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Aromatase and 5 α -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 5 α -reductase (5 α R) catalyze the synthesis of testosterone (T) metabolites: estradiol and 5 α -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 5 α R (5 α R2) 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 5 α R2 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 5 α R2 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.

Keywords

Androgen metabolism; preoptic area; amygdala; ventromedial hypothalamus; *Anolis carolinensis*; lizard

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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 5 α -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 5 α -reductase [5 R; 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.

5 α R has not been studied as extensively as aromatase. It exists in two forms: 5 α R1 and 5 α R2 [35]. In humans, mice, and rats, 5 α R1 mRNA is expressed in diverse neural regions, whereas 5 α R2 mRNA is found in relatively low levels in the adult brain [9]. Expression of both isozymes is greater in the brainstem than forebrain. 5 α R1 has a relatively low affinity for T and is present in both neurons and glial cells [40]. In contrast, 5 α R2 has a higher affinity for the hormone and is found in hypothalamic and hippocampal neurons in the adult brain [44]. In rat prefrontal cortex, 5 α R2 is upregulated after T administration, but 5 α R1 is not [61; 62]. Thus, T appears to upregulate at least one isozyme of 5 α R.

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 5 α R inhibitor showed that the activity of the enzyme is important for the full expression of male green anole sexual behaviors (Rosen and Wade, 2000). Although 5 α R 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, 5 α R1 is not expressed in the forebrain of green anoles, although expression in specific brainstem nuclei is clear. In contrast, 5 α R2 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 5 α R2 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.

Materials and Methods

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 the 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.

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 5 α R 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 $20\text{ }\mu\text{m}$ into four alternate series and thaw mounted onto SuperFrost plus slides (Fisher Scientific; Hampton, NH). Slides were stored at -80°C with dessicant until further processing.

Radioimmunoassay

Plasma samples from each individual were incubated overnight at 4°C with 1000 CPM of 3H-T (80.4 $\mu\text{Ci/ml}$; 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-TR018W; 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).

In situ hybridization

Both aromatase (GenBank ID: XM_003225883) and 5 α R2 (GenBank ID: XM_003215965.1) were cloned previously [10; 12]. Briefly, sense (T7 for aromatase and SP6 for 5 α R2) and antisense (T3 for aromatase and T7 for 5 α R2) probes were transcribed using the Digoxigenin RNA Labeling Kit per manufacturer's instructions (Roche Diagnostics; Indianapolis, IN). Probes were cleaned using G50 sephadex bead columns and stored at -80°C until use. For each gene, one set of slides from each animal was used for the antisense reaction. As a control, another set of slides from one animal from each group was used for the sense reaction. Slides were thawed and then fixed for 10 min in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS; pH 7.4). They were treated with 0.25% acetic anhydride in triethanolamine-HCl with 0.9% NaCl buffer (pH 8.0). Slides then incubated overnight at 55°C with 200 ng/ml (aromatase) or 100 ng/ml (5 α R2) probe in hybridization buffer, which consisted of 50% formamide, 4x SSC, 1x Denhardt's solution, 200 $\mu\text{g/ml}$ fish sperm DNA, 10% dextran sulfate, 20 mM dithiothreitol, 250 $\mu\text{g/ml}$ tRNA, 2 mM EDTA, and 0.1% Tween-20. The following day, the tissue was rinsed in 2x SSC and 0.2x SSC at 60°C . It was then treated with 0.9% H_2O_2 in maleic acid buffer (pH 7.5) with 0.1% Tween-20 (MABT) for 30 mins. The slides were incubated in a blocking solution of 5% normal sheep serum (Jackson Immuno Research; West Grove, PA) in MABT for 30 min, and were treated with 0.5 $\mu\text{l/ml}$ Anti-Digoxigenin-AP Fab fragments (Roche) in MABT. After two hours, the color reaction was conducted by incubating the slides with 4.5 $\mu\text{l/ml}$ NBT and 3.5 $\mu\text{l/ml}$ BCIP (Roche) in 0.1M Tris-HCl and 0.1M NaCl (pH 9.5). The reaction was monitored so that the slides incubated with the antisense probe showed a distinct reaction product within the cytoplasm of individual cells with an absence of labeling on the sense-treated slides (about 10 minutes for aromatase and 5 minutes for 5 α R2), the color reaction was stopped with 1M Tris and 0.5M EDTA (pH 8.0). Slides were dehydrated in increasing concentrations of ethanol and coverslipped.

Stereological analysis

The slides were examined under brightfield illumination using Stereo Investigator software (MicroBrightfield, Inc.; Williston, VT) as in Cohen and Wade [10; 12] by a user blind to experimental group. Estimates of total counts of cells positive for aromatase or 5 α R2 were obtained using the Optical Fractionator function. Brain areas were determined using a green anole atlas [22]. After tracing the outline of a brain region (as defined by aromatase or 5 α R2 expression) in each tissue section in which it existed, the software placed a grid over each area (POA: 100 $\times$ 100 μm^2 , AMY: 40 $\times$ 40 μm^2 , VMH: 80 $\times$ 80 μm^2) and sampling sites (30 $\times$ 30 μm^2) were placed randomly within the defined region. The software calculated a volume for the brain region, and estimated the total number of positive cell based on the overall size of the region and the samples in which manual cell counts were taken. Any cell with distinct blue cytoplasmic labeling found within the defined region was counted, as in Cohen and Wade [10; 12]. A Gundersen coefficient of error at or below 0.1 was confirmed to ensure accurate estimates of cell count. The density of positive cells was determined by dividing the estimated cell count by the calculated volume for the region. For both total counts and densities, values from the left and right halves of the brain were averaged prior to statistical analysis, except in cases where data could only be obtained from one side due to tissue damage. As in our previous studies, aromatase and 5 α R2 were only expressed in the lateral portion of the VMH and thus were only quantified in that region [10; 12]. In all photomicrographs, brightness and contrast was modified to insure that the images matched what was analyzed on the computer screen.

Statistical analysis

Analysis of each brain region and enzyme was conducted separately. Three-way ANOVAs were run to determine the effects of, as well as any interactions among, sex, season, and treatment on the number of positive cells, the density of the cells, and the volume of each region. Interactions were further broken down first by two-way ANOVAs and then pairwise comparisons to evaluate specific effects between pairs of groups, as appropriate. Final sample sizes are indicated in the figures.

Results

Radioimmunoassay

Almost all of the gonadectomized, blank-treated animals (32 of 34 individuals) had values that were below the limit of detectability (7.8 pg/tube T). One male, however, had high plasma androgen and was excluded from the study; examination at the time of euthanasia indicated a remnant of testicular tissue. His androgen level was within the range of those that had been completely gonadectomized and treated with T. Of the 39 T-treated animals, 17 had plasma androgen levels that were slightly above the limits of the standard curve (250 pg/tube T). Thus, it was not possible to get accurate estimates of their circulating levels. Of those that had values we could determine, there were no effects of sex or season, and no interaction between these variables (all $F < 1.87$, $p > 0.189$). Among the individuals from which we could get accurate estimates of androgen levels, the average concentration of the T-treated animals was 48.69 ± 4.78 ng/ml. Substantial variability exists across studies, but under natural conditions, unmanipulated male anoles have between 1 and 20 ng/ml of circulating T in the BS [27; 36; 38]. Thus, the values in the present experiment were generally supraphysiological. However, the fact that a male in the castrated+blank group with some testicular tissue had T in the range of those with T-capsules suggests that the levels were not extreme.

Aromatase expression

In the POA, no effects of sex, season, treatment, or interactions among these factors were detected on the estimated total number of cells expressing aromatase (all $F < 3.63$, $p > 0.064$). Across the groups, the animals had an average of 5678 ± 216 aromatase positive cells in this region. The density of these cells was greater in females than in males ($F = 11.095$, $p = 0.002$; Fig. 1a, b, c), but no other effects on density were detected in this brain area (all $F < 2.90$, $p > 0.097$). POA volume based on the borders defined by aromatase expressing cells was larger in males than females ($F = 35.37$, $p < 0.001$; Table 1a).

In the AMY, no effects were seen on the number of aromatase expressing cells (all $F < 2.10$, $p > 0.157$). On average, this value was 4096 ± 200 cells. Females had a greater density of aromatase expressing cells than males ($F = 11.23$, $p = 0.002$, Fig. 1d, e, f). A trend was detected for a sex by treatment interaction ($F = 4.00$, $p = 0.054$), but no other effects were detected on the density of aromatase positive cells (all $F < 1.98$, $p > 0.169$). There was also a trend for a sex difference in the volume of the AMY ($F = 3.99$, $p = 0.055$) without other effects on the volume of this region (all $F < 0.174$, $p > 0.680$; Table 1a).

In the VMH, no significant effects were detected on the number or density of aromatase expressing cells (all $F < 2.65$, $p > 0.114$). On average, the number of aromatase expressing cells was 6767 ± 276 and the density was $27,226 \pm 827$ cells/mm³. Males had a larger VMH than females ($F = 5.026$, $p = 0.033$), as defined by aromatase labeling, and NBS animals had a larger VMH than those in the BS, regardless of sex ($F = 5.15$, $p = 0.031$; Table 1a). No other effects were detected on the volume of the region (all $F < 0.56$, $p > 0.462$).

5αR2 expression

In the POA, we detected no effects of sex, season, treatment, or interactions among these on the number of 5αR2 expressing cells (all $F < 2.12$, $p > 0.154$). On average, animals had an estimated total of 3408 ± 140 cells. This population of 5αR2 expressing cells was denser in females than males ($F = 5.66$, $p = 0.023$, Fig. 2). No other effects were detected on this variable in the POA (all $F < 2.24$, $p > .143$). The volume of this region defined by 5αR2 expression was greater in males than females ($F = 15.50$, $p < 0.001$). No other effects were detected on POA volume (all $F < 2.96$, $p > 0.093$; Table 1b).

In the AMY, no significant effects were detected on the number or density of 5αR2 expressing cells, nor on the volume of the region (all $F < 2.46$, $p > 0.126$). On average the number of 5αR2 positive cells was 2826 ± 133 cells and the density was $130,329 \pm 3674$ cells/mm³. The volume of the region in males and females is indicated in Table 1b.

In the VMH, males had a greater number of 5αR2 expressing cells than females ($F = 16.22$, $p < 0.001$; Fig. 3a). No main effects of season, treatment or interactions among the three variables were detected (all $F < 0.99$, $p > 0.326$) on the estimate of total 5αR2 cells in this region, although a trend for sex by season interaction was seen ($F = 4.01$, $p = 0.052$). In contrast, a three-way interaction among sex, season, and treatment existed on the density of 5αR2 expressing cells ($F = 5.44$, $p = 0.025$; Fig. 3b–f). A trend for a sex x treatment interaction on the density of cells ($F = 3.81$, $p = 0.058$) was also detected, but no other main effects or interactions were revealed in this analysis (all $F < 1.32$, $p > 0.258$). A two-way ANOVA within females revealed a main effect of hormone treatment such that T-treated individuals had a denser population of 5αR2 cells than blank-treated ones ($F = 4.81$, $p = 0.041$). No other effects were detected within females (all $F < 2.35$, $p > 0.142$). A two-way ANOVA within males showed no main effects (all $F < 0.77$, $p > 0.392$), but did indicate an interaction between season and treatment ($F = 4.37$, $p = 0.05$). Pairwise comparisons within BS males indicated a denser population of cells expressed 5αR2 in blank than T treated animals ($t = 2.64$, $p = 0.027$). T-treatment of males had no effect in the NBS ($t = 0.74$, $p =$

0.56). We also detected a main effect of sex in the three-way ANOVA on the volume of the VMH, as defined by 5 α R2 expression, such that the region was larger in males than females ($F = 29.34$, $p < 0.001$). No other effects were detected on the volume of the VMH (all $F < 3.45$, $p > 0.071$; Table 1b).

Discussion

In this study, we tested the hypothesis that T influences aromatase and 5 α R2 expression in three forebrain regions that control reproductive behavior: the POA, AMY, and VMH. Previous work had shown various sex and seasonal differences in both whole brain activity and the pattern of mRNA expression within these limbic areas. Collectively, the current data confirm the presence of sex differences in the distribution of aromatase and 5 α R2, and suggest that these differences are not entirely due to circulating T. Interestingly, research from other animals (and whole brain activity data from anoles) suggests that T does play a key role in regulating these enzymes (see Introduction). Thus, the present study is an important step in examining how T regulates its own metabolism within specific brain regions and how this control might differ across vertebrate groups. Below, the effects of treatment are discussed. Our present results are then compared with studies on intact male and female green anoles from the BS and NBS. Finally, the results are discussed in context with work from other species.

Effects of T-treatment

Although no main effects of hormone manipulation were detected on aromatase or 5 α R2 mRNA expression in this study, we did find two significant interactions, both involving 5 α R2 expression in the VMH. First, females treated with T had a higher density of 5 α R2 cells in this region than blank-treated females, which suggests that T plays a role in 5 α R2 expression in females specifically, regardless of the environmental conditions. While the estimated total number of cells did not differ among treatment groups, it is possible that the denser population of 5 α R2 expressing cells could produce an increased local concentration of DHT. Previous work on female anoles, using similarly constructed T implants, detected no influence of the hormone on 5 α R activity in whole brain homogenates [11]. Thus, the hormone likely has a relatively local effect on 5 α R2 expression in the VMH. The lack of effects of T in other regions investigated in this study is consistent with this idea. However, it is also possible that the expression levels we detected do not reflect functional protein or activity differences.

There are some distinct possibilities for why we found that T influences 5 α R2 in the female VMH. In this study, females were exposed to a supra-physiological level of T, which may have revealed effects of T that are not as apparent in normal females. For example, under conditions of high hormone, 5 α R2 may serve to reduce excess quantities of T and/or E2. Alternatively, the enzyme could affect reproduction by acting on progesterone, as 5 α R2 metabolizes it into dihydroprogesterone [35]. In anoles, progesterone plays a facilitatory role in female and male anole reproduction [70; 71], and its reduction via 5 α R2 may also play a role in controlling these behaviors. Another possibility is that DHT production itself is important in the female VMH although it is currently unclear what role this hormone might play in that sex. However, the fact that whole brain 5 α R activity does not consistently differ between unmanipulated intact males and females [57] raises the possibility that DHT in the VMH could have a function in both sexes, such as influencing feeding behaviors [VMH function reviewed in 29], although future work will be necessary to determine whether this region is important in the control of reptilian feeding behaviors as well.

The second effect of T that we found was in BS males, such that blank-treated males had a denser population of 5 α R2 expressing cells in the VMH than T-treated males. Again, the

decrease in density of 5 α R2 expressing cells may serve to decrease DHT production in the VMH of BS males. In anoles, T is the primary hormone activating male sexual behaviors [65]. DHT does facilitate them, but is not sufficient for the full expression of male-specific behaviors [56]. Assuming that sufficient DHT is present under conditions of high T to perform this function, 5 α R2 might be selectively diminished to increase the amount of T present in the VMH of BS males. Although the VMH is traditionally associated with the activation of female sexual behaviors, the region is a key element in the limbic circuit that activates reproductive behaviors in both males and females [19; 41], and thus T in this region may play a facilitatory role in males. Decreased 5 α R2 may also serve to increase local E2 metabolized from T. While the specific region of action is not clear, the fact that E2 increases motivation for males to display sexual behaviors [31] is consistent with this idea. Alternatively, the VMH plays a role in non-reproductive behaviors, such as feeding behaviors (indicated above), and this role for the VMH may be influenced by 5 α R2 expression. For example, during the BS, male anoles defend territories and attract mates using stereotypical displays [21; 37; 39]. This added energy expenditure could increase the need to spend time on food acquisition. In fact, green anoles are more active overall in the BS than NBS, and expend 60% more energy during the BS [26; 43]. Thus, increased T or decreased DHT in the breeding male VMH might facilitate activity on a general level.

Comparison with previous work from intact animals

We detected similar patterns of aromatase and 5 α R2 expression in this study as in intact green anoles ([10; 12; Table 2], with a few exceptions including sex differences in the density of aromatase positive cells in the VMH and the total number in the POA. It is possible that the discrepancies between the current and previous experiments were due to the greater number of lizards used in the present experiment compared to our work on intact animals. However, this idea seems unlikely, as there is not a consistent pattern of differences between the intact and treated animals. The more likely source of the variation is the fact that the animals used in this study were gonadectomized, whereas the animals in the previous studies on aromatase and 5 α R2 were unmanipulated [10; 12]. Previous work has demonstrated that gonadectomy can reveal sex differences in soma size and volume of these regions that are not detected in intact anoles [7; 42]. Innervation between the gonads and brain has been described, and the brain can modulate T release from the testes [18; 33; 58]. Thus, the differences we detected may have arisen from the loss of the gonads themselves, factors potentially including innervation or secretion of substances other than T that may have a role in regulating aromatase and 5 α R2 expression. These ideas warrant further investigation.

We determined the volume of the three brain regions using two different markers, aromatase and 5 α R2. The sizes of the regions and patterns of differences between sexes and seasons were largely similar to those found in previous work on mRNA for these enzymes [10; 12]. Both aromatase and 5 α R2 expressing cells define similar regions of the POA and AMY as those based on morphological characteristics seen in Nissl stained tissue [6], suggesting the cells synthesizing these enzymes are relatively evenly distributed throughout these areas. However, this is not the case for the VMH. Unlike Nissl stained tissue [6], only the lateral VMH appears to express aromatase and 5 α R2 mRNAs and we confined our analysis to this portion only. Both androgen and estrogen receptor expression are also confined to the lateral VMH in green anoles [8; 55], which suggests that the production of hormones and their site of action is in the same general location within the VMH.

Broader context and interpretations

While we did detect some selective effects of T in the present study, they were limited to 5 α R2 in the VMH. The role of T in regulating aromatase and 5 α R2 in other regions is

unclear. Previous work on whole brain homogenates has shown that T specifically increases the activity of both of these enzymes in males [11]. It is possible that T may substantially affect their synthesis in different areas than those investigated in the present study, which could cause an overall increase in activity. Alternatively, the effects we saw on whole brain activity may result from the sum of smaller effects distributed across a large number of regions. T does play a role in upregulating aromatase and 5 α R2 in a variety of vertebrates (see Introduction), yet there are also species where T does not regulate these enzymes in every brain area. For example, castration does not change aromatase activity in the guinea pig amygdala or ram VMH or amygdala [13; 53]. Additionally, T does not alter aromatase activity in the POA and AMY of rhesus monkeys, the AMY of rats, or in the POA of hamsters exposed to short days [25; 48; 51]. Thus, aromatase activity in specific areas is not always influenced by T treatment. 5 α R activity is also not increased in Japanese quail by T treatment in the POA, VMH, or nucleus taeniae (analogous to the steroid sensitive portion of the mammalian amygdala; [5]). Although these data suggest that 5 α R is not consistently regulated by T, activity measures do not allow one to distinguish between the contributions of the two isozymes and may not accurately reflect the activity of 5 α R2 specifically.

Another possibility for why we did not detect more effects of T treatment is that activity of the enzymes may not be reflected in the mRNA expression as we measured it. Protein levels might be greater with T treatment due to an increase in translation efficiency or decrease in turnover, and not necessarily be reflected in an increase in mRNA levels. It is also possible that although the amount of enzyme may not change with treatment, the activity may still change with different conditions. For example, Pradhan et al. [45] examined steroid hormone metabolism in song sparrows and found that giving exogenous NAD⁺ (a cofactor for the reaction) eliminated the differences that had been detected when the reaction was incubated without exogenous cofactors. Intracellular calcium can also have an effect on aromatase activity [30]. Thus, varying conditions in anoles could have an effect on aromatase activity, and explain why we did not detect treatment differences in mRNA expression.

Finally, our *in situ* hybridization protocol only allowed us to count the number of cells that expressed aromatase or 5 α R2. It may be that treatment affects the amount of mRNA per cell and not the number, or in some cases density, of cells expressing it. For example, silver grain analysis of aromatase mRNA in rats showed differences between intact and castrated animals [67]. Perhaps this technique would reveal additional effects in anoles as well. Thus, the fact that we did not detect differences in the number of cells expressing mRNA for aromatase or 5 α R2 does not necessarily indicate that T does not upregulate the expression or activity of these enzymes in the POA, AMY, and VMH. More work is required before we can firmly draw such a conclusion. However, the present results provide an important step in quantifying the cells expressing both aromatase and 5 α R2 in the reptilian brain under a variety of conditions influencing reproductive behavior.

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Abbreviations used

5 α R 5 α -reductase

5αR1	5 α -reductase type 1
5αR2	5 α -reductase type 2
AMY	ventromedial nucleus of the amygdala
BS	breeding season
DHT	5 α -dihydrotestosterone
E2	estradiol
NBS	non-breeding season
POA	preoptic area
T	Testosterone
VMH	ventromedial hypothalamus

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- We examined aromatase and 5 α -reductase 2 expression in the anole lizard brain.
- Testosterone has no effect on aromatase mRNA.
- Testosterone increases 5 α -reductase 2 mRNA in the female ventromedial hypothalamus.
- Testosterone decreases 5 α -reductase 2 mRNA in the male ventromedial hypothalamus.
- We conclude that testosterone regulates 5 α -reductase 2 expression in anoles.

Figure 1.

Density of aromatase expressing cells in the POA (a, b, c) and AMY (d, e, f). Females had a greater density than males in the POA, regardless of season or treatment (a). Females also had a greater density of these cells in the AMY (d). Males are depicted in (b) and (e); females are depicted in (c) and (f). All animals are non-breeding and blank-treated. Scale bar = 50 μ m. Final sample sizes are indicated in panels (a) and (d).

Figure 2.

Density of 5αR2 expressing cells in the POA. The photos depict cells in a breeding male (a) and female (b) both T-treated. Females had a greater density of 5αR2 expressing cells in the POA than males, regardless of season or treatment (c). Scale bar = 50 μm. Final sample sizes are indicated in panel (c).

Figure 3.

5αR2 expression in the VMH. The number of 5αR2 expressing cells was greater in males than females (a). The density of these cells was greater T- than blank-treated females, and greater in blank- than T-treated BS males (b). The photos depict T- (c, e) and blank-treated (d, f) animals. Males are shown in (c) and (d), and females in (e) and (f). Note the lower density of 5αR2 cells in the T-treated male (c) and blank-treated females (f) as compared to the rest of the photos. All photos were taken from BS animals. Scale bar = 50 μm. Bl = blank. Final sample sizes are indicated in (a).

Table 1

Volumes of the POA, AMY, and lateral VMH as defined by aromatase (A) and 5 α R2 (B) expression ($\mu\text{m}^3 \times 10^7$).

		POA	AMY	VMH
A. Aromatase	M:	1.97 (0.079)*	2.21 (0.118)	BS: 2.50 (0.135)* NBS: 3.01 (0.190)
	F:	1.38 (0.034)	1.91 (0.081)	BS: 2.08 (0.154) NBS: 2.57 (0.249)
B. 5 α R2	M:	1.95 (0.085)*	2.18 (0.084)	2.67 (0.102)*
	F:	1.47 (0.062)	2.13 (0.131)	1.98 (0.076)

All values are means (S.E.). For both genes, males had a greater volume of the POA and lateral VMH than females, as indicated by (*). No sex difference existed in the AMY. Volume of the lateral VMH volume, as defined by aromatase expression, was greater in the NBS than BS in both males and females. Because no seasonal differences existed in the volumes of the POA and AMY, values were averaged across season. M = male, F = female.

Table 2

Comparison of aromatase (A) and 5 α R2 (B) expression patterns in the POA, AMY, and VMH in intact (data from [10, 12]) and gonadectomized T-treated animals (current study).

A. Aromatase			
	POA	AMY	VMH
Intact ¹	Number: M > F	Number: no effects	Number: no effects
	Density: BS > NBS	Density: F > M	Density: F > M
	Volume: M > F	Volume: no effects	Volume: M > F
Treated ²	Number: no effects	Number: no effects	Number: no effects
	Density: F > M	Density: F > M;	Density: no effects
	Volume: M > F	Volume: no effects	Volume: M > F, NBS > BS
B. 5αR2			
	POA	AMY	VMH
Intact ³	Number: no effects	Number: no effects	Number: no effects
	Density: no effects	Density F > M	Density: no effects
	Volume: M > F	Volume: M > F	Volume: M > F
Treated ²	Number: no effects	Number: no effects	Number: M > F
	Density: F > M	Density: no effects	Density: F: T > Bl; BS M: Bl > T
	Volume: M > F	Volume: no effects	Volume: M > F

Patterns detected in the volumes of these regions based on these markers are also included. The animals from each of the three studies were collected from the field at the same time, but the tissue was processed and analyzed separately. Bl = blank treatment; F = female; M = male; T = T treatment.

¹Data from [12]

²Data from present study

³Data from [10]