, tied with a silk ligature, and all tissue was cauterized. Prior to destruction, gonads were visually inspected to confirm breeding state. During the BS, all males had large, vascularized testes, and females each had at least one yolking follicle. During the NBS, all gonads were fully regressed. At the time of gonadectomy, one blank- or T-filled capsule was inserted subcutaneously. Incisions were closed using silk sutures, which went through both the skin and internal muscle wall.

Implants were constructed from Silastic tubing (0.7 mm inner and 1.65 mm outer diameters) cut to 7 mm in length. Each capsule contained either 5 mm of packed T-propionate or was left empty. This dose of T was selected because it reliably activates sexual behaviors in adult male green anoles (e.g., Neal and Wade, 2007). Both ends of the implants were sealed with silicone adhesive (Dow Corning Corporation, Midland, MI). One week after surgery, the animals were rapidly decapitated. Presence of the capsule and completeness of gonadectomy were confirmed at this time. Brains were flash frozen on dry ice and stored at -80 °C until processed.

2.3. Enzyme assay

Aromatase and 5 α -reductase activities were determined using previously validated procedures (Wade, 1997; Rosen and Wade, 2001). Briefly, brains were thawed and homogenized in sucrose phosphate buffer. Homogenates were incubated in duplicate with a saturating concentration of 3H-T (80.4 μ Ci/ml) and cofactors at 27 °C for 50 min, a time point at which detection of both 3H-E2 and 3H-DHT is increasing linearly (Wade, 1997). Control tubes were included in each assay; these contained all factors other than tissue. Separate sets of tubes with known amounts of 3H-E2 and 3H-T were included for estimation of recovery efficiency.

Reactions were stopped by placing samples in a methanol-dry ice bath. Steroid hormones were extracted three times using ether. Androgens and estrogens were separated by phenolic partition. Individual steroids (estrone, E2, T, and DHT) were further separated in 3:1 ether:hexane using thin layer chromatography (TLC). Androgens were sprayed with primulin and visualized using long-wave (T) and short-wave (DHT) ultra-violet light. Estrogens were visualized with iodine vapors. Bands corresponding to E2, T, and 5 α -DHT were scraped and steroids were eluted from the silica gel using aqueous methanol. Estrone was not evaluated, as none was detected during a pilot study. Samples were counted in Ultima Gold (Perkin-Elmer and Analytical Sciences, Shelton, CT) on a Beckman LS 6500. A Bradford assay (Bio-Rad Kit) was conducted to determine protein content in each homogenate. Final values are reported as fmol/min/mg protein, and are corrected for background counts, recovery efficiency, and volume counted. Aromatase activity is represented by E2; 5 α -DHT production indicates 5 α -reductase activity. T levels were evaluated simply to determine that sufficient substrate remained.

Due to constraints in the number of samples that could be included during a single run, three separate assays were conducted. The primary goal was to determine whether T affected enzyme activity, and whether it did so similarly across the two seasons. We were interested in determining whether patterns were similar in males and females, but as a maximum of 21 individuals could be run in each assay, it was not feasible to directly compare the sexes. Assay 1 included five females, randomly selected from each of the four groups (T-treated vs. control, each in the BS and NBS). Assay 2 consisted of five randomly selected males from each of these four groups. Based on the results (see below) the remainder of the males we had collected were evaluated in Assay 3. Final sample sizes are included in the figures.

2.4. Statistical analysis

Separate two-way ANOVAs were conducted for aromatase and 5 α -reductase to determine whether their activities are influenced by season and/or treatment. The female samples (Assay 1) were analyzed in one test, and based on the results no further work was conducted. The analysis of Assay 2, which contained the first set of male tissue, produced intriguing results (see below), so the sample sizes were increased with the remaining males (Assay 3). Unpaired *t*-tests were first used to confirm that none of the groups differed across Assays 2 and 3 (all $t < 1.97$, $p > 0.102$). Because the aromatase data were not normally distributed in the male samples, the interaction between treatment and season was also evaluated using a non-parametric test for interactions (Adjusted Rank Transformation; Sawilowsky, 1990).

3. Results

3.1. Females

Aromatase activity in females (Assay 1; Fig. 1A) was not influenced by season ($F = 1.72$, $p = 0.207$) or treatment ($F = 1.38$, $p = 0.257$), and no interaction between these variables was detected ($F < 0.001$, $p = 0.997$). Similarly, the effects of season ($F = 1.03$, $p = 0.325$), treatment ($F = 2.016$, $p = 0.175$) and their interaction ($F = 1.01$, $p = 0.330$) on 5 α -reductase activity in the female brain were not statistically significant (Fig. 1B).

3.2. Males

In Assay 2 on the first set of male brains (data not shown), a main effect of treatment existed; T increased 5 α -reductase activity ($F = 5.34$, $p = 0.035$). There was no effect of season ($F = 1.57$, $p = 0.228$) and no interaction ($F = 0.017$, $p = 0.897$). The ANOVA on raw aromatase data from this first assay did not show an effect of treatment ($F = 1.57$, $p = 0.228$), season ($F = 0.62$, $p = 0.441$), or an interaction between the two ($F = 3.21$, $p = 0.092$). However, activity was extremely low in all groups other than T-treated males in the BS. It was undetectable in 75% of the samples from those three groups, so the data were not normally distributed. The Adjusted Rank Transformation test did reveal a significant interaction between season and treatment ($F = 5.52$, $p = 0.032$), such that T-treated animals in the BS had increased aromatase activity.

Following the addition of more individuals (combined analysis of Assays 2 and 3), 5 α -reductase activity continued to be influenced by treatment ($F = 4.18$, $p = 0.049$), but not season ($F = 1.234$, $p = 0.274$); T-treatment resulted in higher levels of 5 α -DHT synthesis than blank treatments (Fig. 2). No interaction between treatment and season was detected ($F = 0.131$, $p = 0.719$). The aromatase data with increased sample sizes also paralleled the first male assay. Main effects of season and treatment were not detected from the raw data (both $F < 2.90$, $p > 0.978$), although with more animals an interaction between season and treatment was revealed ($F = 4.70$, $p = 0.037$). T increased aromatase activity, but only

during the BS (Fig. 3). The same interaction was detected with the Adjusted Rank Transformation ($F = 5.29$, $p = 0.028$).

4. Discussion

The present results indicate that T specifically increases the activity of both aromatase and 5 α -reductase in the adult green anole lizard brain. Both enzymes were modulated by T-treatment only in males, and the effect on aromatase was specific to the breeding season. The results for each enzyme are discussed below in the context of other available data.

4.1. Aromatase

The difference between aromatase activity in T-treated males during the BS and the other groups was striking. Levels were extremely low in T-treated males during the NBS, as well as in castrated males that did not receive hormone replacement in both the BS and NBS. This pattern parallels data collected from intact animals, in which whole brain aromatase activity is increased during the BS compared to the NBS in males, and within the BS is greater in males than females (Rosen and Wade, 2001). Plasma T levels are also greater in males than females during the BS, and in the BS compared to NBS in males (Lovern et al., 2001). Collectively, these data suggest that brain aromatase activity is up-regulated by T specifically during the BS in the adult male green anole.

Seasonal changes in intact animals of other species tend to be consistent with the idea that T up-regulates activity of the enzyme. For example, male song sparrows show an increase during the BS (Soma et al., 2003). Also, both male and female midshipman fish exhibit greater aromatase activity with seasonally increased steroid hormone levels (reviewed in Forlano et al., 2006). However, in male spotted ant birds, a tropical species in which the environment changes very little, natural changes in T across seasons do not alter aromatase mRNA expression in the brain (Canoine et al., 2007).

The function of T-induced up-regulation of aromatase in breeding male anoles is unclear, as activity of this enzyme is not critical for their display of reproductive behaviors (Winkler and Wade, 1998). Aromatization of T (and a T-induced increase in the enzyme) often serves to facilitate the display of male sexual behaviors (see Section 1). Clearly, the mechanisms are available in this reptilian species as well, but they seem to have not been co-opted for this purpose. It has been suggested that in some organisms aromatase serves to decrease the amount of T in the brain. For example, this role of clearing excess androgens has been proposed for the very high level of aromatase in songbird brains (reviewed in Schlinger, 1997b). The role seems less likely for the much lower levels of aromatase activity in the green anole brain as a whole. However, this function may occur on a local level within specific neural regions. Future work is required to determine whether this hypothesis is plausible.

Regardless, the fact that, in male green anoles, a T-induced increase in aromatase occurs specifically during the BS is particularly intriguing, and implies a seasonal change in sensitivity to T. This type of effect has been detected in T-regulation of male sexual behavior (Neal and Wade, 2007), as well as copulatory muscle and hemipene morphology (Holmes and Wade, 2004), in this species. The mechanisms controlling the change in responsiveness are not known, but could involve differences in androgen receptor expression. The idea requires more detailed evaluation in a variety of tissues in green anoles. However, at least in copulatory muscles, T can increase androgen receptor immunoreactive nuclei (Holmes and Wade, 2005). Consistent with this idea, when the androgen receptor blocker flutamide is given to castrated male rats, the T-induced increase in aromatase activity is inhibited (Roselli and Resko, 1984). Further, aromatase is co-localized with

androgen receptors in musk shrews, goldfish, and Japanese quail, which lends support to the idea that they may regulate activity or expression of the enzyme (Veney and Rissman, 2000; Balthazart et al., 1998; Gelinis and Callard, 1997).

Aromatase activity in female green anoles did not differ across treatment or season in the present study. This result suggests that T does not regulate the production of E2 in the female brain, and that the enzyme is equally active in the BS and NBS. This result was unexpected, given that the enzyme facilitates female receptivity (Winkler and Wade, 1998), plasma T is increased in females in the BS compared to the NBS (Lovern and Wade, 2001), and T had clear and specific effects on aromatase in males. It is unknown whether aromatase activity changes seasonally in unmanipulated female anoles, but if so, the present results suggest it would be regulated by a mechanism other than T, perhaps an ovarian hormone. Consistent with the idea that an ovarian hormone is involved, gonadectomy of female midshipman fish reduces aromatase activity in the brain, similar to non-breeding levels (Forlano and Bass, 2005). While there is a significant increase of plasma T during the BS compared to NBS in female green anoles (Lovern and Wade, 2001), the absolute level of T during the BS may not be high enough to induce changes in aromatase activity. Alternatively, perhaps substrate (T) availability is more important for regulation of female sexual behavior than actual aromatase levels.

It is also important to consider that T-regulated changes in aromatase activity might occur within specific brain regions (in either sex). Because the present experiment tested aromatase activity in whole brains, we could not detect changes in the distribution of the enzyme across different nuclei. Female canaries, for example, have higher levels of aromatase activity and mRNA in the caudomedial neostriatum after T-treatment (Fusani et al., 2001). However, if regional differences due to steroid hormone or seasonal effects do not exist, or they exist in areas outside of those that control reproduction, the data might suggest other potential roles for aromatase. For example, the enzyme has been implicated in the regulation of synaptic plasticity, induction of neurogenesis, and neuroprotection after injury (reviewed in Garcia-Segura, 2008; Roselli, 2007).

4.2. 5 α -Reductase

The increased levels of 5 α -reductase activity in T-treated males regardless of the season (BS or NBS) were somewhat unexpected. Previous work on 5 α -reductase in anoles indicated no differences due to season or sex (Rosen and Wade, 2001). In parallel, mammalian 5 α -reductase activity does not differ between the sexes, and seasonal differences are not detected in song sparrows (reviewed in Negricesi et al., 1996; Soma et al., 2003). It is possible that we were able to detect a difference with hormone manipulation in the current study due to larger differences in T levels between blank- and T-treated animals than the natural differences observed between intact BS and NBS animals. This idea is similar to a greater increase in the muscles associated with copulation in T-treated compared to control animals than what was observed in intact animals during the BS and NBS (Holmes and Wade, 2004).

Compared to aromatase, less is known about the role and regulation of 5 α -reductase in the brain. However, it is clear that some species have two isozymes that catalyze the conversion of T to DHT, type I and type II, and they can be differentially regulated. In both male and female adult rats, for example, type I is not affected by T-treatment, whereas type II mRNA is increased (Torres and Ortega, 2003, 2006). Expression of the two forms has not been evaluated in green anoles, but it is possible that we detected type II activity or that type I may be differently regulated in green anoles than other systems. Future work is needed to evaluate these ideas.

Unlike in males, gonadectomy and T replacement in female anoles did not alter 5 α -reductase activity in either BS or NBS. This result parallels the idea that the enzyme has some role in male sexual behavior (see Section 1), whereas no evidence exists for it influencing female receptivity in this species. One possibility is that females may have lower levels of type II than males. Because type I is important for catabolism of excess steroids (Torres and Ortega, 2003), it may be present in equal amounts in males and females, which could account for our ability to detect 5 α -reductase activity in both sexes, in treated and non-treated individuals, and during BS and NBS. 5 α -Reductase also can act on progesterone, which is metabolized to an intermediate product that is then converted to allopregnanolone (Paul and Purdy, 1992). Progesterone, together with estrogen, can induce receptivity in many female vertebrates, including anoles (McNicol and Crews, 1979). Thus, 5 α -reductase may play a role in regulating the effects of progesterone on female reproductive behavior.

5. Conclusion

T appears to regulate its own metabolism in male, but not female, anoles. 5 α -Reductase activity in males is increased by T regardless of season, and aromatase activity is increased by T solely during the BS. In males, both of these enzymes may be mediating other behaviors or processes that are not directly associated with reproductive function. These results seem to be consistent with much of the data from other vertebrate species, and suggest that T can regulate metabolizing enzymes in a variety of different circumstances. It is possible that, similar to other organisms, this modulation occurs through the action of androgen receptors in male green anoles. Thus, T-regulation of aromatase in particular, but also 5 α -reductase to some extent, seems highly conserved for males. While more data need to be collected, this feature appears to exist regardless of the relative importance of aromatase and 5 α -reductase in the activation of sexual behaviors. The results for females are less consistent, and it will be important to determine the functional roles of these two enzymes, as well as what factors (if any) regulate the enzymes in this sex. Understanding the mechanisms associated with the sex differences and how/ whether they differ in reptiles compared to other vertebrate groups will further elucidate the evolutionarily conserved mechanisms operating in the control of reproduction by testosterone metabolizing enzymes.

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Fig. 1. Enzyme activity in manipulated females during the breeding and non-breeding seasons (means + SE). Aromatase activity is indicated in (A) and 5 α -reductase activity in (B). Testosterone refers to gonadectomized + T-treated individuals while blank refers to gonadectomized + blank treated individuals. No significant differences were detected.

Fig. 2. 5 α -Reductase activity in manipulated males during breeding and non-breeding seasons (means + SE); data combined from two assays. Testosterone refers to gonadectomized + T-treated individuals while blank refers to gonadectomized + blank treated individuals. Testosterone treatment resulted in an increase in activity.

Fig. 3.

Aromatase activity in manipulated males during the breeding and non-breeding seasons (means + SE); data combined from two assays. Testosterone refers to gonadectomized + T-treated individuals while blank refers to gonadectomized + blank treated individuals. Testosterone treatment increased activity in the breeding season only.