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Aromatase and 5α-reductase type 2 mRNA in the green anole forebrain: An investigation of the effects of sex, season and testosterone manipulation

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ABSTRACT

Aromatase and 5α-reductase (5αR) catalyze the synthesis of testosterone (T) metabolites: estradiol and 5α-dihydrotestosterone, respectively. These enzymes are important in controlling sexual behaviors in male and female vertebrates. To investigate factors contributing to their regulation in reptiles, male and female green anole lizards were gonadectomized during the breeding and non-breeding seasons and treated with a T-filled or blank capsule. *In situ* hybridization was used to examine main effects of and interactions among sex, season, and T on expression of aromatase and one isozyme of 5αR (5αR2) in three brain regions that control reproductive behaviors: the preoptic area, ventromedial nucleus of the amygdala and ventromedial hypothalamus (VMH). Patterns of mRNA generally paralleled previous evaluations of intact animals. Although no main effects of T were detected, interactions were present in the VMH. Specifically, the density of 5αR2 expressing cells was greater in T-treated than control females in this region, regardless of season. Among breeding males, blank-treated males had a denser population of 5αR2 positive cells than T-treated males. Overall, T appears to have less of a role in the regulation of these enzymes than in other vertebrate groups, which is consistent with the primary role of T (rather than its metabolites) in regulation of reproductive behaviors in lizards. However, further investigation of protein and enzyme activity levels are needed before specific conclusions can be drawn.

1. Introduction

Hormones regulate the production of male- and female-specific sexual behaviors in a wide variety of species [2,14,17,24,59,60]. These hormone-activated sexual behaviors are mediated by several regions of the forebrain. In particular, the preoptic area (POA) and amygdala are critical for male sexual behaviors, while the ventromedial hypothalamus (VMH) is important for female receptivity. Testosterone (T) and/or its metabolites, estradiol (E2) and 5α-dihydrotestosterone (DHT), generally activate these behaviors in adulthood. In the brain, T is metabolized into E2 via the action of aromatase, and into DHT via 5α-reductase (5αR; [34,35]).

Neural aromatase is critical for male sexual behavior in a variety of species including Japanese quail, midshipman fish, zebrafish, musk shrews, rats, and mice [1,4,16,20,46,47,54]. Peripheral and neural aromatase is also important for females. For example, inhibitors of this enzyme decrease female canary sexual behaviors, and aromatizable androgens increase female musk shrew copulatory behaviors [3,32,46,63]. T increases neural aromatase activity or mRNA in male and female rats and Japanese quail, female midshipman fish and male ring doves and zebra finches [3,5,15,49,50,52,64,72]. Thus, T commonly upregulates this enzyme in both males and females of a variety of species.

5αR has not been studied as extensively as aromatase. It exists in two forms: 5αR1 and 5αR2 [35]. In humans, mice, and rats, 5αR1 mRNA is expressed in diverse neural regions, whereas 5αR2 mRNA is found in relatively low levels in the adult brain [9]. Expression of both isozymes is greater in the brainstem than forebrain. 5αR1 has a relatively low affinity for T and is present in both neurons and glial cells [40]. In contrast, 5αR2 has a higher affinity for the hormone and is found in hypothalamic and hippocampal neurons in the adult brain [44]. In rat prefrontal cortex, 5αR2 is upregulated after T administration, but 5αR1 is not [61,62]. Thus, T appears to upregulate at least one isozyme of 5αR.

Abbreviations: 5αR, 5α-reductase; 5αR1, 5α-reductase type 1; 5αR2, 5α-reductase type 2; AMY, ventromedial nucleus of the amygdala; BS, breeding season; DHT, 5α-dihydrotestosterone; E2, estradiol; NBS, non-breeding season; POA, preoptic area; T, testosterone; VMH, ventromedial hypothalamus.

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Green anole lizards (*Anolis carolinensis*) are excellent models for the examination of sex and seasonal differences in T metabolizing enzyme expression. They are seasonally breeding animals, with higher levels of plasma steroid hormones during the breeding season (BS) than non-breeding season (NBS; [36]). Similar to other vertebrates, the POA and a portion of the amygdala (ventromedial nucleus; AMY) in this species are important in the control of male sexual behavior [23,68]. Although the experiment has not been conducted in this species, electrolytic lesioning of the VMH in other lizards has shown that it is critical to female receptivity [28].

Aromatase appears to play less of a role in facilitating male sexual behaviors in anoles than in mammals or birds. T itself is the most potent activator of these displays in green anoles [66]. However, aromatase does enhance behavioral expression. For example, while inhibition of the enzyme's activity in gonadectomized T-treated males did not reduce the display of sexual behavior, additional E2 treatment enhanced sexual motivation in male anoles [31,69]. In addition, data from inhibition of aromatase in ovariectomized, T-treated females suggest that activity of the enzyme is important for female receptivity [31,69]. Whole brain aromatase activity is sexually and seasonally dimorphic; it is elevated in breeding males compared to females, and in males it is greater in the BS than NBS [57]. The effects of T on whole brain aromatase activity are specific; T induces an increase only in the BS and only in males [11]. Aromatase mRNA is expressed in the three regions controlling sexual behaviors (POA, AMY, and VMH) and is sexually dimorphic, such that males have a greater number of aromatase positive cells in the POA than females, but these cells are denser in the AMY and VMH of females [12].

The administration of a 5αR inhibitor showed that the activity of the enzyme is important for the full expression of male green anole sexual behaviors [56]. Although 5αR activity does not consistently differ between sexes or seasons in assays of whole brain homogenates, T-treatment increases activity in males (but not females) regardless of season [11,57]. Unlike in mammals, 5αR1 is not expressed in the forebrain of green anoles, although expression in specific brainstem nuclei is clear. In contrast, 5αR2 mRNA is detected in specific regions throughout the brain; the density of these cells in the AMY is greater in females than in males [10].

The goal of the present study was to determine whether T affects the expression of aromatase and 5αR2 mRNA specifically within brain regions that regulate male and female reproductive behaviors. Because we previously documented seasonal effects of T on aromatase activity (see above), we also examined these enzymes across seasons. As we had done in intact animals [10,12], *in situ* hybridization was used to evaluate the numbers and densities of mRNA-containing cells in the POA, AMY and VMH of male and female green anoles from both the BS and NBS.

2. Materials and methods

2.1. Animals

Wild-caught adult green anole lizards were purchased from Charles Sullivan Co., (Nashville, TN) during the BS (June) and NBS (October). At Michigan State University, the animals were housed individually in 10-gallon aquaria with peat moss, sticks, rocks and water dishes. They were misted daily with water and fed calcium phosphate dusted crickets three (BS) or two (NBS) times a week. During the BS, animals were kept on a 14:10 light/dark cycle with ambient temperatures ranging from 28 °C during the day to 19 °C at night. During the NBS, animals were kept on a 10:14 light/dark cycle, with ambient temperature ranging from 24 °C during the day to 15 °C at night. Relative humidity was maintained at approximately 70% during both seasons. In addition to full spectrum lamps above each cage, heat lamps were also provided, which allowed than animals to bask in temperatures up to 10 °C above ambient.

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

2.2. Treatment and tissue collection

One week after arriving in lab, animals were anesthetized by hypothermia and gonadectomized. A small incision was made on each side of the animal. The gonads were gently removed from the body cavity, ligated with silk and fully destroyed by cauterization. The incisions were closed using silk sutures that went through the skin and internal muscle wall. Gonads were visually inspected at the time of surgery to confirm breeding state. During the BS, males had large, fully vascularized testes, and females had large oviducts and at least one large yolking follicle. During the NBS, gonads were fully regressed, with males having small, non-vascularized testes and females having small oviducts and tiny follicles (all <1 mm in diameter).

At the time of gonadectomy, one blank- or T-filled implant was inserted subcutaneously into each animal. The implants were constructed from Silastic tubing (0.7 mm inner and 1.65 outer diameters) cut to 7 mm in length and were either packed with 5 mm of T-propionate (Steraloids Inc., Wilton, NH) or left empty. Both ends were sealed using silicone adhesive (Dow Corning Corporation, Midland, MI). This dose of T was used because it reliably activates male sexual behaviors and increases neural aromatase and 5αR activities in this species [11,39].

One week after surgery, animals were rapidly decapitated. The presence of the capsule and the completeness of gonadectomy were both confirmed at this time. One individual was removed from the study due to a testicular remnant (see below). Blood was collected from the trunk and head of each animal and kept on ice until centrifuged (10,000 rpm for 10 min). The plasma was stored at −80 °C until assayed to confirm effectiveness of treatment. Brains were immediately frozen in methyl butane on dry ice and stored at −80 °C until processed. They were sectioned coronally at 20 μm into four alternate series and thaw mounted onto SuperFrost plus slides (Fisher Scientific; Hampton, NH). Slides were stored at −80 °C with desiccant until further processing.

2.3. Radioimmunoassay

Plasma samples from each individual were incubated overnight at 4 °C with 1000 CPM of 3H-T (80.4 μCi/ml; PerkinElmer, Boston, MA) for recovery determination. They were extracted twice with diethyl ether and dried under nitrogen gas. The samples were then reconstituted with 500 μl of phosphate-buffered saline and stored at 4 °C. The next day, duplicate samples were incubated overnight with 3H-T (4000 CPM) and T antibody [1:10,000; 20R-TR018 W; originally produced by Wien Laboratories, sold by Fitzgerald, Concord, MA; as in 38]). To remove unbound hormone, samples were incubated with dextran-coated charcoal (Sigma, St. Louis, MO) for 15 min. They were then centrifuged (3000 RPM for 25 min), and the supernatant was mixed with 3.5 ml of UltimaGold scintillation fluid (PerkinElmer, Shelton, CT) and counted on a Beckman LS 6500. Samples were adjusted for initial sample volume and recovery and compared to a standard curve run in triplicate. Average recovery efficiency was 92% and the intra-assay CV was 13%. Before running the samples, parallelism and accurate detection of known T concentrations were confirmed (data not shown).