STEROID METABOLISM IN THE BRAIN OF GREEN ANOLE LIZARDS (*ANOLIS CAROLINENSIS*): EFFECTS OF SEX, SEASON AND TESTOSTERONE ON AROMATASE AND 5α-REDUCTASE

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ABSTRACT

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Steroid hormones are critical for proper brain development, as well as the expression of sexual behaviors in adulthood. In the brain, testosterone (T) can be metabolized into estradiol via the action of the aromatase enzyme, or 5α-dihydrotestosterone via the action of the 5α-reductase (5αR) enzyme. Aromatase and 5αR are critical for sexual behaviors in a variety of species, including the green anole lizard. In this dissertation, I began to evaluate the hypothesis that T regulates its own metabolism in the brain of green anoles. I tested this idea using two types of experiments: comparisons across groups of animals under naturally varying T levels and gonadectomized animals with or without hormone replacement. In Chapter 1, I examined the whole brain activity of aromatase and 5αR in gonadectomized males and females that were treated with either a T- or blank-capsule. In Chapters 2 and 3, I cloned the anole-specific aromatase and both isozymes of 5αR (5αR1 and 5αR2) and examined their expression in the brain. In Chapter 4, I described the expression of aromatase and 5αR2 in the forebrain of gonadectomized males and females that were treated with either a blank or T-filled capsule. Finally, in Chapter 5, I examined aromatase, 5αR1, and 5αR2 expression at two different stages in development. This body of work has expanded knowledge on steroid metabolizing enzymes to a reptilian species, and begun to address the idea that T can influence its own metabolism in multiple vertebrate taxa.

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Chapter 1: Introduction

Hormonal regulation of sexually dimorphic behaviors

In many species, male and female animals have very different behavioral repertoires. Specifically, reproductive behaviors differ between the sexes, including courtship and copulatory behaviors in males, and proceptive and receptive behaviors in females. Gonadal steroid hormones are key factors in controlling these sexually dimorphic functions. For example, estradiol (E2) and progesterone are important for the expression of female receptive and proceptive behaviors in many different species (Ball and Balthazart, 2002; Fabre-Nys and Martin, 1991; Steel, 1981; Tennent et al., 1980). In male birds and rodents, testosterone (T) and its metabolites, E2 and 5α-dihydrotestosterone (DHT), are important for the expression of male courtship and copulatory behaviors (Fusani, 2008; Hull and Dominguez, 2007). DHT has a higher affinity for androgen receptor (AR) than does T, and thus is generally considered a more potent androgen (Negri-Cesi et al., 1996).

Hormonal effects that elicit sexual behavior in adults are classified as 'activational', while those that induce permanent changes in the capacity to display sexually dimorphic behaviors during development are considered 'organizational' (Arnold and Breedlove, 1985). Activational roles of hormones are perhaps most clearly demonstrated in animals that breed seasonally. It is often the case that hormones that produce sexual behavior during the breeding season (BS) do not activate sexual behavior and are at lower concentrations in plasma during the non-breeding season (NBS; Fitzgerald et al., 2000; Hau, 2001). Not only do T and its metabolites play important roles in the regulation of adult sexually dimorphic behaviors, they are

also very important in the early organization of these behaviors. For example, treating female guinea pigs and rats with T during development masculinizes their adult behaviors (Phoenix et al., 1959; Wolf et al., 2002). Thus, hormones play a pivotal role in the production of sexual behaviors, both during development and after sexual maturation.

Brain regions involved in sexual behaviors

Brain regions that are particularly important in the control of sexual behaviors include the preoptic area (POA), ventromedial hypothalamus (VMH), and amygdala (AMY). The POA has been implicated in the control of male sexual behavior in a variety of vertebrates, including ferrets, Japanese quail, lizards, and European starlings (Balthazart and Surlemont, 1990; Crews and Moore, 2005; Riters et al., 2000; Tobet et al., 1986). Additionally, portions of this area are larger in males than in females, and T treatment during development in rats and during adulthood in Japanese quail can increase the volume of the region in both sexes (Gorski et al., 1978; Panzica et al., 1987). The VMH is critical for the display of female receptivity, and females of some species, including rats and whiptail lizards, have a larger VMH than males (Emery and Moss, 1984; La Vaque and Rodgers, 1975; Wade and Crews, 1992). Estrogen implants into the VMH produce estrous behaviors in female rats, and lesioning this area decreases sexual behaviors (Emery and Moss, 1984; Rubin and Barfield, 1980). The AMY is involved in the control of male sexual behaviors in both rodents and birds; lesioning or inhibiting E2 synthesis in the region decreases these behaviors (Huddleston et al., 2006; Kostarczyk, 1986; Thompson et al., 1998). Although each of these brain regions have been implicated in controlling male- or female-specific sexual behaviors, both male and female animals have these regions at least in part because each of them is important for various other functions that are not necessarily

sexually dimorphic (for example, the POA is involved in thermoregulation, VMH in regulation of feeding, and AMY in emotional learning; King, 2006; McGaugh, 2004; Morrison et al., 2008).

Neural steroid metabolism

In the brains of both male and female animals, T is commonly metabolized into E2 via the action of the aromatase enzyme, or into DHT by the 5α-reductase enzyme (5αR). The synthesis of DHT and E2 in the brain is important for male-specific behaviors in species including Japanese quail, hamsters, rats, and guinea pigs (Balthazart, 1991; Hull and Dominguez, 2007; Romeo et al., 2001). Both of these enzymes play important roles in the activation and organization of sexual behaviors.

Aromatase in adults

Aromatase is a member of the cytochrome P450 family and converts T into E2 in a variety of tissues, including ovaries, testes, placenta and adipose, as well as the brain (reviewed in Lephart, 1996). Male sexual behaviors in many mammals are influenced by neural aromatase (Roselli, 2007). For example, male aromatase knock-out mice do not display complete sexual behavior as adults unless given estrogens (Bakker et al., 2004). Similarly, aromatase inhibitors block male sexual behavior in Japanese quail (Balthazart, 1991). In songbirds, estrogens and androgens that can be aromatized are important for song production (Balthazart, 1991). Aromatase also plays a role in female sexual behavior. For example, copulatory behavior in female musk shrews is induced by aromatizable androgens but not DHT, which suggests that aromatase in the brain may be controlling female sex behavior (Rissman, 1991). Also, female canaries injected with aromatase inhibitors showed decreased sex behavior (Leboucher et al.,

1998). Thus, aromatase is important for sexual behaviors in a wide variety of both male and female animals.

Aromatase has been found in the brains of all vertebrate species studied to date, including lampreys, teleost fishes, reptiles, birds, and mammals (Callard, 1977; Forlano et al., 2006). In general, relatively high levels of the enzyme are detected in the hypothalamus and limbic regions of the brain in rats (Roselli et al., 1985; Wagner and Morrell, 1996), monkeys (Roselli and Resko, 2001), whiptail lizards (Dias et al., 2009), and opossums (Fadem et al., 1993). In birds, aromatase is also found in the hypothalamus and limbic system; however, songbirds also have high levels of aromatase in portions of the telencephalon that are analogous to the mammalian cortex (Silverin et al., 2000). There are differences in aromatase expression between males and females such that males of some species have higher aromatase activity in particular regions of the brain (Balthazart et al., 1990; Negri-Cesi et al., 1996; Roselli et al., 1985). In rodents, this sex difference appears to be due to a higher number of cells in the male brain that express aromatase (Balthazart, 1991; Negri-Cesi et al., 1996; Wagner and Morrell, 1996). In zebra finches, adult males have higher aromatase expression than females at synapses in the posterior telencephalon (Rohmann et al., 2007).

Aromatase activity and expression are commonly regulated by T availability. For example, T treatment of castrated males results in increased numbers of cells expressing the protein in specific brain regions in birds such as Japanese quail, zebra finches, and ring doves (Balthazart, 1991; Balthazart and Foidart, 1993; Voigt et al., 2011). T also increases aromatase activity in the hypothalamus of rats, and this increase is greater in males than in females (Balthazart et al., 1990; Roselli et al., 1996; Roselli and Resko, 1984; 1997; Zhao et al., 2008). T treatment can also influence aromatase in the female brain. For example, it upregulates

aromatase mRNA in the POA and sonic motor nucleus of female midshipman fish (Forlano and Bass, 2005) and treatment increases aromatase mRNA and activity in the caudomedial neostriatum in female canaries, an area involved in song perception (Fusani et al., 2001). Additional studies support the idea of androgenic regulation of the aromatase enzyme. For example, in musk shrews, aromatase is extensively co-localized in the hypothalamus with AR and not with estrogen receptor (Veney and Rissman, 1998; 2000a). In addition, aromatase activity in the hypothalamus is decreased in the male rat brain following administration of an AR antagonist, which would suggest that ARs play a role in regulating aromatase (Roselli and Resko, 1984).

5α-Reductase in adults

5αR has been far less studied than aromatase. Two isozymes of 5αR exist: 5αR1 and 5αR2 (Lephart et al., 2001). In humans, mice and rats, 5αR1 is expressed primarily throughout the brain, whereas 5αR2 is found in very low concentrations in the adult brain, and expression is greater overall for both isozymes in the brainstem (Celotti et al., 1997). 5αR1 has a low affinity for T and is present in both neurons and glial cells (Negri-Cesi et al., 2008). In contrast, 5αR2 has a high affinity for T and is found in hypothalamic and hippocampal neurons in the adult brain (Poletti and Martini, 1999). Thus, these two isozymes may play different roles in the adult brain.

Unlike aromatase, the distribution and activity of 5αR does not appear to differ between the sexes. In the mammalian species studied so far, 5αR activity does not differ between males and females, and is not regulated by steroid hormones (Negri-Cesi et al., 1996). In addition, 5αR activity in male and female Japanese quail does not differ in specific brain nuclei, including the

avian equivalent of the AMY (nucleus taeniae), POA, and VMH (Balthazart et al., 1990). Although T treatment does not seem to affect overall 5αR activity (Negri-Cesi et al., 1996), one study suggested that T treatment increases 5αR2 activity and does not affect 5αR1 (Torres and Ortega, 2003).

As DHT acts at androgen receptors (AR), one should expect some degree of co-localization of AR with 5αR. Very few studies have examined this issue, although 5αR2 was found in LHRH-secreting neurons that also contain AR in the rat brain (Poletti and Martini, 1999). In addition to acting at ARs, DHT can be further metabolized into 5α-androstane-3β, 17β-diol, which can act at estrogen receptor (ER) β (Handa et al., 2008). Thus DHT production may potentially be influenced by both ARs and ERs.

Aromatase in development

Aromatase plays various roles in sexual differentiation during development in many vertebrate species. Teleost fish have two isoforms of aromatase, one expressed in the brain and one in the ovary (Esterhuyse et al., 2008). However, in teleost species studied to date, aromatase is not expressed in a sexually dimorphic manner in the brain during development (Blaquez et al., 2008; Esterhuyse et al., 2008; Kallivretaki et al., 2007; Patil and Gunasekera, 2008). Similarly, no sex differences have been detected in aromatase expression in developing South African clawed frogs (Urbatzka et al., 2007). In these species, aromatase seems to be more important for gonadal than brain differentiation.

Similarly, in those reptilian species for which aromatase has been studied during development, the enzyme has been investigated more in gonadal than neural differentiation. For example, in the reptiles that undergo temperature-dependent sexual differentiation (turtles,

crocodilians, and some lizards), increased aromatase activity in the gonads during development results in ovarian differentiation, and inhibiting aromatase activity results in the production of testes (Belaid et al., 2001; Lance, 2009; Pieau et al., 1999; Wibbels and Crews, 1994). Aromatase also regulates gonadal differentiation in lizards with genetic sex determination; inhibition of aromatase activity produces testicular development in females (Ganesh et al., 1999; Wennstrom and Crews, 1995). The few studies that exist on aromatase in the developing reptile brain were conducted in species with temperature dependent sexual determination. Aromatase is expressed in the developing brain of alligators, leopard geckos, and red-eared slider turtles, and appears sexually monomorphic (Crews et al., 2001; Endo et al., 2008; Milnes et al., 2002).

Aromatase has also been studied during development in birds. As in reptiles, administration of aromatase inhibitors to developing eggs causes sex reversed gonads in genetically female chicken and zebra finch hatchlings (Elbrecht and Smith, 1992; Gong et al., 1999; Holloway and Clayton, 2001; Vaillant et al., 2001; Wade, 2001). E2 administration can masculinize parts of the female zebra finch song system, suggesting that neural estrogens might play a role in masculinization of the brain (Bender and Veney, 2008; Foidart and Balthazart, 1995; Holloway and Clayton, 2001; Tang and Wade, 2009). However, much of the data on the role of neural aromatase during development has been inconclusive in zebra finches (reviewed in Wade, 2001). In Japanese quail, aromatase expression in the hypothalamus and prosencephalon varies with age, T increases aromatase activity, and both of these are not sexually dimorphic (Aste et al., 2008; Schumacher and Hutchison, 1986). T treatment also increases the number of cells expressing aromatase in newly hatched Japanese quail (Bardet et al., 2010).

In mammals, the role of aromatase has been extensively studied during development. Estrogens play key roles in both masculinizing and defeminizing the brain (Bakker et al., 2006).

A protein called α-fetoprotein binds estrogens in the plasma, which stops estrogens produced in the gonads from entering the brain (Bakker et al., 2006). The testes of males, however, produce T during critical periods. This hormone is not bound by α-fetoprotein. Thus, T can enter the male brain and be aromatized in particular brain regions into E2. Aromatase activity and mRNA expression is higher in developing male brains than female brains in mice and rats (Hutchison et al., 1994; Hutchison et al., 1997; Hutchison et al., 1999). E2 synthesized in the brain is important for masculinization, and males have more E2 production because they have higher circulating T (as substrate) and more neural aromatase. However, aromatase most likely plays a role in females as well as males. Studies of female aromatase knockout mice showed that females need E2 during development in order to display normal female sexual behavior (Bakker et al., 2003). Aromatase inhibition during development decreases female attractiveness to males as adults in prairie voles (Northcutt and Lonstein, 2008). Furthermore, exposing female rat fetuses and newborns to androgens can masculinize adult aromatase expression in the brain (Roselli and Klosterman, 1998). Androgens can also regulate aromatase expression and activity during development such that T treatments increase both expression and activity in male and female rodents (Hutchison et al., 1997; Hutchison et al., 1996; Romeo et al., 1999). Thus, aromatase plays a crucial role in the development of most vertebrate species studied.

5α-Reductase in development

Similar to the situation in adults, 5αR is much less studied during development than is aromatase. In those organisms in which it has been studied, the two different isozymes of 5αR are expressed differently in the brain during development, such that 5αR1 mRNA levels are higher than 5αR2 levels and consistent across ages, whereas 5αR2 levels show a peak early in

development, and then decrease steadily to low adult levels (Poletti et al., 1998; Urbatzka et al., 2007). Because the expression of the two isozymes differs, it is potentially important to examine where in the brain they are acting during development. For example, because of their expression patterns in the brain, 5αR2 may be important for sexual differentiation and 5αR1 might not.

In general, no sex differences in the brain during development have been detected in the expression of the two isozymes or overall 5αR activity in various species, including South African clawed frogs, Japanese quail, mice, and rats (Hutchison and Schumacher, 1986; Jacobson et al., 1997; Karolczak et al., 1998; Urbatzka et al., 2007). Interestingly, T regulates the two isozymes of 5αR differently between the sexes. 5αR1 expression is not regulated by T in the whole brain or hypothalamus of either male and female newborn rats or mice (Karolczak et al., 1998; Poletti et al., 1998). On the other hand, 5αR2 expression is regulated by T only in male rats, such that treatment with T increases 5αR2 in the hypothalamus and treatment with an AR antagonist decreases 5αR2 (Poletti et al., 1998). Neither of these treatments affects 5αR2 expression in female rodents. It is possible that 5αR plays an important role in proper male development. For example, by inhibiting 5αR in juvenile male zebra finches, the brain areas that control song are smaller than those of controls (Grisham et al., 1997). Additionally, DHT treatment in hatchling female zebra finches can induce some modest masculinization of the song system (Schlinger and Arnold, 1991). In male rats, those that were treated with a 5αR inhibitor during development showed higher levels of female sexual behavior than control animals (Ribeiro and Pereira, 2005). However, aromatase tends to play a larger role than 5αR in sexual differentiation of brain and behavior. One hypothesis to explain the roles of the two isozymes is that 5αR1 (which exhibits widespread neural expression) serves to protect the brain from excess steroid hormones (Melcangi et al., 1998). 5αR2, on the other hand, may be involved in the

sexual differentiation of the male nervous system, which is suggested by the high levels found in the brain during a very brief period of development and the fact that T regulates expression in males.

Green Anole Lizards

Green anole lizards (*Anolis carolinensis*) are found throughout the southeastern United States in forested habitats. They are sexually dimorphic such that males are larger than females. Males have a large, red throat fan (dewlap), which they use to court females and in territorial defense. Even controlling for body size, this structure is far smaller in females, who use it rarely (Lovern et al., 2004). These lizards are seasonal breeders, and the breeding season (BS) lasts from April to August. Females lay one egg (from alternating ovaries) about every 7 to 10 days and may mate multiple times throughout the season (Lovern and Wade, 2003). Juveniles become sexually mature by the next BS. Once the BS is over, gonads in both sexes regress and these animals enter a refractory period during which time they cannot produce sexual behavior, and their gonads cannot be stimulated to recrudesce.

During the BS, male anoles defend large territories that overlap the territories of several females (Greenberg and Noble, 1944). Males have distinct courtship displays, which consist of extending the dewlap, headbobs, and pushups (Orrell and Jensson, 2003). If a female is sexually receptive, she will allow the male to mount, and he will grip her neck and insert one of his two bilateral hemipenes into her cloaca. If the female is not receptive, she will run to escape (Greenberg and Noble, 1944).

As in other vertebrates, specific limbic brain regions are important for male- and female-specific reproductive behaviors in the green anole. The POA is important for green anole male

sexual behaviors such that if this area is lesioned, these behaviors are absent (Wheeler and Crews, 1978). Similarly, if the AMY is lesioned, courtship displays are decreased and neck grips are not achieved (Greenberg et al., 1984). Lesion studies have not been conducted in the VMH in green anoles, but in other lizards, female sexual behaviors are absent after destruction of the VMH (Kendrick et al., 1995). Because these three regions play such an important role in reproductive behaviors in anoles, it is interesting that the cytoarchitecture of these brain regions differs among the sexes and seasons. For example, the POA is larger in males than females, and is larger in the BS than NBS (Beck et al., 2008). In addition, the VMH is larger in breeding animals, regardless of sex, and the AMY has more cells during the NBS. Thus these brain regions are influenced by different seasonal conditions and show plasticity in adulthood.

As in other vertebrates, male green anoles have much higher levels of plasma T than females, and breeding males have much higher levels of T than non-breeding males (Lovern et al., 2001). Although females have less T than males, breeding females do have higher plasma T levels than do non-breeding females. Somewhat surprisingly, during the BS, females have higher levels of AR mRNA than males in brain nuclei that control reproductive behaviors (POA, AMY; Rosen et al., 2002). ERα mRNA is also higher in females in the POA and the VMH, although there were no sex differences detected in the AMY and there were no effects of season in any of these three limbic regions (Beck and Wade, 2009c).

Whole brain 5αR activity is greater than aromatase activity in adults of both sexes (Wade, 1997). However, aromatase activity is highest in the hypothalamus and 5αR activity is highest in the brainstem (Wade, 1997). Brains of breeding males have higher levels of aromatase activity than either breeding females or non-breeding males, and there is no difference in aromatase activity between males and females during the NBS (Rosen and Wade, 2001). In

contrast, whole brain 5αR activity appears not to differ between the sexes or the seasons in gonadally intact adult green anoles.

Similar to other species, aromatase and 5αR play roles in anole sexual behavior. In males, aromatase inhibitors do not affect the displays of males, although the production of DHT is necessary for the full potential of these behaviors (Rosen and Wade, 2000; Winkler and Wade, 1998). Castrated males and those castrates treated with E2 do not engage in sexual activity, while those treated with T do (Crews, 1980; Mason and Adkins, 1976). Sexual behaviors in adult male green anoles are primarily regulated by T itself, although recent evidences suggests that E2 increases sexual motivation (Latham and Wade, 2010). In addition, castrated males treated with T, E2, or DHT directly in the POA can display normal sexual behavior, suggesting that direct application of high enough doses can facilitate male sexual behavior (Crews and Morgentaler, 1979). In females, E2 in the plasma is at moderate levels during periods of sexual receptivity, but increases during non-receptive periods, perhaps to control ovulation and oviposition (Jones et al., 1983). Receptivity in gonadectomized females is increased by E2, E2 + progesterone, or T, and is decreased by antiestrogen treatments (Crews, 1980; Mason and Adkins, 1976; Tokarz and Crews, 1980). Additionally, inhibition of aromatase activity in T-treated females reduces receptivity, suggesting that neural E2 synthesis is important for this behavior (Winkler and Wade, 1998).

Conclusion

This dissertation aims to further explore the expression and regulation of T-metabolizing enzymes in the forebrain of green anole lizards. Specifically, I test the hypothesis that T

modulates aromatase and/or 5αR in the green anole brain, and that this occurs locally in brain regions important for the display of male and female sexual behaviors. The first four studies address this question in adults, and the last investigates developing anoles. The experiment in Chapter 1 directly investigates whether T induces changes in whole brain aromatase and 5αR activity, and whether it does so differently between the sexes and seasons. This study follows directly from work described above documenting enzyme activity between groups of animals naturally experiencing differences in circulating T (males and females; BS and NBS males). The remaining chapters follow a similar approach but specifically investigate expression of the enzymes in the POA and AMY (critical for male sexual behavior) and VMH (female sexual behavior). Chapter 2 (aromatase) and Chapter 3 (5αR1 and 2) evaluates untreated adults of both sexes and season, and, in Chapter 4, T is experimentally manipulated to determine both its specific effects and whether sensitivity to the hormone differs between the sexes and seasons. In Chapter 5, the brain regions are investigated in males and females at a time when circulating T is equivalent (day of hatching) and after it has increased substantially in males only (day 50). This last study will provide the first steps in determining whether aromatase and 5αR may play roles in anole sexual differentiation, as the enzymes have not yet been investigated in the brains of developing anoles. Overall, my work will expand knowledge of the regulation of T metabolism to a reptilian species.

Chapter 2: Testosterone selectively affects aromatase and 5α-reductase activities in the green anole lizard brain.

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 5α-reductase (5αR) converts T to 5α-dihydrotestosterone (DHT). Male green anole reproduction depends on androgens, yet 5αR 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 5αR 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 5αR 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. 5αR can be mediated by T availability, but this regulation may not serve a sex- or season-specific purpose. *Key Words: Steroid hormone, steroid metabolism, sexual dimorphism, seasonal difference*

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 5α-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 (Bakker et al., 2004; Schlinger, 1997b; Timonin and Wynne-Edwards, 2008). Aromatase levels in the brain are often sexually dimorphic, with male individuals having increased activity compared to females in species including rats, Japanese quail, gray short-tailed opossums, zebrafish and European sea bass (Balthazart, 1991; Fadem et al., 1993; Forlano et al., 2006; Roselli and Resko, 1997). 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 5α-reductase (5αR) 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). 5α-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 Balthazart, 1991; Balthazart et al., 1990; Celotti et al., 1997). 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, 5αR is generally not affected by T

administration (reviewed in (Balthazart et al., 1990; Negri-Cesi et al., 1996). However, one of the two isozymes of 5αR (5αR2) present in the brain shows an increase in mRNA with T treatment (Negri-Cesi et al., 2008; Torres and Ortega, 2003).

Aromatase and 5αR 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, 5αR 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 (Crews, 1980; Lovern et al., 2001). Anoles have distinct male courtship displays consisting of head-bobbing, pushups, and repeated extension of a red throat fan, or dewlap (Orrell and Jensson, 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, 5αR 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 5αR 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.

Methods

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-gallon 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 ºC warmer than ambient. During the BS, the animals were kept on a 14:10 light/ dark cycle with temperatures ranging from 28 ºC during the day to 19 ºC at night. During the NBS the animals were kept on a 10:14 light/ dark cycle with room temperatures ranging from 24 ºC during the day to 15 ºC 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.

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-proprionate 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, 2007b). 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 ºC until processed.

Enzyme Assay

Aromatase and 5αR activities were determined using previously validated procedures (Rosen and Wade, 2001; Wade, 1997). Briefly, brains were thawed and homogenized in sucrose phosphate buffer. Homogenates were incubated in duplicate with a saturating concentration of 3H-T (80.4 μCi/ml) and cofactors at 27°C for 50 minutes, a time point at which detection of both 3H-E2 and 3H-DHT are 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. 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 5α-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 (PerkinElmer and Analytical Sciences, Shelton, CT) on a Beckman LS 6500. A Bradford assay (BioRad 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; 5α-DHT production indicates 5αR 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.

Statistical Analysis

Separate two-way ANOVAs were conducted for aromatase and 5αR 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).

Results

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 ($F < 0.01$, $p = 0.997$). Similarly, the effects of season ($F = 1.03$, $p = 0.325$), treatment ($F = 2.02$, $p = 0.175$) and their interaction ($F = 1.01$, $p = 0.330$) on 5αR activity in the female brain were not statistically significant (Fig. 1b).

Males

In assay 2 on the first set of male brains (data not shown), a main effect of treatment existed; T increased 5αR activity ($F = 5.34$, $p = 0.035$). There was no effect of season ($F = 1.57$,

p = 0.228) and no interaction (F = 0.02, 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), 5αR activity continued to be influenced by treatment (F = 4.18, p = 0.049), but not season (F = 1.23, p = 0.274); T treatment resulted in higher levels of 5α-DHT synthesis than blank treatments (Fig. 2). No interaction between treatment and season was detected (F = 0.13, p = 0.719). The aromatase data with increased sample sizes were also parallel to 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).

Discussion

The present results indicate that T specifically increases the activity of both aromatase and 5αR 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.

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 the 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 Introduction). 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, 1997a). 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, 2007b), 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 (Balthazart et al., 1998; Gelinas and Callard, 1997; Veney and Rissman, 2000a).

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).

5α-reductase

The increased levels of 5αR activity in T-treated males regardless of the season (BS or NBS) was somewhat unexpected. Previous work on 5αR in anoles indicated no differences due

to season or sex (Rosen and Wade, 2001). In parallel, mammalian 5αR activity does not differ between the sexes, and seasonal differences are not detected in song sparrows (reviewed in (Negri-Cesi 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 5αR in the brain. However, it is clear that some species have two isozymes that catalyze the conversion of T to DHT, 5αR1 and 5αR2, and they can be differentially regulated. In both male and female adult rats, for example, 5αR1 is not affected by T treatment, whereas 5αR2 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 5αR2 activity or that 5αR1 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 5αR activity in either BS or NBS. This result parallels the idea that the enzyme has some role in male sexual behavior (see Introduction), whereas no evidence exists for it influencing female receptivity in this species. One possibility is that females may have lower levels of 5αR2 than males. Because 5αR1 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 5αR activity in both sexes, in treated and non-treated individuals, and during BS and NBS. 5αR 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, 5αR may play a role in regulating the effects of progesterone on female reproductive behavior.

Conclusion

T appears to regulate its own metabolism in male, but not female, anoles. 5αR 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 5αR 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 5αR 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.

Acknowledgements

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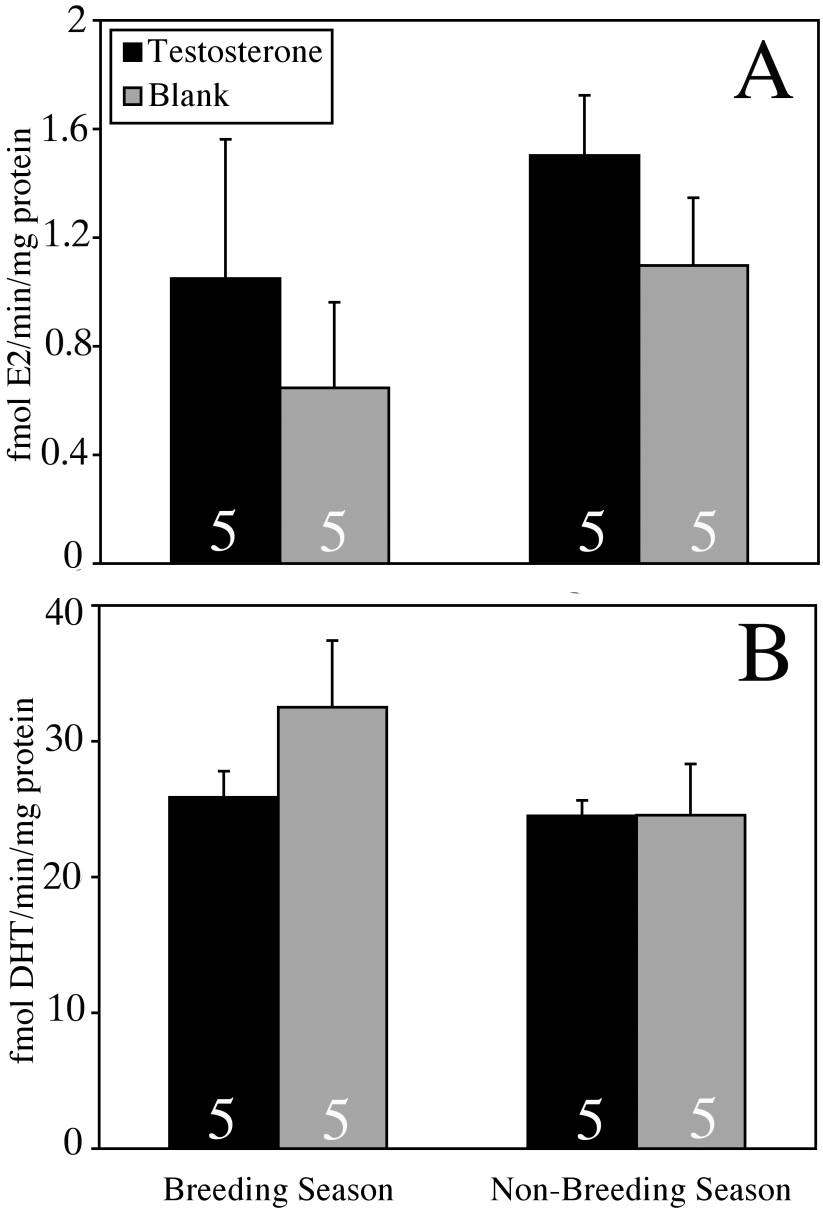

Figure 1: Enzyme activity in manipulated females during the breeding and non-breeding seasons (means + S.E.). Aromatase activity is indicated in (A) and 5α-reductase activity in (B). Testosterone refers to gonadectomized + T treated individuals while Blank refers to gonadectomized + blank treated individuals.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

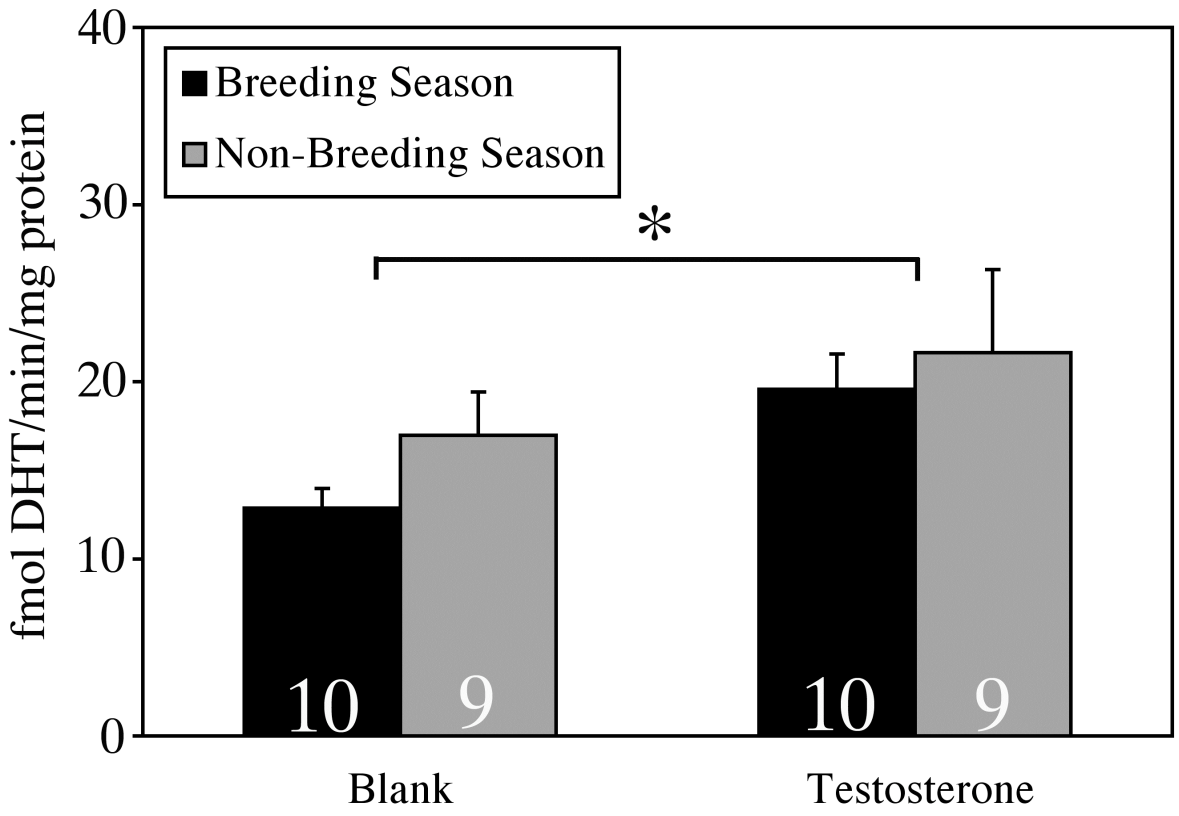

Figure 2: 5α-reductase activity in manipulated males during breeding and non-breeding seasons (means + S.E.); data combined from two assays. Testosterone refers to gonadectomized + T treated individuals while Blank refers to gonadectomized + blank treated individuals. Testosterone treatment resulted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

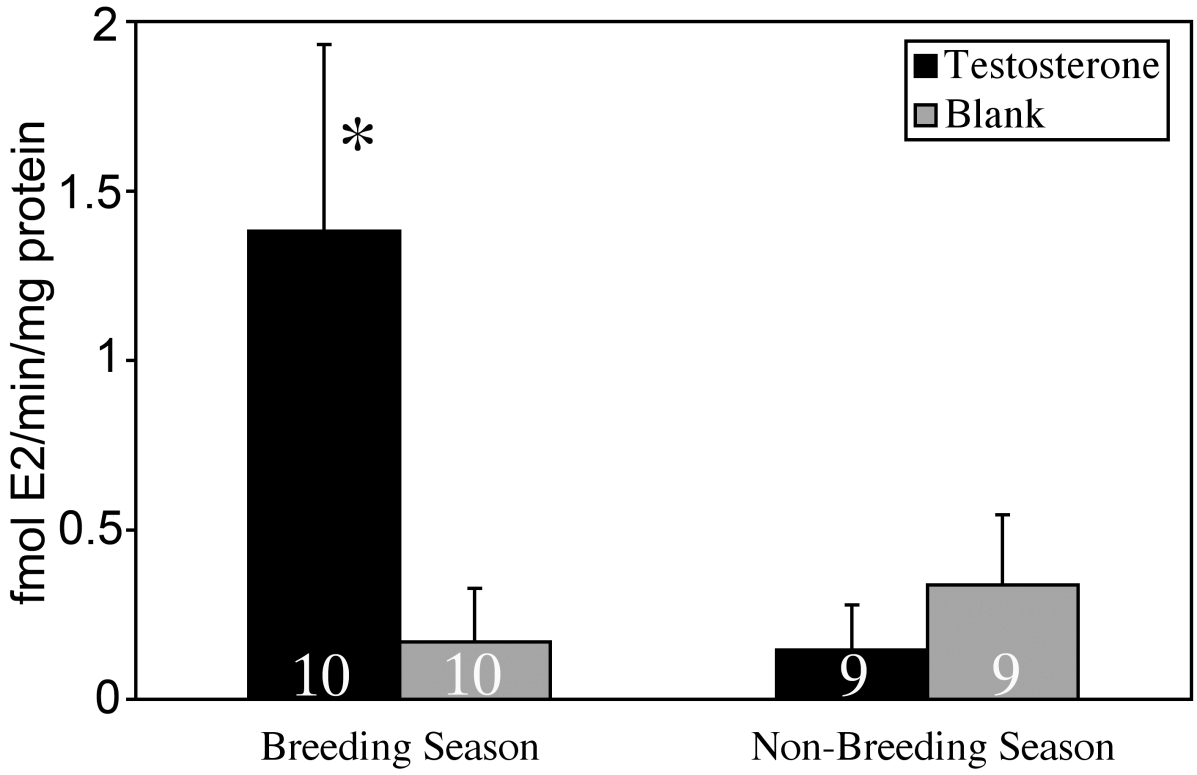

Figure 3: Aromatase activity in manipulated males during the breeding and non-breeding seasons (means + S.E.); data combined from two assays. Testosterone refers to gonadectomized + T treated individuals while Blank refers to gonadectomized + blank treated individuals. Testosterone treatment increased activity in the breeding season only.

Chapter 3: Aromatase mRNA in the brain of adult green anole lizards: effects of sex and season

Abstract

Neural testosterone (T) metabolism, particularly the synthesis of estradiol (E2) via the aromatase enzyme, is important for sexual behaviors in many vertebrates. In green anole lizards, E2 metabolized from T facilitates female receptivity and increases sexual motivation in males. T treatment increases aromatase activity in whole brain homogenates of gonadectomized male, but not female, anoles, which is an effect limited to the breeding season (BS). To investigate the potential for local effects of this enzyme in reproductive behavior, we used *in situ* hybridization for aromatase mRNA to examine expression during the BS and non-breeding season (NBS) in areas of the brain that control male sexual behaviors (preoptic area and amygdala; POA and AMY), as well as one regulating female reproductive behaviors (ventromedial hypothalamus, VMH). Males had a greater total number of aromatase-expressing cells in the POA than females, and the density of aromatase-expressing cells (number per unit volume) was greater in the VMH and AMY of females. This density was also higher during the BS than NBS in the POA. Expression of aromatase in the AMY appeared lateralized, as trends were detected for the left side to have more total cells and more cells per unit volume than the right. These results suggest that, similar to other vertebrates, regional aromatization of T may be important for control of sex-specific reproductive behaviors. *Key words: testosterone metabolism, preoptic area, amygdala, ventromedial hypothalamus*

Introduction

Steroid hormones are important for the expression of sexual behaviors in a variety of animals. Specifically, the neural metabolism of testosterone (T) to estradiol (E2) by aromatase regulates expression of these behaviors in vertebrates including Japanese quail, midshipman fish, musk shrews, songbirds, and mice (Bakker et al., 2004; Balthazart and Foidart, 1993; Forlano et al., 2006; Rissman, 1991; Riters et al., 2000). In males, inhibition of aromatase blocks normal copulatory behaviors in Japanese quail, and aromatase knock-out mice do not display complete sexual behavior unless given estrogens (Bakker et al., 2004; Balthazart, 1991). Aromatase is also important for the expression of female behaviors. Inhibitors of this enzyme decrease female canary sex behaviors and aromatizable androgens facilitate copulatory behavior in female musk shrews (Leboucher et al., 1998; Rissman, 1991).

In most species, aromatase is concentrated in limbic regions of the brain, including those important for the expression of male specific sexual behaviors such as the preoptic area (POA) and amygdala (AMY), as well as the ventromedial hypothalamus (VMH), which is important for female receptivity (Balthazart, 1991; Wagner and Morrell, 1996). Aromatase is commonly upregulated by androgens and is found in cells that also express androgen receptor (Balthazart et al., 1998; Gelinas and Callard, 1997; Veney and Rissman, 2000a). Estrogen receptors are commonly located in brain regions that express aromatase, but cellular co-localization varies substantially, suggesting that in at least some areas (such as the POA) autocrine regulation of the enzyme by E2 is unlikely (Balthazart et al., 1996; Veney and Rissman, 1998). Males generally express higher levels of neural aromatase than females (Balthazart et al., 1990; Negri-Cesi et al., 1996; Rohmann et al., 2007; Roselli et al., 1985). A sex difference in responsiveness to T also

exists; the steroid can have a greater effect on aromatase activity and mRNA expression in males than in females (Balthazart et al., 1990; Roselli et al., 1996; Roselli and Resko, 1997). Although expression of aromatase activity has been documented in the reptilian brain (Callard, 1977), less is known about its distribution, especially in regions that control sex behaviors.

Green anole lizards are seasonally breeding animals, with the breeding season (BS) lasting typically from April to August. Males have higher levels of circulating T than females, and both sexes have increased plasma T during the BS compared to the non-breeding season (NBS). For example, males during the BS have on average about 20ng/ml T, whereas the mean value in females is less than 1ng/ml. In both sexes, BS levels of T are at least twice those detected in the NBS (Lovern et al., 2001). The same limbic brain regions are critical to the display of reproductive behaviors in reptiles as mammalian and avian systems. Lesions to the POA and AMY in green anoles impair male sexual behaviors (Greenberg et al., 1984; Wheeler and Crews, 1978). Although the experiment has not been conducted in this species, lesions to the VMH inhibit receptivity in other female reptiles, including whiptail lizards (e.g. Kendrick et al., 1995). Also as in other vertebrates, T is critical for the display of male sexual behaviors, and E2 activates receptivity in anoles (Mason and Adkins, 1976; Tokarz and Crews, 1980). Unlike rodents and birds, aromatase is not critical for the expression of male sexual behaviors in green anoles (Winkler and Wade, 1998). Experiments in gonadectomized anoles, however, have documented facilitation of sexual motivation in males by E2 and a role for aromatization of T in female receptivity (Latham and Wade, 2010; Winkler and Wade, 1998).

Aromatase activity is relatively high in tissue containing the POA and hypothalamus compared to other parts of the anole brain (Wade, 1997). Males have greater whole brain aromatase activity than females, and breeding males have higher aromatase activity than do

males during the NBS (Rosen and Wade, 2001). Exogenous T increases whole brain aromatase activity only in BS males and has no effect in females, which suggests sexually and seasonally dimorphic regulation of this enzyme in the entire brain (Cohen and Wade, 2010b). However, specific distributions and relative patterns of expression of aromatase in areas likely to control reproductive behaviors of males and females are unknown.

This study was designed to begin to test the hypothesis that natural variations in T levels between the sexes and seasons mediate local synthesis of aromatase in the brain. To our knowledge, this is the first study to quantify aromatase expression in three regions controlling sex behavior of reptiles, examining potential sex and seasonal differences in these regions. We investigated numbers of cells containing aromatase mRNA in areas of the brain that control sexual behaviors (POA, AMY and VMH) under conditions when T normally differs.

Methods

Animals and tissue processing

Adult male and female green anole lizards were purchased from Charles Sullivan (Nashville, TN) during the BS and NBS (April and November, respectively). They were wild-caught and shipped within a few days to our animal facilities, where they were housed individually in 10-gallon aquaria that contained peat moss for substrate, rocks, sticks for climbing, and water dishes. During the BS, animals were kept on a 14:10 light/dark cycle and ambient temperatures ranged from 28 ºC during the day to 19 ºC at night. During the NBS, animals were kept at a 10:14 light/dark cycle and ambient temperatures ranged from 24 ºC during the day to 15 ºC at night. Under these conditions, previous studies have determined that

plasma T levels are high during the BS and low during the NBS (See above; Lovern et al., 2001). Full spectrum and heat lamps were provided above each cage to allow lizards to bask in temperatures 10 ºC above ambient. Relative humidity was maintained at approximately 70% throughout both seasons. Cages were misted daily with water and the animals were fed crickets or mealworms three (BS) or two times a week (NBS).

After being in the lab for 15 to 24 days, animals were rapidly decapitated, and the brains were frozen in methylbutane and kept at –80 ºC until processing. At this time, breeding state was confirmed by visual inspection of the reproductive system of each lizard. During the BS, females had thick, convoluted oviducts and at least one yolking follicle, both of which are indicative of high E2 and reproductive activity. All males had large, well-vascularized testes and milky, thick vasa deferentia, consistent with high T and sperm production. During the NBS, females had small oviducts with no or one tiny (< 1 mm in diameter) yolking follicle, and all males had small, non-vascularised testes and thin, clear vasa deferentia.

Brains were sectioned coronally at 20 μm into four alternate series of sections and thaw-mounted onto SuperFrost Plus (Fisher Scientific; Hampton, NH) slides. Slides were stored at –80 ºC with dessicant until further processing. All procedures adhered to NIH guidelines and were approved by the Michigan State University IACUC.

Cloning of aromatase

Whole brain RNA was extracted from two breeding males. Tissue was homogenized in Trizol (Invitrogen Corporation; Carlsbad, CA), and RNA was separated using chloroform. Then, the RNA was isolated using RNeasy minicolumns (Qiagen Sciences; Valencia, CA) and concentrated using ethanol precipitation. It was reconstituted in DEPC-treated water and stored

at –80 °C. RNA was converted into cDNA with the SuperScript III Reverse Transcriptase kit (Invitrogen) per manufacturer's instructions, and stored at –20 °C until use.

Searches of available databases using a variety of relevant key words did not provide a sequence for aromatase in the green anole genome, which is publicly available but not fully annotated. Therefore, we identified an appropriate sequence by blasting the aromatase cDNA sequence from a whiptail lizard, *Cnemidophorus uniparens* (NCBI: EU310875) from Dias et. al. (2009), to the anole genome using the Ensembl Genome Broswer. Primers were designed using the Oligo Analysis Tool program (Eurofins MWG Operon; Huntsvile AL; Table 1) and purchased from Invitrogen. PCR reactions included 1 unit of Platinum TAQ High Fidelity DNA polymerase (Invitrogen), 0.2 mM dNTP mixture, 0.2 μM primer mix, 2 mM $MgSO_4$, and template cDNA. The PCR reaction went through 40 cycles of 94 °C for 15 seconds, 50 °C for 30 seconds, and 68 °C for 1 minute. Aromatase was amplified twice using the same primers, first by using cDNA from brain, and then using the PCR product from the first reaction as the template for the second reaction.

The amplicon was cloned following Zhou and Gomez-Sanchez (2000). Briefly, glycerol stocks of *E. coli* cells containing pBluescript II Exo/Mung DNA (Stratagene; La Jolla, CA) were cultured, and plasmids were isolated using Wizard *Plus* Miniprep kits (Promega Corporation, Madison, WI) per manufacturer's instructions. Vectors were digested overnight at 4 °C with a blunt-end restriction enzyme (EcoRV, New England BioLabs; Ipswich, MA) and purified using the QIAquick PCR Purification Kit (Qiagen Sciences). T-overhangs were added to the blunt-ended plasmid vector using a terminal transferase reaction (Roche Diagnostics; Indianapolis, IN), incubated at 37 °C for 1.5 hours. The T-tailed vector was repurified, and the A-tailed aromatase PCR product was ligated to the vector using T4 DNA ligase as per manufacturer's

instructions (Promega).

One Shot TOP10 Chemically Competent *E. coli* cells (Invitrogen) were transformed with the ligated vector. The cells were grown on LB agar plates containing 100 μg/ml of ampicillin and spread with 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal). White colonies were selected and grown overnight in LB broth containing 100 μg/ml of ampicillin. Vector DNA was isolated using Wizard *Plus* Miniprep kits (Promega), and the sequence of the insert was confirmed in both directions on a Perkin Elmer/Applied Biosystems 3100 capillary sequencer. The 181bp green anole insert (GenBank HM585359) shared 86.7% identity with the whiptail sequence. The insert was also 88% identical to the aromatase sequences of both leopard geckos and Italian wall lizards, and ranged from 78-82% identical to aromatase in a variety of avian and mammalian species. Vector DNA was then isolated using Wizard *Plus* Maxiprep kits (Promega), linearized using restriction enzymes (Xho1 and BamH1; New England BioLabs) and stored at –20 °C.

In situ hybridization

Sense (T7) and antisense (T3) probes were transcribed using the Digoxigenin RNA Labeling Kit per manufacturer's instructions (Roche), which labels RNA with digoxigenin-UTPs. Probes were cleaned using a G50 sephadex bead column prior to use.

All slides were processed at the same time. 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. No labeling was detected in tissue exposed to the sense probes. Slides were allowed to thaw and then fixed for 10 mins in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS; pH 7.4). They were treated with 0.25% acetic anhydrase in

triethanolamine-HCl with 0.9% NaCl buffer (pH 8.0). Next, slides were incubated overnight at 55 °C in hybridization buffer (50% formamide, 4x SSC, 1x Denhardt's solution, 200μg/ml fish sperm DNA, 10% dextran sulfate, 20 mM dithiothreitol, 250 μg/ml tRNA, 2 mM EDTA, and 0.1% Tween-20) with 200 ng/ml probe. The following day, slides were rinsed in 2x SSC and 0.2x SSC and then treated with 0.9% H_2O_2 in maleic acid buffer (pH 7.5) with 0.1% Tween-20 (MABT) for 30 mins. They were incubated in a blocking solution of 5% normal sheep serum (Jackson Immuno Research; West Grove, PA) in MABT for 30 mins, and treated with 0.5 μl/ml Anti-Digoxigenin-AP Fab fragments (Roche Diagnostics; Mannheim, Germany) in MABT. After two hours, slides were treated with 4.5μl/ml NBT and 3.5 μl/ml BCIP (Roche) in 0.1M Tris-Hcl and 0.1M NaCl (pH 9.5) for color detection. The color reaction was stopped after 10 mins with 1M Tris and 0.5M EDTA (pH 8.0).

Aromatase analysis

The slides were examined under brightfield illumination using Stereo Investigator software (MicroBrightfield, Inc.; Williston, VT) following Beck et al. (2008) by a user blind to experimental group. Estimates of aromatase expressing cells were determined using the Optical Fractionator function within the POA, VMH, and AMY. Both the left and right sides of each region were analyzed. After tracing the outline of one side of a brain region in each tissue section in which it existed, the software placed a user-defined grid over each area (POA: 100x100 μm^2, AMY: 40x40 μm^2, VMH: 80x80 μm^2) and sampling sites (30x30 μm^2) were placed randomly within the defined region. The software calculated a volume for the brain region, and computed the number of aromatase-positive cells based on it and the samples in which manual counts were taken. To further characterize the patterns of expression, densities of

aromatase-positive cells were determined by dividing the number of cells by the calculated volumes of each brain region. Final sample sizes are indicated in the figure captions.

Statistical Analysis

Analysis was conducted separately for each brain region. The left and right sides of the POA and VMH did not differ in volume, estimated number of aromatase positive cells, or density of these cells (all $F < 2.13$, $p > 0.155$). Thus, the average of both sides for these two regions was used for statistical analyses reported for these brain regions. Within the POA and VMH, two-way ANOVAs were used to compare main effects of and interactions between sex and season. Interactions were further broken down by one-way ANOVAs and pairwise comparisons as appropriate.

Unlike the POA and VMH, paired t-tests indicated that the left and right sides of the AMY differed in the number of aromatase expressing cells ($F = 5.45$, $p = 0.03$). Thus, we accounted for side in all subsequent analysis by using 3-way mixed model ANOVAs.

Results

We detected aromatase mRNA in a variety of diencephalic and telencephalic areas in the green anole lizard brain, a pattern very similar to the distribution found in whiptail lizards (Dias et al., 2009) and red-sided garter snakes (Krohmer et al., 2002).

POA

Similar to previous work using Nissl-stained tissue from other individuals (Beck et al.,

2008), the POA defined by aromatase positive cells was larger in males than females ($F = 6.848$ $p = 0.014$, data not shown). There was a trend for males to have more aromatase-expressing cells than females ($F = 4.04$, $p = 0.054$; Fig. 4). Effects of season and interactions between sex and season were not detected on the number of aromatase-expressing cells (all $F < 0.95$, $p > 0.339$). The density of aromatase cells was higher during the BS than the NBS ($F = 5.77$, $p = 0.023$; Fig. 4). There was no effect of sex or an interaction for the density of aromatase cells (all $F < 1.47$, $p > 0.236$).

AMY

Parallel to the analysis of Nissl-stained tissue (Beck et al., 2008), volume of this region did not differ among the groups (all $F < 2.73$, $p > 0.116$, data not shown). There also were no effects of sex or season on the number of aromatase-positive cells (all $F < 0.14$, $p > 0.713$, Fig. 5). However, the left side of the AMY had a trend for more aromatase expressing cells than the right side ($F = 4.18$, $p = 0.056$). Females had a higher density of aromatase expressing cells than males ($F = 17.04$, $p = 0.001$, Fig. 5). A trend for greater density of aromatase cells on the left was also detected ($F = 4.19$, $p = 0.056$), as well as a trend for an interaction between side of the brain and season ($F = 3.79$, $p = 0.067$). There were no other effects detected on the density of aromatase expressing cells (all $F < 2.03$, $p > 0.171$).

VMH

Although Nissl stains show cell bodies throughout (Beck et al., 2008), we only detected aromatase in the lateral portion of the VMH. Lesions to this portion of the VMH abolished female sexual behaviors in a different species of lizard and seems likely to be the portion of this

region important in the control of female reproductive behaviors (Kendrick et al., 1995). Thus, we confined our analysis of the VMH to this region.

The lateral VMH was larger in volume in males than females ($F = 4.76$, $p = 0.041$, data not shown). There were no effects detected on number of aromatase cells (all $F < 0.07$, $p > 0.800$; Fig. 6). However, the density of aromatase expressing cells was greater in females than males ($F = 9.24$, $p = 0.006$; Fig. 6). Neither a main effect of season nor an interaction between sex and season were detected on the density of these cells (all $F < 0.35$, $p > 0.563$).

Discussion

This is the first study in anoles and one of the few in reptiles to quantify aromatase expression in three brain regions that are important in the control of sexual behaviors. We document a number of effects of sex and season on aromatase expression in these three areas, with specific patterns differing among them. In the POA, the estimated number of aromatase expressing cells was greater in males than females, and their density was increased in the BS compared to NBS. In the AMY, we found that females had a higher density of aromatase expressing cells than males and detected a trend for more cells with aromatase mRNA on the left side of the brain. Females had a higher density of aromatase expressing cells in the VMH. These results allow us to formulate hypotheses about potential roles and regulation of aromatase within these specific brain regions.

Aromatase in the POA

The increase in aromatase-expressing cells in the POA of males compared to females is

consistent with a variety of other vertebrates. Males have more aromatase expressing cells, as well as more activity, in the POA in musk shrews, zebra fish, guinea pigs, Japanese quail, cowbirds, and rats (Balthazart et al., 1990; Connolly et al., 1990; Goto-Kazeto et al., 2004; Roselli et al., 1985; Saldanha and Schlinger, 1997; Veney and Rissman, 2000a). Additionally, parthenogenic whiptail lizards show increased aromatase mRNA in the POA after ovulation, when male-like sexual behaviors occur in these genetic females (Dias et al., 2009). Collectively, the data suggest that higher aromatase expression in the POA in males may be important for sex-specific behaviors. In green anoles, this function is likely focused on motivation to display reproductive behaviors, as E2 appears mainly to increase mount attempts (Latham and Wade, 2010).

Consistent with a role of the POA in male sexual behavior, the volume of this region is sexually dimorphic, determined both from borders defined with Nissl-staining and labeling of aromatase mRNA (see above). While there were no seasonal differences in the volume of the region or number of cells, we did detect an effect of season on the density of aromatase cells. This increased density of aromatase-expressing cells in the BS compared to NBS is probably due to subtle differences in aromatase expression and volume that were undetected in our previous analysis. This result does support the idea that aromatase in the POA is important for sexual behaviours. Seasonal differences in aromatase expression have been studied (e.g., Silverin et al., 2004; Soma et al., 2003), although relatively few studies have examined both sex and seasonal differences. Male little brown bats have more overall neural aromatase activity than females during the BS, and this activity does not differ between males and females prior to emergence from hibernation (Callard et al., 1983). This is consistent with activity data from green anoles (see above), as well as the results from the present study.

Aromatase in the AMY

The lack of effects in aromatase expressing cells due to sex and season is consistent with previous studies that have found no differences in aromatase activity between males and females in similar brain areas in Japanese quail, rats, and guinea pigs (Connolly et al., 1990; Roselli and Resko, 1997), although male turtles (Chrysemys picta) have higher aromatase activity in this general region than do females (Callard, 1977). Activity does not necessarily parallel mRNA expression such that male rats appear to express more aromatase mRNA (represented by silver grains) per cell even though activity does not differ (Wagner and Morrell, 1996; 1997). In the present study, we cannot detect relative quantities of aromatase mRNA in each cell due to the constraints of the labeling procedure, which facilitated counting of individually labeled cells. However, that type of analysis could be undertaken in the future.

We also found a trend for lateralization, such that a greater number of more densely packed cells appeared to be present on the left than the right. In other species, morphology and neurochemistry of the AMY is lateralized (Cooke et al., 2007; Morris et al., 2008). However, straightforward hypotheses have not been suggested regarding the roles of these lateralizations, and one hesitates to speculate based on values that only approach statistical significance.

We found that the density of aromatase cells was higher in females than in males and a trend for lateralization existed on this measure. Density was calculated from the number of cells and the volume of the region, neither of which differed between the sexes. However, in both the BS and NBS, more cells were present in a smaller volume in females compared to males, on average. The biological relevance of the sexual dimorphism in density is unclear, but could reflect an increased need for sensory integration in females (perhaps of courtship cues from

males; reviewed in McDonald, 1998; Moreno and Gonzalez, 2007). While it is difficult to speculate on whether the trend for lateralization is associated with a particular function, it is consistent with what we observed for the number of aromatase-expressing cells.

Aromatase in the VMH

The similarity in aromatase expression in the VMH of males and females suggests that it may be important for both sexes. This result differs somewhat from other species, although relatively few studies have examined aromatase distribution in both males and females. For example, more VMH cells express aromatase mRNA and activity is higher in male than in female rats (Roselli et al., 1985; Wagner and Morrell, 1996; 1997). Male turtles also have more aromatase activity in the entire hypothalamus than do females (Callard, 1977). However, no sex difference was detected in activity in the VMH equivalent in Japanese quail (Balthazart et al., 1990). Much of the data on aromatase in the VMH represents regional activity rather than quantification of cell number. The former technique does not differentiate between portions of the VMH (lateral vs. medial). Our analysis of only the lateral portion of this region may relate to some of the differences with other species. It also is likely to be the reason we found a novel sex difference in volume compared to earlier work on intact adult green anoles, which quantified the entire VMH (Beck et al., 2008). Similar to the present study, however, a larger sample size in gonadectomized animals (some E2-treated) did reveal a male-biased sex difference in the volume of the VMH in Nissl-stained tissue (Beck and Wade, 2009a).

Although the number of aromatase expressing cells does not differ, female anoles may have proportionally more aromatase in the lateral VMH than do males, as the density of aromatase expressing cells is higher in females than in males. This result is consistent with the

idea that E2 production via aromatase in the VMH is important for female receptivity. Similar to the current data, female anoles also express more estrogen receptor α in the VMH than do males (Beck and Wade, 2009c). Thus, locally produced E2 could have more of an effect in the VMH in females.

Conclusion

Aromatase is expressed in the brain of vertebrates, specifically in portions that control sexual behaviors. The present data indicate that this pattern holds for green anole lizards. There were more cells that expressed aromatase in the POA of males than in females, and the density of VMH cells expressing aromatase was greater in females. These data follow the idea that aromatase in the POA is important for male specific behaviors, and aromatase in the VMH is important for female specific behaviors. Collectively, the present data considered in the context of those available from other taxa on aromatase suggest evolutionary conservation. However, at least in terms of male sexual behavior, estrogens appear to have a larger role in birds and mammals (Bakker et al., 2004; Schlinger, 1997a). As these groups both evolved from reptilian ancestors (Richman et al., 2006), investigations in reptiles such as green anoles can elucidate the evolution of mechanisms regulating reproduction. Increasing our understanding of changes in the role of aromatase will be particularly informative.

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Table 1. Primers used to clone aromatase from the green anole brain. Melting temperatures (T_M) are indicated.

Primer Name	Sequence (5' to 3')	T_M (°C)
Forward	GACATGCCGAAGCTGAA	56.72
Reverse	TTGGGAAGAACTCAAGCCGA	63.12

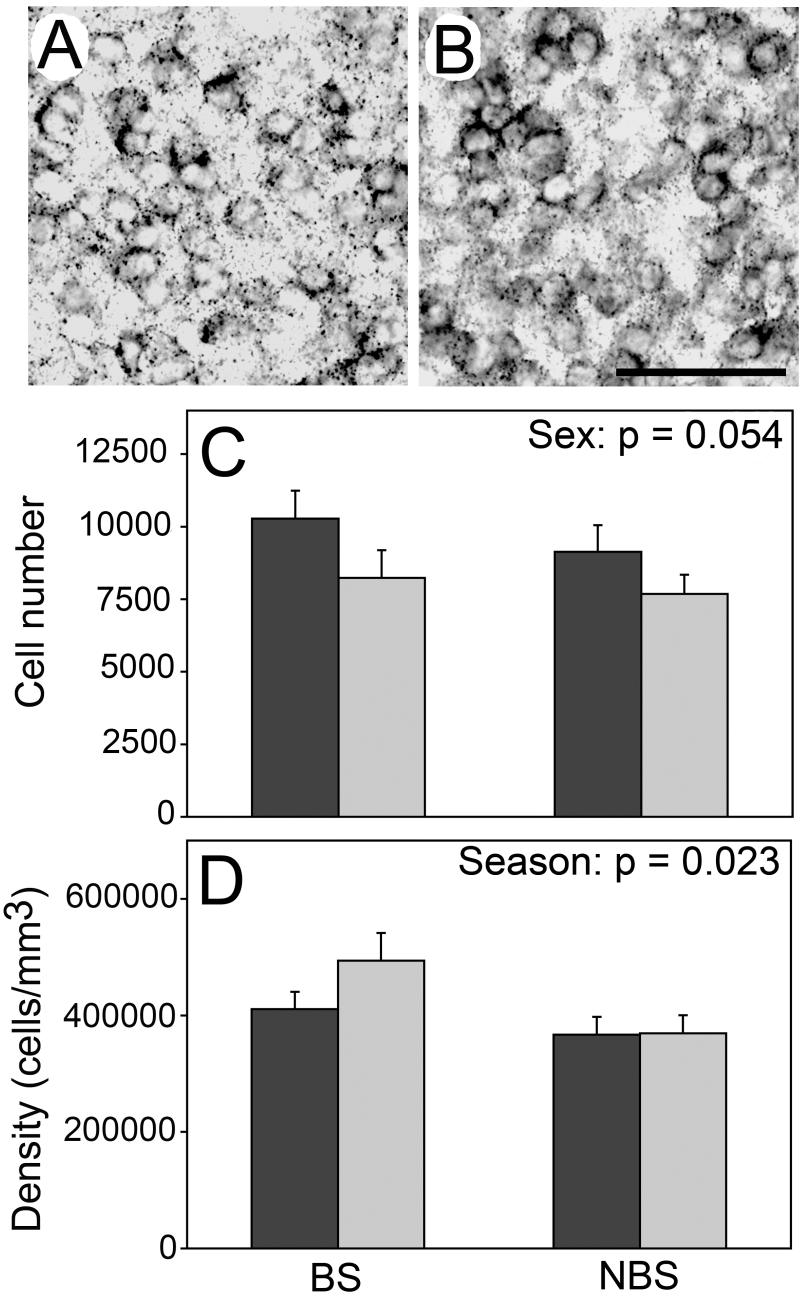

Figure 4. Aromatase expression in the POA (mean + S.E.). The photograph in panel (A) depicts a BS male, and (B) shows a NBS male. Panel (C) indicates the number of aromatase expressing cells; (D) shows the increased density of aromatase expressing cells overall in the BS compared to NBS. Scale bar = 50 μm. n = 9 NBS females, 8 for the rest of the groups. Males = black; females = grey.

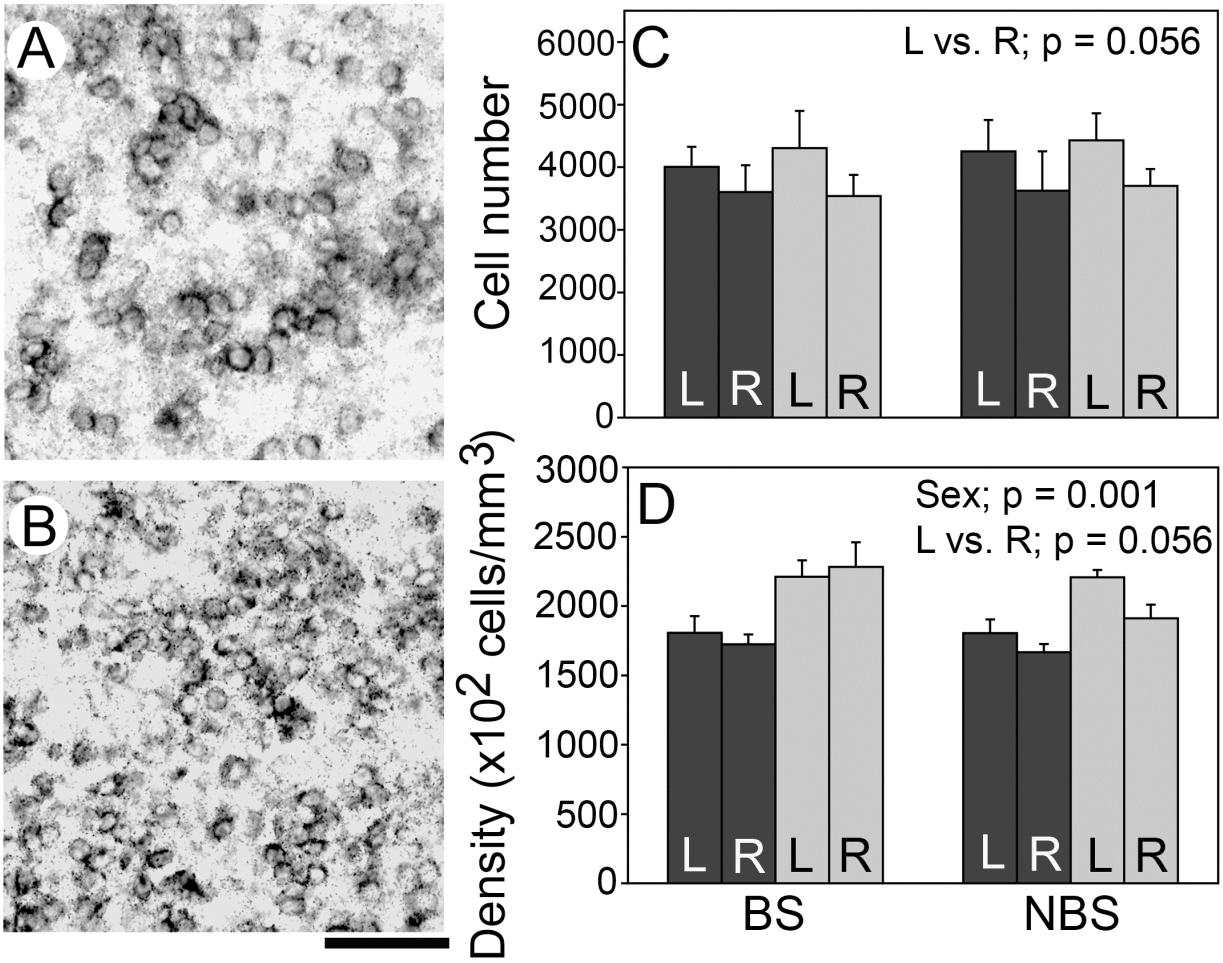

Figure 5. Aromatase mRNA in the AMY. Panel (A) is from the left AMY of a male. Panel (B) shows the left AMY of a female. Breeding animals are represented in all panels. Mean + SE of the number of aromatase expressing cells is indicated in (C). The density of aromatase expressing cells is in (D). Scale bar = 50 μm. n = 5 BS and NBS males, 6 BS females, and 8 NBS females. Black bars = males; grey bars = females.

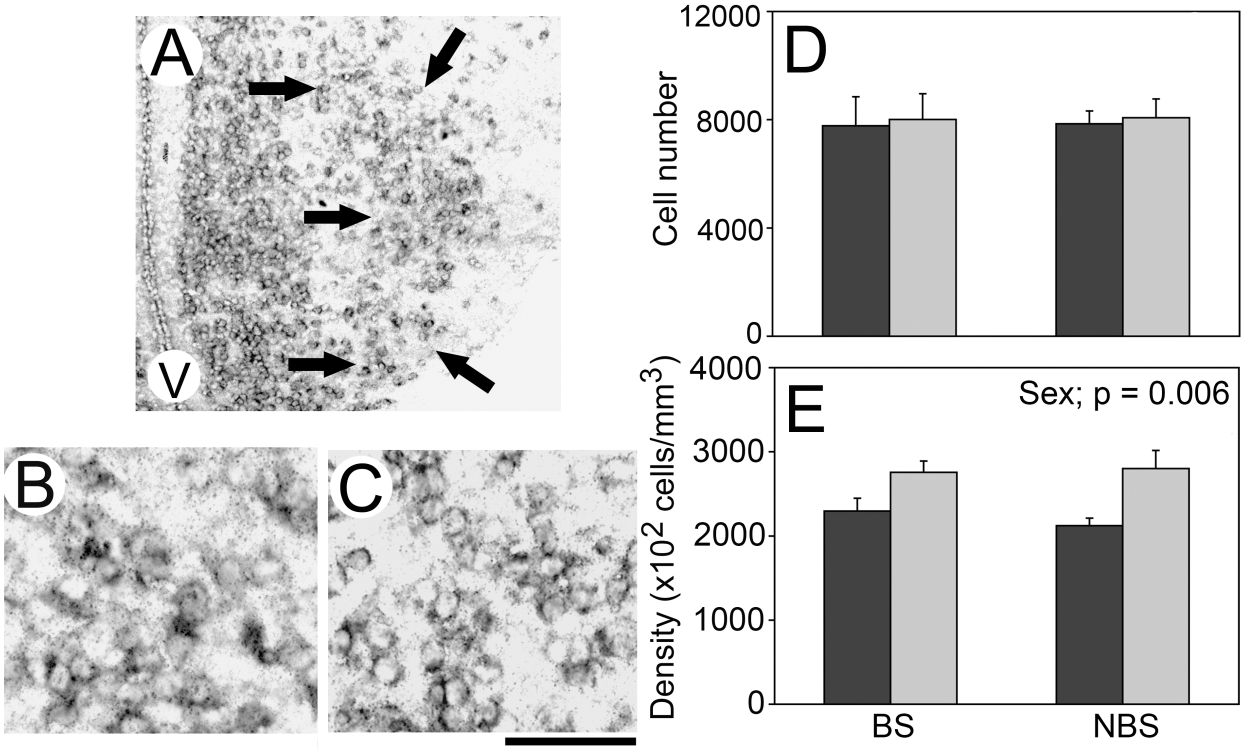

Figure 6. Aromatase mRNA expression in the VMH. Panel (A) is a photomicrograph of the VMH from a BS female with arrows indicating the boundary of the region quantified. The photograph in panel (B) depicts a BS male, and panel (C) depicts a BS female. No differences were detected in the total number (mean + S.E.) of aromatase expressing cells (D). Females have a greater density of aromatase cells than males (E). Scale bar = 150 μm (A) and 50 μm (B and C). V = 3rd ventricle. n = 7 BS males, 5 NBS males, 4 BS females, and 8 NBS females. Black bars = males; grey bars = females.

Chapter 4: Distribution of two isozymes of 5α-reductase in the brains of adult male and female green anole lizards

Abstract

The 5α-reductase (5αR) enzyme converts testosterone to 5α-dihydrotestosterone. This local metabolism within the brain is important for the full expression of male sexual behavior in many species, including green anole lizards. Two isozymes of 5αR exist and little is known about their specific distributions. We conducted *in situ* hybridization for both isozymes in intact male and female green anole brains during the breeding (BS) and non-breeding (NBS) seasons. 5αR1 mRNA was only detected in the brainstem, while 5αR2 was expressed in specific areas throughout the brain. As our primary interest was evaluating the potential role of 5αR in forebrain regulation of reproductive behavior, we quantified 5αR2 expression in the preoptic area (POA), amygdala (AMY), and ventromedial hypothalamus (VMH). More 5αR2 cells were detected during the NBS than BS in the AMY, and the density of these cells was greater in females than males. In the VMH, the right side contained more 5αR2 cells than the left, an effect driven by a lateralized increase in the NBS. These data expand understanding of the distribution and potential roles of both isozymes in the adult brain, and differences in expression patterns with mammals and birds suggest that they may have been co-opted for different functions later in evolution. *Key words: amygdala, androgen metabolism, preoptic area, testosterone, ventromedial hypothalamus*

Introduction

Steroid hormones are important for the expression of sexual behaviors in many vertebrate species (Ball and Balthazart, 2002; Fabre-Nys and Martin, 1991; Fusani, 2008; Hull and Dominguez, 2007; Steel, 1981; Tennent et al., 1980). These hormones act at brain regions that are critical for the expression of sexual behaviors, including the preoptic area (POA) and amygdala (AMY), which control male behaviors (courtship and copulatory behaviors; Balthazart and Surlemont, 1990; Crews and Moore, 2005; Kostarczyk, 1986; Thompson et al., 1998; Tobet et al., 1986), and the ventromedial hypothalamus (VMH), which controls female behaviors (proceptive and receptive behaviors; Emery and Moss, 1984; La Vaque and Rodgers, 1975; Wade and Crews, 1992). Neural metabolism of gonadal hormones is often critical. For example, local conversion of testosterone (T) into estradiol (E2) and 5α-dihydrotestosterone (DHT) facilitates production of the full suite of male reproductive behaviors in many organisms, including Japanese quail, hamsters, rats, and guinea pigs (Balthazart, 1991; Hull and Dominguez, 2007; Romeo et al., 2001). Aromatase is the enzyme that converts T to E2, and 5α-reductase (5αR) converts T to DHT. While much is known about aromatase (reviewed in Lephart, 1996), much less information is currently available for 5αR.

Two isozymes of 5αR exist: 5αR1 and 5αR2 (Lephart et al., 2001). In mammals, neural 5αR1 is generally more abundant than 5αR2 (Celotti et al., 1997). Expression is greater overall for both isozymes in the brainstem than in the forebrain, however 5αR2 is found in hypothalamic and hippocampal neurons in adult rodents (Poletti and Martini, 1999). 5αR1 has a lower affinity for T and is present in both neurons and glial cells, whereas 5αR2 has a higher affinity for T and is found primarily in neurons (Negri-Cesi et al., 2008). Thus, these two isozymes may serve

somewhat different functions.

In general, whole brain 5αR activity does not differ between males and females, and is not typically modulated by steroid hormones. However, evidence in adult rats suggests that 5αR2 mRNA, but not 5αR1, can be upregulated by T (Negri-Cesi et al., 1996; Torres and Ortega, 2003; 2006). Specific distributions and relative patterns of expression of 5αR1 and 5αR2 in areas likely to control reproductive behaviors are unknown. Additionally, expression of these isozymes has not yet been examined in reptilian species.

Green anole lizards are seasonally breeding animals native to the southeastern United States, with the breeding season (BS) lasting from approximately April to August. During the non-breeding season (NBS) gonads regress and circulating steroid hormone levels decrease (Lovern et al., 2001). Thus, these animals offer an excellent natural experiment to examine how circulating T levels across the year might influence 5αR1 and 5αR2 expression.

Similar to other species, DHT is important for the full expression of male reproductive behaviors (Mason and Adkins, 1976; Rosen and Wade, 2000). 5αR activity in anoles is also similar to what has been observed in other species. Activity is higher in the brainstem than the forebrain, and whole brain activity does not differ between the sexes or the seasons in gonadally intact animals (Rosen and Wade, 2001; Wade, 1997). However, T treatment increases whole brain 5αR activity in gonadectomized males (Cohen and Wade, 2010b).

Although it is known that 5αR activity is concentrated in the brainstem of many species, including anoles (Wade, 1997), detailed expression patterns have not been reported. Thus, this experiment was designed to determine the distribution of both isozymes of 5αR in the green anole brain, and to investigate local changes in expression due to sex and season. This study also begins to address the hypothesis that T can influence the distribution of the two isozymes by

examining the brain under naturally occurring differences in T levels (male vs. female, BS vs. NBS).

Methods

Animals and tissue processing

Male and female green anole lizards were purchased from Charles Sullivan Co. (Nashville, TN) during the BS (May) and NBS (November). These animals were wild-caught and sent to our animal facilities within a few days of capture. Animals were housed individually in 10 gallon aquaria. Peat moss was provided as substrate, and cages contained rocks, sticks, and water dishes. Aquaria were misted daily, and crickets dusted with calcium phosphate powder were provided three times (BS) or two times (NBS) per week. During the BS, animals were kept on a 14:10 light/dark cycle and ambient temperatures ranged from 28 ºC during the day to 19 ºC at night. During the NBS, animals were kept at a 10:14 light/dark cycle and ambient temperatures ranged from 24 ºC during the day to 15 ºC at night. Heat lamps and full spectrum lights were provided above the cages, which allowed the animals to bask at temperatures 10 ºC above ambient temperatures. Relative humidity was maintained at approximately 70 % throughout both seasons.

At least two weeks after arrival in the lab, animals were rapidly decapitated. Breeding state was confirmed at this time, with animals in the NBS having fully regressed gonads. Males in the BS had large, vascularized testes, and females had at least one yolking follicle. Brains were collected, immediately frozen in methyl butane on dry ice, and stored at –80 °C until processing. Brains were sectioned coronally at 20 μm into four alternate series (three were used

for the present study, see below) and thaw-mounted onto SuperFrost plus slides (Fisher Scientific; Hampton, NH). Slides were stored with dessicant at –80 ºC until further processing.

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

Cloning of 5αR1 and 5αR2

Testes were taken from two breeding males and homogenized in Trizol (Invitrogen Corporation; Carlsbad, CA). RNA was separated from the homogenate using chloroform, then isolated using RNeasy minicolumns (Qiagen Sciences; Valencia, CA) and concentrated using ethanol precipitation. It was reconstituted in DEPC-treated water and stored at –80°C. RNA was converted into cDNA with the SuperScript III Reverse Transcriptase kit (Invitrogen) per manufacturer's instructions, and stored at -20°C until use.

Anole-specific 5αR1 and 5αR2 cDNA sequences were found using the Ensembl Genome Browser (ENSACAG00000000672 and ENSACAG00000000551, respectively). The top 25 results from a BLASTx (NCBI) search of the green anole 5αR1 represented diverse vertebrates with 53-61% sequence identity. Parallel results were obtained for 5αR2, with somewhat higher identity (57-79%). Primers were designed based on the anole sequences for both genes using the Oligo Analysis Tool program (Eurofins MWG Operon; Huntsvile AL; Table 2) and purchased from Invitrogen. PCR reactions included 1U Platinum TAQ High Fidelity DNA polymerase (Invitrogen), 0.2 mM dNTP mixture, 0.2 μM primer mix, 2 mM $MgSO_4$, and template cDNA from the testes. The PCR reaction went through 40 cycles at 94°C for 15 seconds, 50°C for 30 seconds, and 68°C for 1 minute. 5αR2 was amplified once, purified using the QIAquick PCR purification kit (Qiagen) and concentrated using ethanol precipitation. 5αR1 was amplified

twice, using the same primers and the product from the first reaction as the template for the second. The pGEM-T Easy Vector System I (Promega Corporation, Madison, WI) was used to ligate the A-tailed PCR products to vectors as per manufacturer's instructions.

We transformed One Shot TOP10 Chemically Competent *E. coli* cells (Invitrogen) with the ligated vectors and grown on LB agar plates containing 100 μg/ml of ampicillin and 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal). White colonies were selected and grown overnight in LB broth containing 100 μg/ml of ampicillin. Vector DNA was isolated using Wizard *Plus* Miniprep kits (Promega), and the sequences of the inserts were confirmed in both directions on a Perkin Elmer/Applied Biosystems 3100 capillary sequencer. After sequence confirmation, vector DNA was isolated using Wizard *Plus* Maxiprep kits (Promega) and stored at –20 °C.

In situ hybridization

Briefly, sense (SP6) and antisense (T7) probes were transcribed for both genes using the Digoxigenin RNA Labeling Kit per manufacturer's instructions (Roche Diagnostics; Indianapolis, IN), which labels RNA with digoxigenin-UTPs. Probes were cleaned using a G50 sephadex bead column 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 anhydrase in triethanolamine-HCl with 0.9% NaCl buffer (pH 8.0). Slides then incubated overnight at 55 °C with 200 ng/ml (5αR1) or 100 ng/ml (5αR2) probe in hybridization buffer, which consisted of 50 % formamide, 4x SSC, 1x Denhardt's solution, 200

μg/ml fish sperm DNA, 10% dextran sulfate, 20 mM dithiothreitol, 250 μg/ml tRNA, 2 mM EDTA, and 0.1% Tween-20. The next day, slides were rinsed in 2x SSC and 0.2x SSC at 60 °C. They were then treated with 0.9% H_2O_2 in maleic acid buffer (pH 7.5) with 0.1% Tween-20 (MABT) for 30 mins. They were incubated in a blocking solution of 5% normal sheep serum (Jackson Immuno Research; West Grove, PA) in MABT for 30 mins, and were treated with 0.5 μl/ml Anti-Digoxigenin-AP Fab fragments (Roche) in MABT. After two hours, the color reaction was conducted by incubating the slides with 4.5 μl/ml NBT and 3.5 μl/ml BCIP (Roche) in 0.1M Tris-HCl and 0.1M NaCl (pH 9.5). Reaction time was titrated so that the slides incubated with the antisense probe showed distinct reaction product within the cytoplasm of individual cells with an absence of labeling on the sense-treated slides (about 9 minutes for 5αR1 and 5 minutes for 5αR2; Fig. 1), the color reaction was stopped with 1M Tris and 0.5M EDTA (pH 8.0).

5αR1 and 5αR2 mapping

We mapped the expression of both 5αR1 and 5αR2 in the brain. Regions in the anole forebrain were identified using a green anole atlas (Greenberg, 1982). Brainstem regions were identified using ten Donkelaar and Nieuwenhuys (1979) and Barbas-Henry and Lohman (1984). Three different labeling intensities were determined: light, medium and dark (Fig. 7). The light labeling was characterized by the blue reaction product that was confined to the cytoplasm, but was somewhat punctuate and did not fill the entire cellular compartment. Dark labeling was characterized by labeling that was intense and specifically filled the entire cytoplasm of individual cells. Medium labeling was intermediate; the labeling was clearly cytoplasmic, filled most of that portion of the cell and was darker than the light labeling.

5αR2 quantification

Because 5αR1 was not expressed in the forebrain regions mediating sexual behavior (POA, AMY, and VMH), only 5αR2 was quantified. These three regions were chosen because previous work has documented that exhibit sexual and seasonal dimophrisms (e.g. Beck et al., 2008), and are critical to the display of masculine and feminine sexual behaviors (see above). Similar to previous work on steroid receptors and aromatase in green anoles, we found that only the lateral portion of the VMH expressed 5αR2 (Beck and Wade, 2009c; Rosen et al., 2002; Cohen and Wade, unpublished). Thus, we confined our analyses to this region. The slides were examined under brightfield illumination using Stereo Investigator software (MicroBrightfield, Inc.; Williston, VT) following Beck and Wade (2009a) by a user blind to experimental group. Estimates of total counts of cells positive for the steroid metabolizing enzyme were counted using the Optical Fractionator function. After tracing the outline of a brain region in each tissue section in which it existed, the software placed a user-defined grid over each area (POA: 100x100 μm^2, AMY: 40x40 μm^2, VMH: 80x80 μm^2) and sampling sites (30x30 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 and the samples in which manual counts were taken. A Gundersen coefficient of error (indicates precision of the estimated population size) at or below 0.1 was confirmed to ensure accurate estimates of cell count. The density of 5αR2 cells was determined by dividing the estimated cell count by the calculated volume for the region.

Brain region boundaries based on enzyme expression were similar to those indicated by Nissl staining, except for the VMH, where expression of 5αR2 was only present in the lateral

portion of the nucleus (for Nissl-stained examples of each region see Beck et al., 2008). Volumes described here are based on the area defined by 5αR2 expression.

Statistical analysis of stereological measurements (5αR2 only)

Analysis was conducted separately for each brain region. The left and right sides of the POA and AMY did not differ in the volume, estimated number of positive cells, or density of the cells per unit volume (all $F < 0.34$, $p > 0.566$). Thus the average of the two sides were used for statistical analysis. Two-way ANOVAs were used to compare main effects of, and interactions between, sex and season. Interactions were further broken down by one-way ANOVAs and pairwise comparisons, as appropriate.

Paired t-tests detected a side difference for cell number and density in the VMH (both $F > 4.44$, $p < 0.048$). We thus accounted for side in all subsequent analysis by using 3-way mixed model ANOVAs.

Results

5αR1 and 5αR2 mapping

The distribution and relative intensities of both 5αR1 and 5αR2 did not differ qualitatively between the sexes or seasons, so the composite data are indicated on a single map (Fig. 8). 5αR1 was expressed primarily in the brainstem, especially in motor nuclei (Fig. 9). The darkest labeling of 5αR1 mRNA was found in the oculomotor and trochlear nuclei (nIII/IV), abducens nucleus (nVI), glossopharyngeal portion of the nucleus ambiguus and the ventral motor nucleus of the facial nerve (Amb IX/VII), and reticular nucleus (R1). In contrast, dark 5αR2

staining was detected in specific regions throughout much of the brain, including those regions important in controlling sexual behaviors (POA, AMY, and VMH; Fig. 9). 5αR2 was lightly expressed in the forebrain, and exhibited much higher intensity in the brainstem. The darkest labeling of 5αR2 expression was observed in nIII/IV, Amb IX/VII, and nVI.

5αR2 in the POA

We detected no effects of, or interactions between, sex and season on the number of 5αR2 expressing cells (all $F < 3.12$, $p > 0.090$; Table 3). No effects were detected on the density (cell number per unit volume) for any of these variables (all $F < 1.59$, $p > 0.219$).

Similar to previous work, the volume of the POA was larger in males compared to females ($F = 12.51$, $p = 0.002$; Table 4A).

5αR2 in the AMY

No effects were detected on the number of 5αR2 expressing cells (all $F < 3.70$, $p > 0.07$, Fig. 10), although on average more 5αR2 expressing cells were present in the NBS. A sex difference was detected in the density of 5αR2 cells, such that more were present per unit volume in females compared to males ($F = 4.61$, $p = 0.046$; Fig. 10). No other main effects or interactions were detected for this measure (all $F < 0.04$, $p > 0.847$).

We also found a sex difference in the volume of the AMY; the region was larger in males than in females ($F = 6.47$, $p = 0.02$; Table 4B).

5αR2 in the lateral VMH

The estimated total number of cells that expressed 5αR2 in this brain region was

lateralized ($F = 5.60$, $p = 0.030$; Fig. 11), such that the right side expressed more 5αR2 than the left. There was also an interaction between side and season ($F = 5.89$, $p = 0.027$), which was driven by an increase in 5αR2 expression in the right lateral VMH during the NBS ($F = 9.24$, $p = 0.007$). The expression in the left VMH did not change with season ($F = 0.65$, $p = 0.431$).

Parallel to what we found for the number of cells, the density of 5αR2 expressing cells was lateralized ($F = 5.06$, $p = 0.038$; Fig. 11), again with the right VMH containing more than the left. No other effects were detected on all density measures (all $F < 2.32, 3.15$, $p > 0.146$).

Similar to previous work [unpublished observations], we found that males had a larger lateral VMH than females ($F = 15.68$, $p = 0.001$; Table 4C). We also found a seasonal difference in volume, such that the lateral VMH is larger during the NBS than BS ($F = 11.01$, $p = 0.004$).

Discussion

We report here for the first time the distribution of two isozymes of 5αR in the brain of a reptile. The patterns of distribution for 5αR1 and 5αR2 differed from one another. 5αR1 was detected only in the brainstem, while 5αR2 was found in particular regions throughout the brain, although it was concentrated in the brainstem. This result is consistent with previous work showing higher 5αR activity in the brainstem than the rest of the brain in these lizards (Wade, 1997). Thus, it appears that, in the anole, both 5αR1 and 5αR2 contribute to the high level of activity detected in the brainstem, while the majority of activity detected throughout the rest of the brain is due to 5αR2. Our results also parallel data suggesting that the mammalian brainstem expresses higher levels of both isozymes than the forebrain (Celotti et al., 1997). The fact that both 5αR1 and 5αR2 are expressed in the brainstem of lizards as well as mammals, suggests a

potentially conserved function for this enzyme in that portion of the brain.

Expression of these two isozymes has not been completely mapped in other species. In adult rats, 5αR1 mRNA is expressed at higher levels in whole brain homogenates, than 5αR2 mRNA (Negri-Cesi et al., 1996; Poletti et al., 1998). Immunohistochemistry in rats for 5αR1 has detected the protein in the limbic system, as well as the cortex (Pelletier et al., 1994; Tsuruo et al., 1996). 5αR2 was not investigated in the same way, although 5αR2 is expressed in the cortex in rats (Sanchez et al., 2008) and transiently expressed at higher levels than 5αR1 in homogenates of the entire brain during early development (Poletti et al., 1998). In birds, 5αR activity has been detected throughout the brain, including the song control system, although the assay technique does not distinguish between the different isozymes (Balthazart, 1991).

It has been suggested that these enzymes play different roles in mammals: 5αR1 may serve to catabolize excess T, whereas 5αR2 may be important for sexual differentiation and male-specific behaviors (Melcangi et al., 1998). Because it is not expressed as prevalently in the lizard brain, it is possible that 5αR1 was co-opted for clearing excess androgen from the brain later in evolution. In anoles, T itself is more important for the expression of male sexual behaviors, while the metabolites of T (estradiol and DHT) are more important for these behaviors in mammalian and avian species (Bakker et al., 2004; Mason and Adkins, 1976; Schlinger, 1997a). Thus, 5αR1 may not be as abundant in the anole brain, as compared to the mammalian brain, because T itself is the more active hormone.

In the present study, 5αR2 expression did not differ between the sexes or seasons in the POA or the VMH, which suggests that 5αR2 may not be important for the sexually dimorphic behaviors regulated by these areas. We did detect a lateralized effect of season on the number of 5αR2 cells in the VMH, although at this time, the function of this difference remains unclear. In

the AMY, more cells expressed 5αR2 during the NBS than in the BS, and the population of these cells was denser in females than in males. These results are inconsistent with the idea that increased regional enzyme expression facilitates male sexual behavior. Across vertebrates, the AMY is a sensory integration site, which gets input from the auditory, visual, and olfactory systems (reviewed in McDonald, 1998; Moreno and Gonzalez, 2007). The region projects to other limbic regions, including the POA and VMH (reviewed in Goodson, 2005; Newman, 1999). Assuming these differences in AMY mRNA due to sex and season result in functional changes in local enzyme activity, at least two interpretations are possible. First, like 5αR1 in higher vertebrates, 5αR2 in the anole AMY may begin to break down excess T, creating a mechanism through which endocrine function is limited in regions in which T likely facilitates sexual behaviors. Alternatively, increased T during the BS and in males could serve to down-regulate AMY 5αR2, preserving sufficient levels of DHT in this region. However, it is difficult to speculate, on the function(s) that this DHT may serve, and the pattern is not consistent with data from rat prefrontal cortex (Torres and Ortega, 2003; 2006).

Similar to previous work (Beck et al., 2008, unpublished observations based on labeling of aromatase mRNA), we found that male anoles have a larger POA and lateral VMH than females. We detected a novel sex difference in AMY size (males larger than females) as defined by 5αR2 expression, which suggests that the borders defined by 5αR2 mRNA labeling compared to Nissl staining and aromatase mRNA are not exactly the same (Beck et al., 2008). Because the 5αR2 expressing cells existed in greater density in females and without a sex difference in their overall number, the larger volume of the AMY in males is most likely due to cells that are more spread out. This change might reflect increased arborization, which could be evaluated in future studies.

We also detected a seasonal difference in the VMH, with the volume of the region larger during the NBS than the BS. Because the number of cells expressing 5αR2 is equivalent across the seasons, perhaps the cells are further spread apart during the NBS. Additionally, we detected a lateralized expression of 5αR2 in the VMH, although the function of this lateralization also remains unclear. We previously identified lateralized expression of aromatase in the AMY [unpublished observations]. Unlike eutherian mammals, reptiles and birds do not have a corpus callosum, and the absence of this structure limits the extent of communication between the two hemispheres of the brain (Mihrshahi, 2006). This limited communication may allow for more lateralized functions of reptilian hypothalamic regions that should be further investigated.

Conclusions

To our knowledge, this is one of the first studies to examine expression of both isozymes in discrete brain regions in vertebrates and the first to examine the pattern in a reptile. The present data show that 5αR1 is mainly present in the brainstem, while 5αR2 is more widespread in the anole brain. This distribution pattern departs from what has been reported in mammals, where 5αR1 is the isozyme that is expressed at higher levels throughout the brain. It is possible that these two isozymes have different functions in the anole than in mammalian species. However, in both groups the brainstem expresses the highest levels of these isozymes, which suggests that there may be a similar function for 5αR in that portion of the brain across species. Expression of 5αR2 in the POA and VMH did not differ between the sexes and seasons, although we did find that 5αR2 expression was higher in the AMY of NBS animals and females. This suggests that 5αR2 may act to break down excess T in those groups that do not need high

amounts of T in the AMY, or produce sufficient amounts of DHT during times when T levels are low.

Collectively, our results highlight the importance of examining these enzymes in multiple taxa. These data help expand current understanding of the roles for both isozymes in the adult brain, and differences in expression patterns with mammals suggest that these enzymes may have been co-opted for different functions later in evolution.

Acknowledgements

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Table 2. Primers used to clone 5αR1 and 5αR2 from the green anole. Melting temperatures (T_M) are indicated.

Primer Name	Sequence (5' to 3')	T_M (°C)
5αR1 Forward	TGATGCTGCCGCTGAGCAA	67.73
5αR1 Reverse	TTCCTGTTGCGTGGATAGT	56.67
5αR2 Forward	CTTGGTTCCTGCAGGAGTT	57.86
5αR2 Reverse	GGTAGCTGCTGAATGTCCT	55.38

Table 3. 5αR2 expression in the POA: The estimated total number (A) and density (B; cells per mm^3) of positive cells. No effects of sex, season or interactions were detected.

	A. Number	B. Density
Female BS	6018 (1050)	422771 (53666)
Female NBS	7670 (611)	457455 (25878)
Male BS	8334 (739)	399982 (18570)
Male NBS	8055 (412)	401162 (35465)

Table 4. Volume (mm^3) of three regions important for sexual behavior (POA, AMY, and VMH) as defined by 5αR2 expression. All regions were larger in males than in females, regardless of season. Values are means (S.E.).

A. POA	Males	Females
BS	0.021 (0.001)	0.014 (0.002)
NBS	0.021 (0.002)	0.017 (0.001)
B. AMY	**Males**	**Females**
BS	0.019 (0.001)	0.015 (0.001)
NBS	0.020 (0.002)	0.017 (0.001)
C. VMH	**Males**	**Females**
BS	0.023 (0.002)	0.018 (0.001)
NBS	0.028 (0.001)	0.022 (0.002)

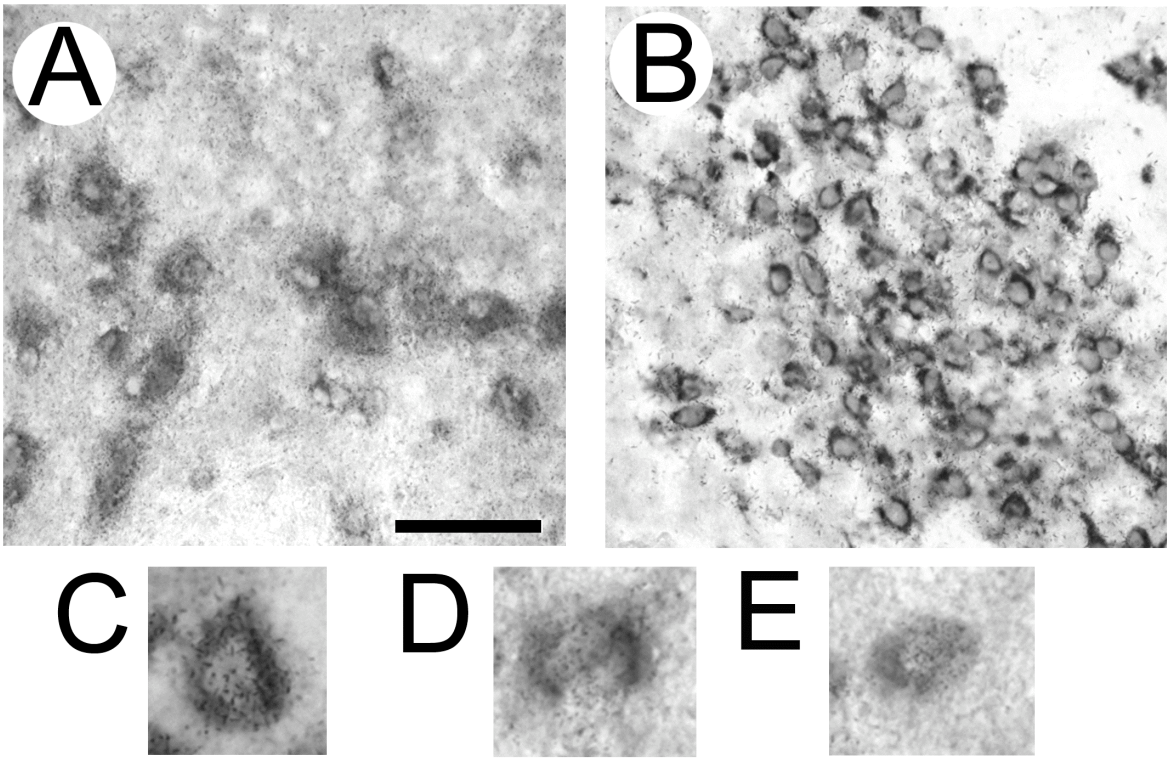

Figure 7. Validation of techniques. (A) 5αR1 antisense. (B) 5αR2 antisense. Labeling in tissue exposed to sense probes was completely absent. The panels below indicate examples of dark (C), medium (D), and light (E) labeling within individual cells. (A), (C), (D), and (E) are from the nucleus of the oculomotor cranial nerve. (B) is from the VMH. Scale bar = 50 μm. (C), (D), and (E) were enlarged 2.5 fold from (A).

Figure 8.

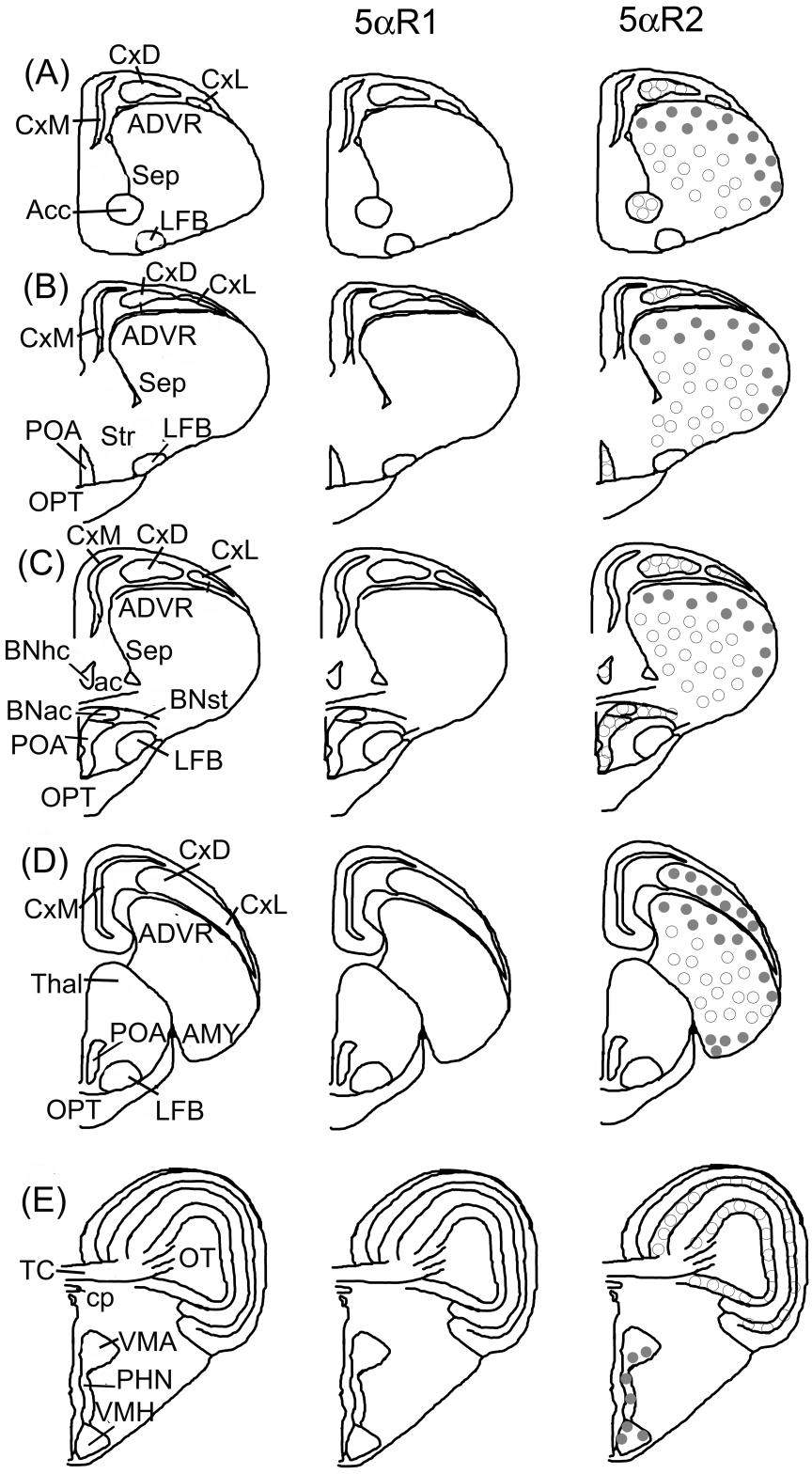
5αR1
5αR2
(A)
CxD
CxL
CxM
ADVR
Sep
Acc
LFB
(B)
CxD
CxL
CxM
ADVR
Sep
POA
Str
LFB
OPT
(C)
CxM
CxD
CxL
ADVR
BNhc
Sep
Jac
BNst
BNac
POA
LFB
OPT
(D)
CxD
CxM
CxL
ADVR
Thal
POA
AMY
OPT
LFB
(E)
TC
OT
cp
VMA
PHN
VMH

Figure 8. Continued

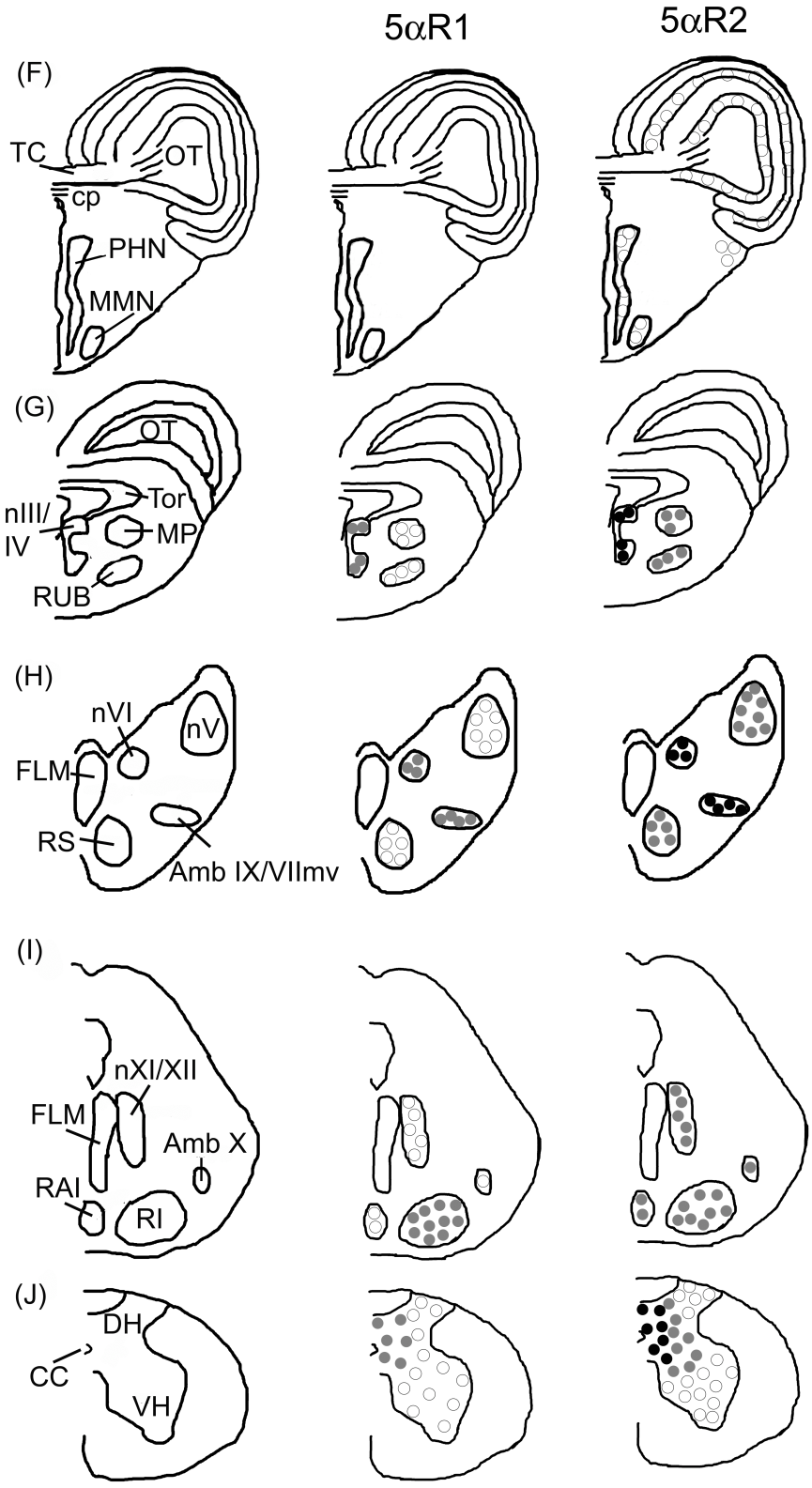

Figure 8. Camera lucida drawings depicting patterns of 5αR1 and 5αR2 expression throughout the anole brain (the distribution was symmetrical; only half the brain is depicted). Open circles = light labeling, grey circles = medium labeling, and dark circles = dark labeling. Abbreviations: Acc = nucleus accumbens; AC = anterior commissure; ADVR = anterior dorsal ventricular ridge; Amb X = vagal portion of nucleus ambiguus; Amb IX/VII = glossopharyngeal portion of the nucleus ambiguus and the ventral motor nucleus of the facial nerve; AMY = amygdala; BNac = bed nucleus of the anterior commissure; BNhc = bed nucleus of the hippocampal commissure; BNST = bed nucleus of the stria terminalis; cc = central canal; CP = posterior commissure; CxD = dorsal cortex; CxL = lateral cortex; CxM = medial cortex; DH = dorsal horn of the spinal cord; FLM = medial longitudinal fasciculus, LFB = lateral forebrain bundle; MMN = mammillary nucleus; MP = medial pretectal nucleus; nIII/IV = oculomotor and trochlear nuclei; nV = trigeminal nucleus; nVI = abducens nucleus; nXI/XIImv = accessory and hypoglossal nuclei; OPT = optic tract; OT = optic tectum; PHN = posterior hypothalamic nucleus, POA = preoptic nucleus; RAI = inferior raphe nucleus; RI = reticular nucleus; RUB = red nucleus; RS = superior reticular nucleus; Sep = septum; Str = striatum; TC = tectal commissure; Thal = thalamus; Tor = torus semicircularis; VH = ventral horn of the spinal cord; VMH = ventromedial hypothalamus.

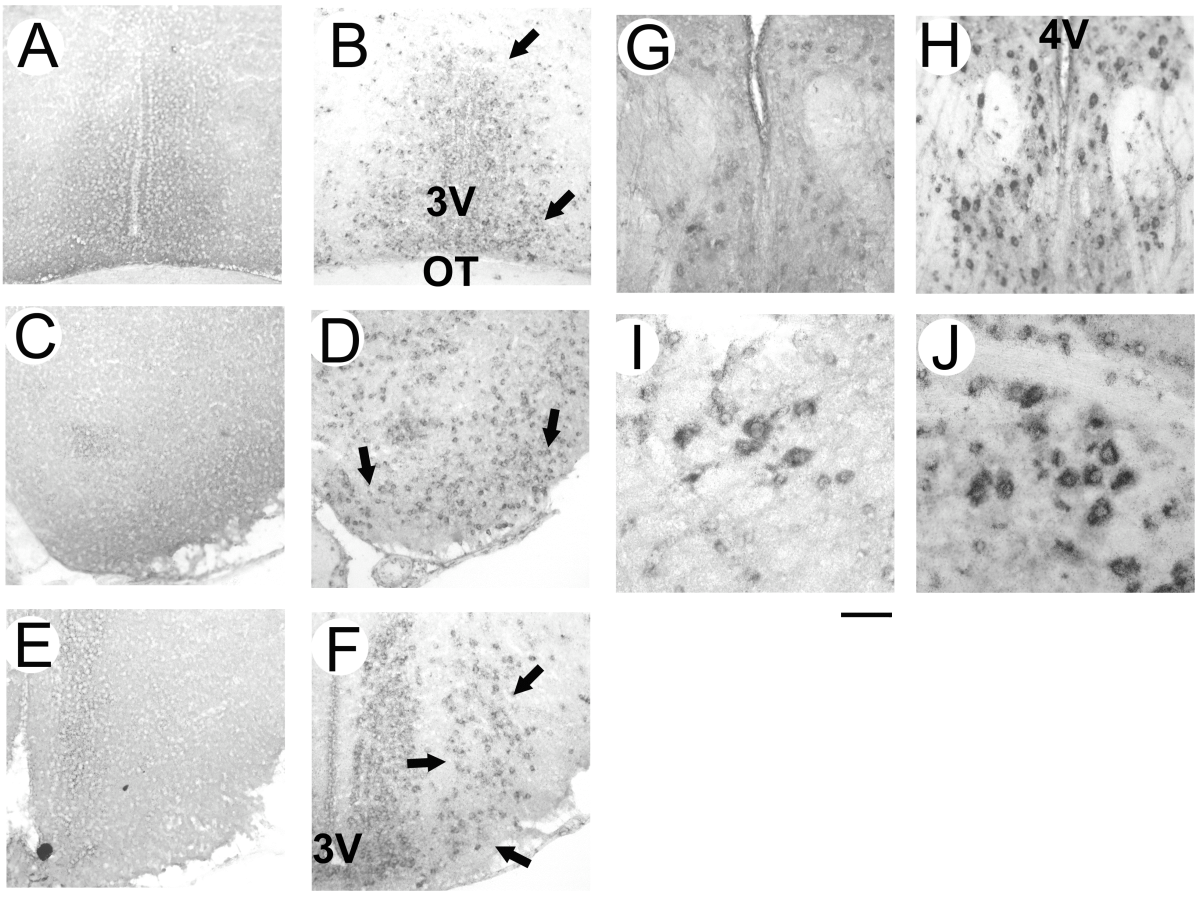

Figure 9. Representative photographs highlighting the distributions of 5αR1 (A, C, E, G, I) and 5αR2 (B, D, F, H, J). The regions are depicted from rostral to caudal: POA (A, B), AMY (C, D), VMH (E, F), nIII (G, H), and Amb XI/VII (I, J). 5αR1 is only present in the brainstem nuclei, while 5αR2 is present throughout the brain. Arrows indicate the boundary of the brain regions in (B), (D), and (F). Scale bar = 100 μm (A-H) or 50 μm (I, J). OT = optic tract; 3V = 3rd ventricle; 4V = 4th ventricle.

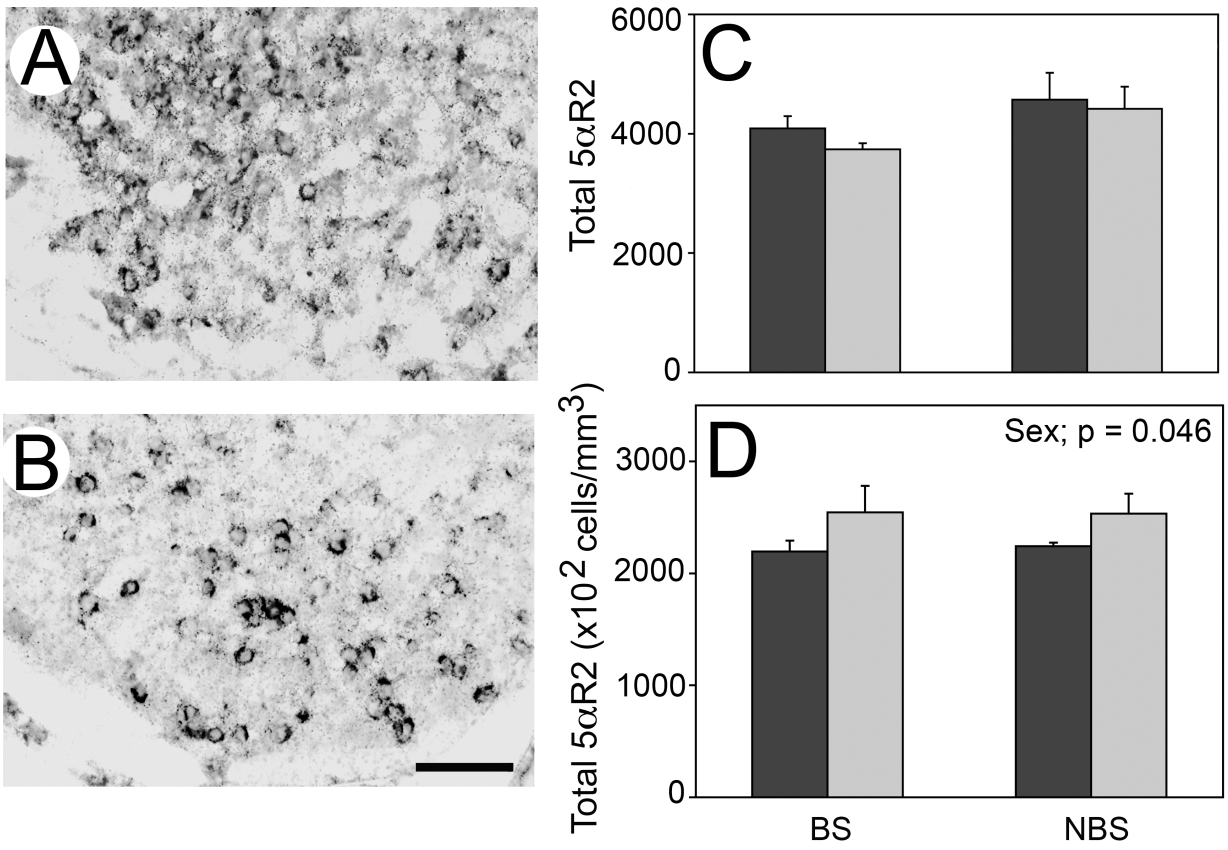

Figure 10. 5αR2 expression in the AMY. A female is depicted in (A) and a male is depicted in (B). A greater number of 5αR2 cells were detected during the NBS than BS (C). Females had a greater density of 5αR2 expressing cells than males (D). Males are depicted with black bars and females are depicted with grey bars. Scale bar = 50 μm for both photographs.

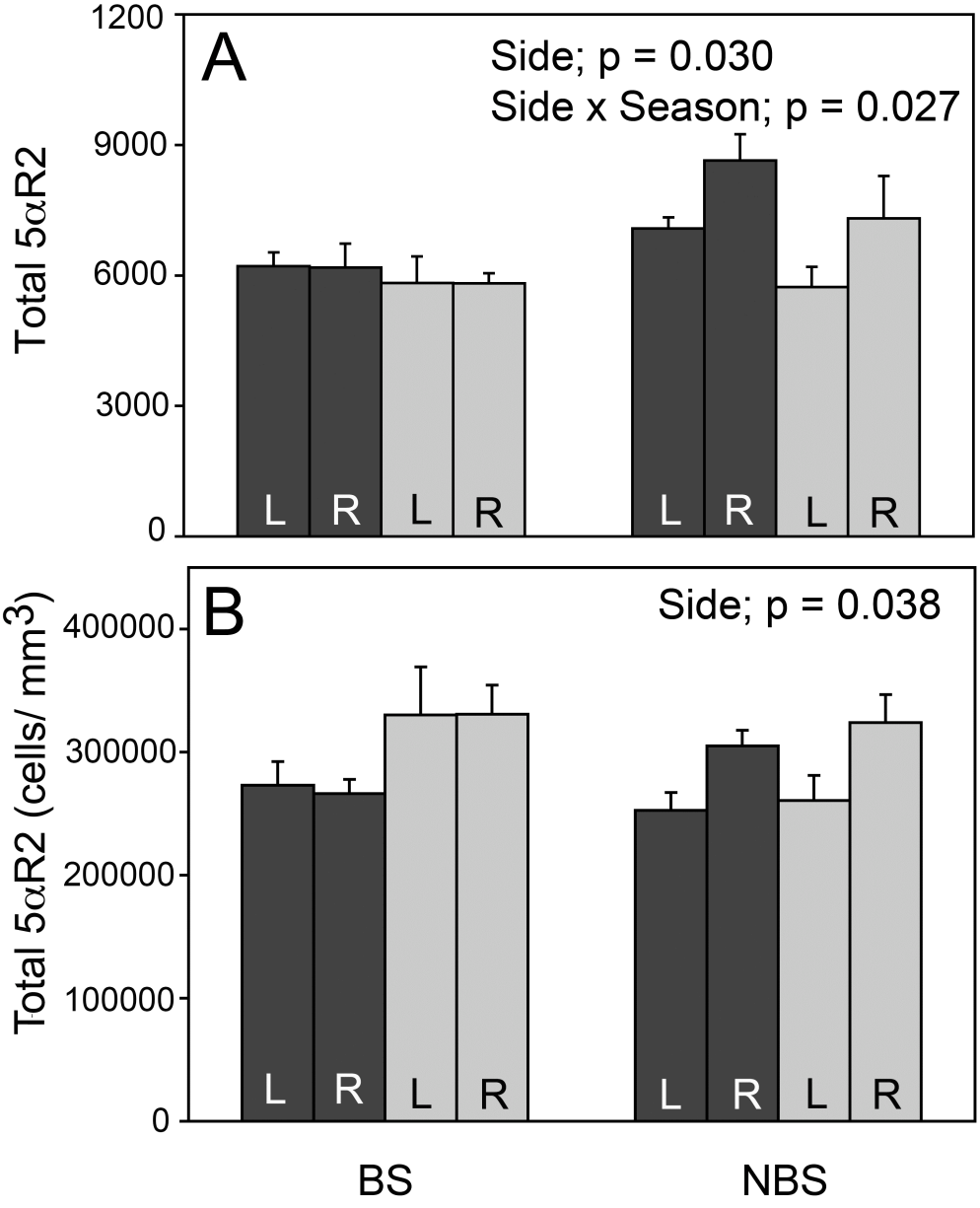

Figure 11. 5αR2 expression in the VMH showing number (A) and density (B) of cells. The right VMH expressed more 5αR2 cells than the left, with the number of 5αR2 positive cells increasing during the NBS (A). Parallel to the number of 5αR2 cells, the right side had a denser population of 5αR2 cells than the left (B). Males are depicted with black bars and females are depicted with grey bars.

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

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 activity levels are needed before specific conclusions can be drawn. *Key words: Androgen metabolism, preoptic area, amygdala, ventromedial hypothalamus, Anolis carolinensis, lizard*

Introduction

Hormones regulate the production of male- and female-specific sexual behaviors in a wide variety of species (Tennent et al., 1980, Steel, 1981, Fabre-Nys and Martin, 1991, Ball and Balthazart, 2002, Hull and Dominguez, 2007, Fusani, 2008). 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; Lephart, 1996, Lephart et al., 2001).

Neural aromatase is critical for male sexual behavior in a variety of species including Japanese quail, midshipman fish, zebrafish, musk shrews, rats, and mice (Rissman, 1991, Balthazart and Foidart, 1993, Riters et al., 2000, Bakker et al., 2004, Goto-Kazeto et al., 2004, Forlano et al., 2006, Roselli, 2007). Peripheral and neural aromatase is also important for females. For example, inhibitors of this enzyme decrease female canary sex behaviors, and aromatizable androgens increase female musk shrew copulatory behaviors (Balthazart, 1991, Rissman, 1991, Leboucher et al., 1998, Veney and Rissman, 2000). 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 (Roselli and Resko, 1984, Balthazart et al., 1990, Balthazart, 1991, Roselli et al., 1996, Roselli and Resko, 1997, Forlano and Bass, 2005, Zhao et al., 2008, Voigt et al., 2011). 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 (Lephart et al., 2001). 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 (Celotti et al., 1997). 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 (Negri-Cesi et al., 2008). In contrast, 5αR2 has a higher affinity for the hormone and is found in hypothalamic and hippocampal neurons in the adult brain (Poletti and Martini, 1999). In rat prefrontal cortex, 5αR2 is upregulated after T administration, but 5αR1 is not (Torres and Ortega, 2003, 2006). Thus, T appears to upregulate at least one isozyme of 5αR.

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; Lovern et al., 2001). 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 (Wheeler and Crews, 1978, Greenberg et al., 1984). 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 (Kendrick et al., 1995).

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 (Wade, 2011). However, aromatase does enhance behavioral expression. For example, while inhibition of the enzyme's activity in gonadectomized T-treated males did not significantly affect the display of sexual behavior, additional E2 treatment enhanced sexual motivation in male anoles (Winkler and Wade, 1998, Latham and Wade, 2010). In addition, data from inhibition of

aromatase in ovariectomized, T-treated females suggest that activity of the enzyme is important for female receptivity (Winkler and Wade, 1998, Latham and Wade, 2010). 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 (Rosen and Wade, 2001). The effects of T on whole brain aromatase activity are specific; T induces an increase only in the BS and only in males (Cohen and Wade, 2010b). 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 (Cohen and Wade, 2011).

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 (Rosen and Wade, 2000). 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 (Rosen and Wade, 2001, Cohen and Wade, 2010b). Unlike 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 (Cohen and Wade, 2010a).

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 (Cohen and Wade, 2010a, 2011), *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.

Experimental Procedures

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.

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-proprionate (Steraloids Inc., Wilton, NH) or left empty. Both ends were sealed using silicone adhesive (Dow Corning Corporation, Midland, MI). This dose of T reliably activates male sexual behaviors and increases neural aromatase and 5αR activities in this species (Neal and Wade, 2007b, Cohen and Wade, 2010b).

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 dessicant until further processing.

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-TR018W; originally produced by Wien Laboratories, sold by Fitzgerald, Concord, MA; as in Neal and Wade, 2007a). 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 5αR2 (GenBank ID: XM_003215965.1) were cloned previously (Cohen and Wade, 2010a, 2011). Briefly, sense (T7 for aromatase and SP6 for 5αR2) and antisense (T3 for aromatase and T7 for 5αR2) 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 anhydrase

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 (5αR2) probe in hybridization buffer, which consisted of 50% formamide, 4x SSC, 1x Denhardt's solution, 200 μg/ml fish sperm DNA, 10% dextran sulfate, 20 mM dithiothreitol, 250 μg/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 μl/ml Anti-Digoxigenin-AP Fab fragments (Roche) in MABT. After two hours, the color reaction was conducted by incubating the slides with 4.5 μl/ml NBT and 3.5 μl/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 5αR2), 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 (2010a, 2011) by a user blind to experimental group. Estimates of total counts of cells positive for aromatase or 5αR2 were obtained using the Optical Fractionator function. Brain areas were determined using a green anole atlas (Greenberg, 1982). After tracing the outline of a brain region (as defined by aromatase or 5αR2 expression) in each tissue section in which it existed, the software placed a

grid over each area (POA: 100x100 μm^2, AMY: 40x40 μm^2, VMH: 80x80 μm^2) and sampling sites (30x30 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, 2010a, 2011). 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 5αR2 were only expressed in the lateral portion of the VMH and thus were only quantified in that region (Cohen and Wade, 2010a, 2011). 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.

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.12a, 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 5a).

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. 12d, 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 5a).

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 ± 827 cells/mm^3. 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 5a). 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. 13). 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 5b).

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 ± 3674 cells/mm^3. The volume of the region in males and females is indicated in Table 5b.

In the VMH, males had a greater number of 5αR2 expressing cells than females ($F = 16.22$, $p < 0.001$; Fig. 14a). 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 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. 14b-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 5αR2 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 5b).

Discussion

In this study, we tested the hypothesis that T influences aromatase and 5αR2 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 5αR2, 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 5αR2 expression in this study, we did find two significant interactions, both involving 5αR2 expression in the VMH.

First, females treated with T had a higher density of 5αR2 cells in this region than blank-treated females, which suggests that T plays a role in 5αR2 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 5αR2 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 5αR activity in whole brain homogenates (Cohen and Wade, 2010b). Thus, the hormone likely has a relatively local effect on 5αR2 expression in the VMH. The lack of effects of T in other regions investigated in this study are 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 5αR2 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, 5αR2 may serve to reduce excess quantities of T and/or E2. Alternatively, the enzyme could affect reproduction by acting on progesterone, as 5αR2 metabolizes it into dihydroprogesterone (Lephart et al., 2001). In anoles, progesterone plays a facilatory role in female and male anole reproduction (Wu et al., 1985, Young et al., 1991), and its reduction via 5αR2 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 5αR activity does not consistently differ between unmanipulated intact males and females (Rosen and Wade, 2001) raises the possibility that DHT in the VMH could have a function in both sexes, such as influencing feeding behaviors (VMH function reviewed in King, 2006).

The second effect of T that we found was in BS males, such that blank-treated males had a denser population of 5αR2 expressing cells in the VMH than T-treated males. Again, the decrease in density of 5αR2 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 (Wade,

2005). DHT does facilitate them, but is not sufficient for the full expression of male-specific behaviors (Rosen and Wade, 2000). Assuming that sufficient DHT is present under conditions of high T to perform this function, 5αR2 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 (Newman, 1999, Goodson, 2005), and thus T in this region may play a facilitatory role in males. Decreased 5αR2 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 (Latham and Wade, 2010) 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 5αR2 expression. For example, during the BS, male anoles defend territories and attract mates using stereotypical displays (Greenberg and Noble, 1944, Mason and Adkins, 1976, Neal and Wade, 2007b). 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 (Jenssen et al., 1996, Orrell et al., 2004). 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 5αR2 expression in this study as in intact green anoles (Cohen and Wade, 2010a, 2011; Table 6), 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 5αR2 were unmanipulated (Cohen and Wade, 2010a, 2011). 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 (O'Bryant and Wade, 2002, Beck and Wade, 2009a). Innervation between the gonads and brain has been described, and the brain can modulate T release from the testes (Lee et al., 2002, Gerendai et al., 2005, Selvage et al., 2006). 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 5αR2 expression. These ideas warrant further investigation.

We determined the volume of the three brain regions using two different markers, aromatase and 5αR2. 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 (Cohen and Wade, 2010a, 2011). Both aromatase and 5αR2 expressing cells define similar regions of the POA and AMY as those based on morphological characteristics seen in Nissl stained tissue (Beck et al., 2008), 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 (Beck et al., 2008), only the lateral VMH appears to express aromatase and 5αR2 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 (Rosen et al., 2002,

Beck and Wade, 2009b), 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 5αR2 in the VMH. The role of T in regulating aromatase and 5αR2 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 (Cohen and Wade, 2010b). 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 5αR2 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 (Connolly et al., 1990, Roselli et al., 1998). 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 (Roselli and Resko, 1989, Hutchison et al., 1991, Roselli et al., 1997). Thus, aromatase activity in specific areas is not always influenced by T treatment. 5αR activity is also not increased in Japanese quail by T treatment in the POA, VMH, or nucleus taeniae (analogous to the mammalian amygdala; Balthazart et al., 1990). Although these data suggest that 5αR 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 5αR2 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. (2010) 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 (Konkle and Balthazart, 2011). 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 5αR2. 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 (Wagner and Morrell, 1996). 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 5αR2 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 5αR2 in the reptilian brain under a variety of conditions influencing reproductive behavior.

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Table 5: Volumes of the POA, AMY, and lateral VMH as defined by aromatase (A) and 5αR2 (B) expression (μm^3 X 10^7). 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. M = male, F = female.

		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. 5αR2	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)

Table 6. Comparison of aromatase (A) and 5αR2 (B) expression patterns in the POA, AMY, and VMH in intact (data from Cohen and Wade 2010a, 2011) and T-treated animals (current study). 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.

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

[1]Data from (Cohen and Wade, 2011)

[2] Data from present study

[3] Data from (Cohen and Wade, 2010a)

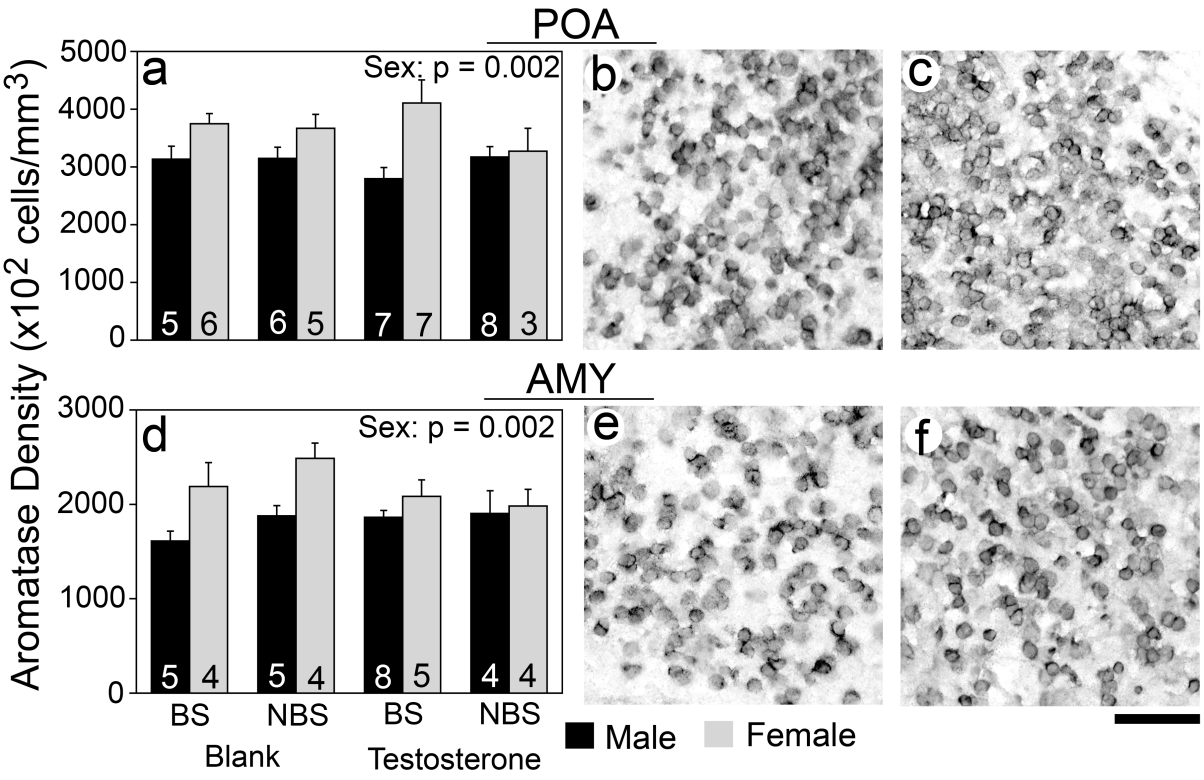

Figure 12. Density of aromatase expressing cells in the POA (a, b, c) and AMY (d, e, f). Females had a greater density than males in the POA, regardless of season or treatment (a). Females also had a greater density of these cells in the AMY (d). Males are depicted in (b) and (e); females are depicted in (c) and (f). All animals are non-breeding and blank-treated. Scale bar = 50 μm. Final sample sizes are indicated in panels (a) and (d).

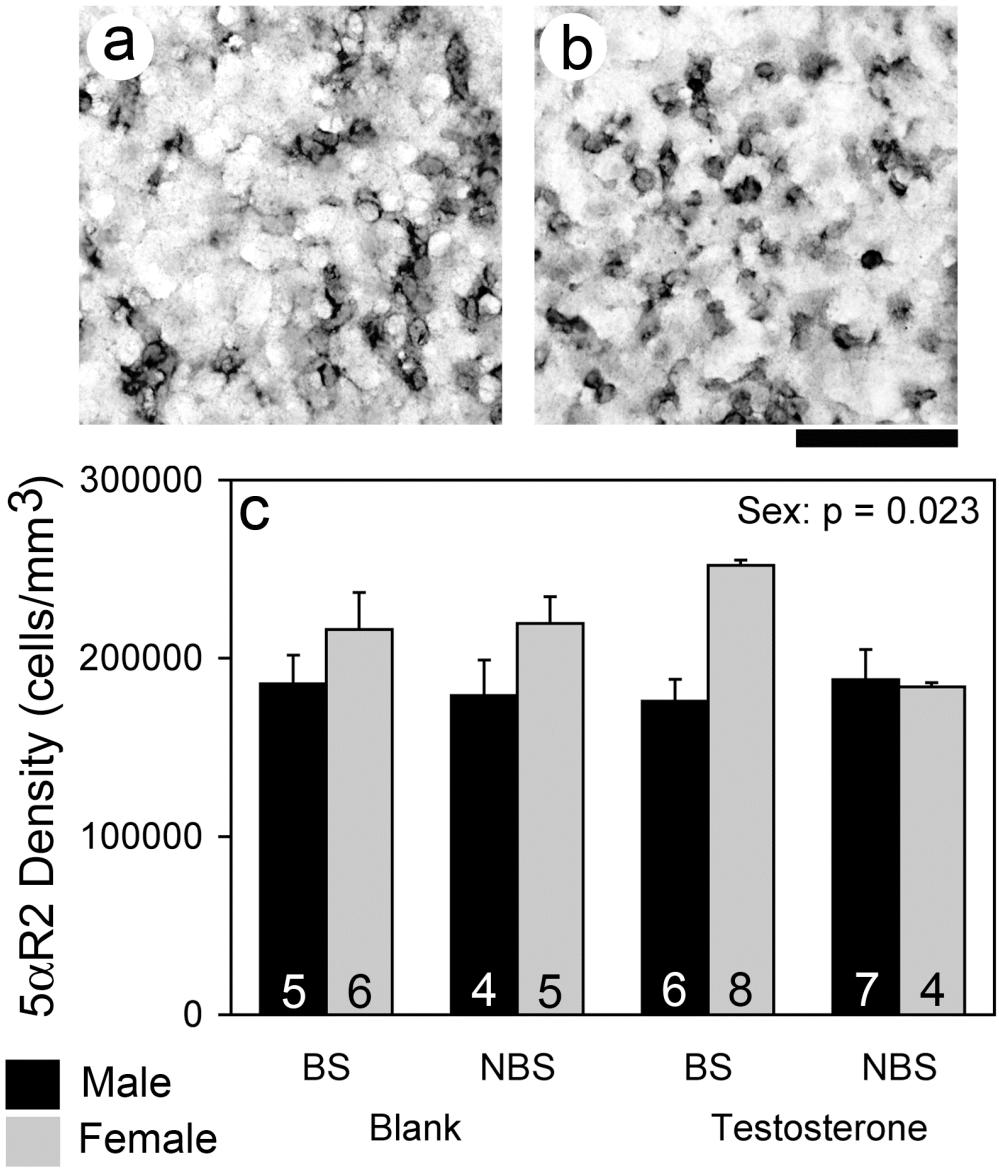

Figure 13. Density of 5αR2 expressing cells in the POA. The photos depict a breeding male (a) and female (b) both T-treated. Females had a greater density of 5αR2 expressing cells in the POA than males, regardless of season or treatment (c). Scale bar = 50 μm. Final sample sizes are indicated.

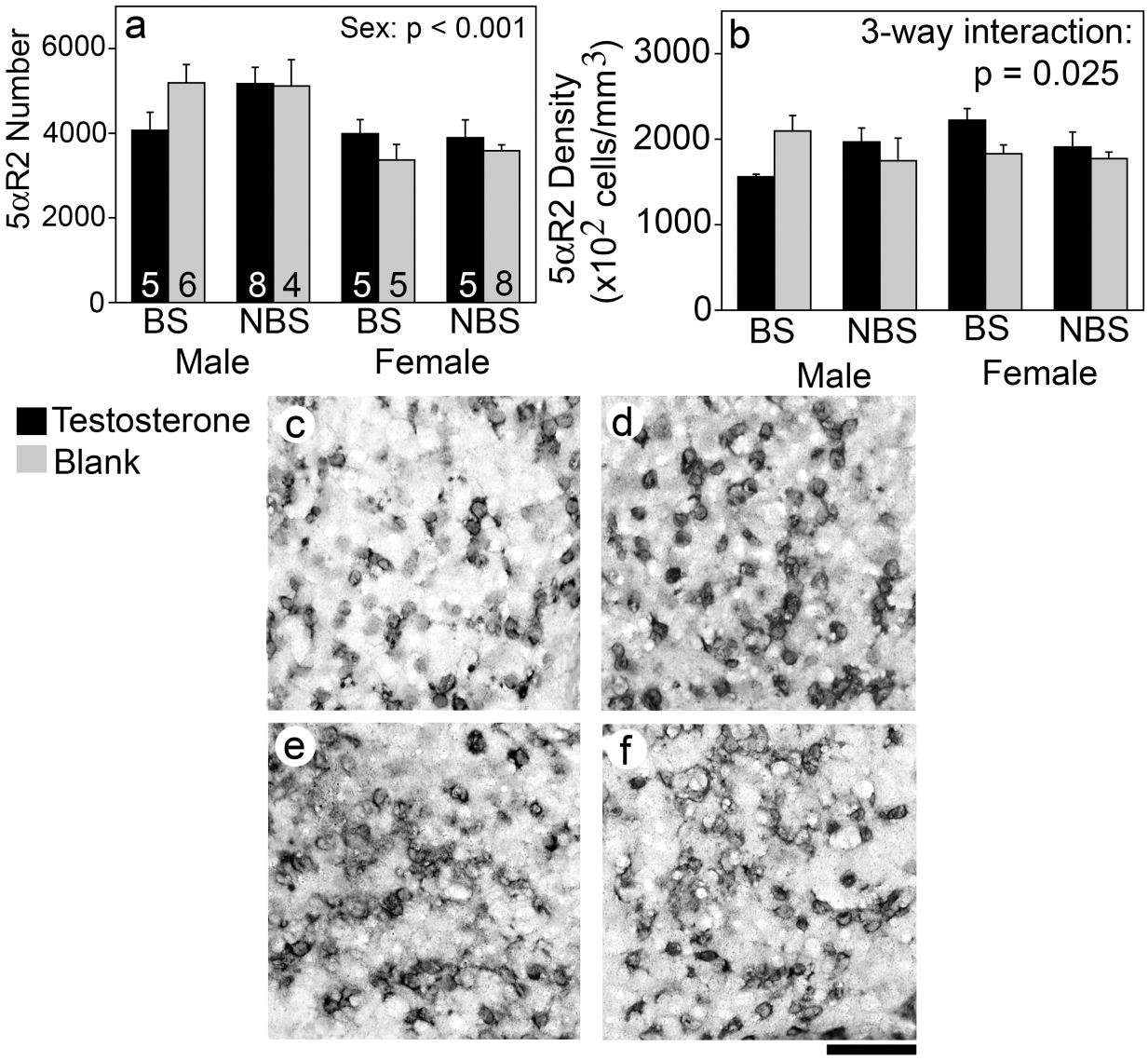

Figure 14. 5αR2 expression in the VMH. The number of 5αR2 expressing cells was greater in males than females (a). The density was greater in T- than blank-treated females ($p = 0.041$), and in blank- than T-treated BS males ($p = 0.027$; b). The photos depict T- (c, e) and blank-treated (d, f) animals. Males are shown in (c, d), and females in (e, f). Note the lower density of 5αR2 cells in the T-treated male (c) and blank-treated female (f) as compared to the rest of the photos. All photos were taken from the BS. Scale bar = 50 μm. Bl = blank. Final sample sizes are indicated in (a).

Chapter 6: Expression of aromatase and two isozymes of 5α-reductase in the developing green anole forebrain

Abstract

Neural steroids, and the enzymes that produce these hormones, are important for sexual differentiation of the brain during development. Aromatase converts testosterone into estradiol. 5α-reductase converts testosterone to 5α-dihydrotestosterone and occurs in two isozymes: type 1 (5αR1) and type 2 (5αR2). Each of these enzymes is present in the developing brain in many species, although no work has been done examining expression of all three enzymes in reptiles with genetic sex determination. In this study, we evaluated mRNA expression of neural aromatase, 5αR1 and 5αR2 on the day of hatching and at day 50 in one such lizard, the green anole. We describe the distribution of these enzymes throughout the brain and quantification of expression in three regions that control adult sexual behaviors: the preoptic area (POA) and ventromedial amygdala (AMY), which are involved in male displays, as well as the ventromedial hypothalamus, which regulates female receptivity. Younger animals had a greater number (POA) and density (AMY) of 5αR1 expressing cells, consistent with a role for this isoform in early post-hatching development of regions important for masculine function. Unlike 5αR1, we detected no effects of sex or age on 5αR2. Aromatase expression in the POA was highly variable at day 50, suggesting that it could be differentiating during the ages examined. Compared to data from adults, the present results support the idea that the green anole forebrain is still differentiating 50 days after hatching, and that 5αR1 may play a role in early development. *Key words: Androgen metabolism, preoptic area, amygdala, ventromedial hypothalamus, Anolis carolinensis, lizard*

Introduction

Steroid hormones are involved in the regulation of adult reproductive behaviors (activation), and play key roles in the developing brain (organization; Arnold and Breedlove, 1985; Phoenix et al., 1959). Local metabolism of these hormones within the brain is in many cases a necessary step in these processes, particularly the conversion of testosterone (T) into estradiol (E2) via the aromatase enzyme and to 5α-dihydrotestosterone (DHT) by 5α-reductase (5αR; Lephart, 1996; Lephart et al., 2001).

Brain aromatase is present during development in many species such as Japanese quail, where it is not sexually dimorphic (Aste et al., 2008; Schumacher and Hutchison, 1986) and mice and rats, where aromatase expression and activity in the hypothalamus is higher in males than females (Hutchison et al., 1994; Hutchison et al., 1997; Hutchison et al., 1999). Furthermore, T treatment during development can increase both expression and activity of neural aromatase in rodents and quail (Bardet et al., 2010; Hutchison et al., 1997; Hutchison et al., 1996; Romeo et al., 1999). Aromatase and/or estrogenic metabolites during development are required for organizing appropriate adult sexual behavior in rodents and Japanese quail (Bakker et al., 2003; Brock et al., 2011; Hosokawa and Chiba, 2009; Houtsmuller et al., 1994). In developing reptiles, the role of aromatase has largely been investigated in gonadal rather than brain differentiation, and the majority of this work has been done in reptiles with temperature-dependent sex determination (e.g. Belaid et al., 2001; Lance, 2009; Pieau et al., 1999; Wibbels and Crews, 1994; but see Ganesh et al., 1999; Wennstrom and Crews, 1995). Among these animals, aromatase is expressed in the developing brains of alligators, leopard geckos, and red-eared

slider turtles, and both whole brain activity and mRNA appears to be sexually monomorphic (Crews et al., 2001; Endo et al., 2008; Milnes et al., 2002).

Much less work has been done on 5αR, with none on developing reptiles. Two isozymes exist, 5αR1 and 5αR2. They are differentially expressed during brain development, such that in rodents 5αR1 mRNA levels are higher and consistent across ages, whereas 5αR2 levels show a peak early in development, and then decrease to relatively low adult levels (Poletti et al., 1998; Urbatzka et al., 2007). Interestingly, T during development selectively regulates the two isozymes of 5αR, such that 5αR2 expression is increased by T in males only (Poletti et al., 1998) and 5αR1 is not regulated by T (Karolczak et al., 1998; Poletti et al., 1998). Inhibiting 5αR can reduce masculinization of the brain and/or behavior in male rats and zebra finches (Grisham et al., 1997; Ribeiro and Pereira, 2005), while treatment with DHT in females can cause modest masculinization of the brain in zebra finches (Schlinger and Arnold, 1991). Thus, 5αR in the brain is involved in normal sexual differentiation.

Green anole lizards (*Anolis carolinensis*) offer an excellent model to examine the expression of aromatase, 5αR1 and 5αR2 during development. These lizards are common in the southeastern United States, and adult aromatase and 5αR2 mRNAs are sexually dimorphic in three brain areas critical for reproduction (Cohen and Wade, 2010a; 2011): the preoptic area (POA) and ventromedial amygdala (AMY), which are important in male reproductive behaviors, and the ventromedial hypothalamus (VMH), which is important in female behaviors (Wade, 2011). In adults, E2 production is important for female receptivity and plays a role in sexual motivation in males (Latham and Wade, 2010; Winkler and Wade, 1998). 5αR activity is critical to the full expression of male sexual behaviors (Rosen and Wade, 2000), although T itself is likely the primary activator of male behaviors (Wade, 2011). T treatment in adulthood

increases whole brain activities of aromatase and 5αR, as well as 5αR2 mRNA (Cohen and Wade, 2010b; 2011). 5αR1 is not expressed in the adult hypothalamus, although it is expressed in the brainstem (Cohen and Wade, 2011). It is currently unknown when sexually dimorphic expression of these genes begins.

This study examines the patterns of expression of aromatase, 5αR1 and 5αR2 mRNA in the brain during two periods in development, the day of hatching and on day 50. The two ages include a time prior to (day 0; P0) and after (day 50; P50) males have higher levels of circulating T than females (sex difference occurs by 35 days after hatching; Lovern et al., 2001). Thus, these two ages also allow us to determine whether changing T levels between males and females are likely to influence steroid metabolizing enzyme expression in the developing green anole forebrain.

Methods

Animals

During the breeding season (April), adult males and females were purchased from Charles Sullivan (Nashville, TN). They were group housed with one male and 5 females in each 29 gallon terrarium. These glass tanks contained peat moss as substrate, as well as water dishes, sticks and rocks. To facilitate egg laying, nest boxes filled with damp peat moss were provided. Ambient temperatures ranged from 28 ºC during the day to 19 ºC at night (14:10 light/dark cycle). Full spectrum bulbs and heat lamps were provided directly above the cages to allow basking temperatures of 10 ºC above ambient. Relative humidity was maintained at about 70%. Animals were fed crickets three times a week and misted daily with water.

Nest boxes were checked daily, and eggs were individually placed in moistened vermiculite (1:1 by mass with dH_20) in a cup covered with plastic wrap to maintain moisture. The eggs were incubated at 27 ºC until hatching, which took an average of 33 days in this study. Hatchlings were placed in 10-gallon tanks (set up similarly to those used for adults) until experimental use. Up to 10 hatchlings were housed together following unique toe-clipping for identification. They were fed fruit flies and misted with water daily.

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

Tissue Collection

On P0 and P50, lizards were rapidly decapitated and their whole heads collected, similar to Beck and Wade (2009). The heads were immediately frozen in cold methyl-butane and stored at –80 ºC until processing. They were sectioned coronally at 16 µm into four alternate series and thaw mounted onto SuperFrost Plus slides (Fisher Scientific; Hampton, NH). The slides were stored at –80 ºC with dessicant until further processing.

Gonadal sex of the hatchlings was determined through visual inspection at the time of euthanasia. In addition, torsos were collected and fixed in Bouin's solution (9% formaldehyde, 0.9% saturated picric acid, 5% glacial acetic acid). They were then dehydrated and embedded in paraffin. The torsos were sectioned at 10 µm, stained with hematoxylin and eosin, and sex was confirmed via microscopic examination of the gonads.

In situ hybridization

Aromatase, 5αR1 and 5αR2 were cloned and linearized previously (Table 1; Cohen and

Wade, 2010a; 2011). Sense (T7 for aromatase and SP6 for 5αR1 and 5αR2) and antisense (T3 for aromatase and T7 for 5αR1 and 5αR2) probes were transcribed using the Digoxigenin RNA Labeling Kit per manufacturer's instructions (Roche Diagnostics; Indianapolis, IN). Probes were cleaned using a G50 Sephadex bead column and stored at –80 °C until use. For each gene, one set of slides from each animal was used for the antisense reaction. As controls, 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 0.1M phosphate buffered saline (PBS; pH 7.4). They were treated with 0.25% acetic anhydrase in triethanolamine-HCl with 0.9% NaCl buffer (pH 8.0). Slides then incubated overnight at 55 °C with 200 ng/ml (aromatase and 5αR1) or 100 ng/ml (5αR2) probe in hybridization buffer (50% formamide, 4x 3M NaCl and 0.4M Na Citrate [SSC], 1x Denhardt's solution, 200 μg/ml fish sperm DNA, 10% dextran sulfate, 20 mM dithiothreitol, 250 μg/ml tRNA, 2 mM EDTA, and 0.1% Tween-20). The next day, slides were rinsed in 2x SSC and 0.2x SSC at 60 °C. They were then treated with 0.9% H_2O_2 in maleic acid buffer (pH 7.5) with 0.1% Tween-20 (MABT) for 30 min. 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 μl/ml Anti-Digoxigenin-AP Fab fragments (Roche) in MABT. After two hours, the color reaction was conducted by incubating the slides with 4.5 μl/ml NBT and 3.5 μl/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 distinct reaction product within the cytoplasm of individual cells with an absence of labeling on the sense-treated slides (about 10 minutes for each gene; Fig. 1), and the reaction was stopped with 1M Tris and 0.5M EDTA (pH 8.0). Slides were dehydrated with increasing concentrations of ethanol and treated with citrisolv (Fisher Scientific). They were coverslipped with VectaMount

mounting medium (Vector Laboratories; Burlingame, CA) and allowed to dry.

Stereological analysis

The slides were examined under brightfield illumination using Stereo Investigator software (MicroBrightfield, Inc.; Williston, VT) following Cohen and Wade (2010a; 2011) by a user blind to experimental group. Estimates of total counts of cells positive for each gene were determined using the Optical Fractionator function. After tracing the outline of a brain region (as defined by gene expression) in each tissue section in which it existed, the software placed a grid over each area (POA: 100x100 μm^2, AMY: 40x40 μm^2, VMH: 80x80 μm^2) and sampling sites (30x30 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 counts were taken. 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. The brightness and contrast was modified for all photomicrographs included in the figures only to insure that the images matched what was analyzed on the computer screen.

Nissl analysis

Due to sex and age differences in enzyme expression in the POA and AMY (see Results), one series of sections from the animals was stained with thionin. The number of total cells, cell density and volume of the POA and AMY were analyzed using the stereological procedures described above.

Statistical Analysis

Analysis of each gene and brain region was conducted separately using data averaged from the two sides of the brain within each individual. Two-way ANOVAs were performed on the means of individuals to determine the effects of sex and age on the expression of aromatase, 5αR1 and 5αR2 in the POA and VMH. Interactions were broken down with Bonferroni-corrected t-tests as appropriate. Final sample sizes ranged from 4 to 8 animals per group, and are included in the figures.

Due to tissue damage from processing, individual sections from a number of AMYs in P0 animals were damages. We therefore could not perform stereological analysis on cells expressing each of the enzymes in the AMY of that age group. Instead, we analyzed sections from P0 animals as they were available (using identical procedures as above), and the software calculated the number of cells and the volume of the AMY in each section analyzed. Density estimates were obtained for each section from these values and averages for each individual were included in statistical analyses. This procedure was also used for all P50 animals in order to compare densities per section across ages. Two-way ANOVAs were conducted on the density per section to determine the effects of sex and age. Because the AMY was intact in a greater number of the P50 animals, we were able to perform stereological analysis on them and therefore also used t-tests to compare the effects of sex on the number of cells and density of cells expressing all three enzymes at that age.

Results

Distribution of aromatase, 5αR1 and 5αR2

Aromatase and 5αR2 were similarly distributed in the developing anole brain as in adults (Cohen and Wade, 2010a; 2011). 5αR1, on the other hand, was more widely expressed in the forebrain of developing anoles than in adulthood (Cohen and Wade, 2010a). Its distribution appeared similar to that of 5αR2 in both development and adulthood. Aromatase, 5αR1 and 5αR2 were expressed in discrete regions throughout the brain (Fig. 2). Overall, aromatase expression was highest in the VMH, AMY and the anterior dorsal ventricular ridge (ADVR; Table 2). 5αR1 expression was generally diminished compared to aromatase, with the lowest levels present in the nucleus accumbens and septum, and no expression in the bed nucleus of the stria terminalis. 5αR2 expression was greatest in the ADVR and dorsal cortex.

Aromatase

In the POA, the number and density of aromatase positive cells, as well as the volume of the region defined by aromatase labeling, were statistically equivalent between the sexes and ages (all $F < 3.42$, $p > 0.08$). However, in one P50 male the estimated total number of aromatase expressing cells was 1.8 standard deviations below the mean. Due to variability across all data points in this group, the value was not a statistical outlier (Dixon's test; Sokal and Rohlf, 1981). However, if it is removed from analysis, P50 animals had more cells expressing aromatase than P0 animals ($F = 7.41$, $p = 0.014$; Fig. 3). Additionally, omitting that animal revealed a sex by age interaction on the number of cells expressing aromatase ($F = 4.94$, $p = 0.039$). P50 males had more aromatase expressing cells than did P50 females ($t = 3.21$, $p = 0.008$, $\alpha = 0.0125$), and P50 males had a greater number of aromatase expressing cells than P0 males ($t = 3.33$, $p = 0.01$, $\alpha = 0.0125$).

In the AMY, no effects of sex among P50 animals were detected on the number or density of cells, or the volume of the region (all $t < 1.25$, $p > 0.240$; Table 3). In the analysis that included both ages, no effects of sex or age, or interaction between the variables were detected on the average density per section (all $F < 0.25$, $p > 0.626$).

We also found no significant main effects of sex or age, or any interactions on the number or density of cells expressing aromatase, or on volume of the VMH as determined with labeling of aromatase mRNA expressing cells (all $F < 2.84$, $p > 0.109$; Table 3).

5αR1

In the POA, a greater number of 5αR1 expressing cells was detected at P0 than P50 ($F = 4.69$, $p = 0.046$; Fig. 4). P0 animals also had a larger volume of the region as defined by 5αR1 expression than P50 animals ($F = 7.19$, $p = 0.016$). No other effects were detected on these variables (all $F < 1.53$, $p > 0.235$). We saw no effects of sex, age, or an interaction on the density of 5αR1 expressing cells (all $F < 1.34$, $p > 0.263$). In Nissl-stained tissue, no significant effects were detected on the number and density of cells overall, or on the volume of the region (data not shown; all $F < 3.31$, $p > 0.089$), suggesting that the differences in cell number and volume of this brain region are based on changes in 5αR1 expression within existing cells.

In the AMY, the number and density of 5αR1 expressing cells at P50 was greater in females than males (number: $t = 2.48$, $p = 0.042$; density: $t = 3.30$, $p = 0.013$; Fig. 5b, c). There was no effect of sex in the volume of P50 animals (data not shown; $t = 1.46$, $p = 0.188$). Analysis of thionin-stained tissue revealed no effects of sex on cell number, density or region volume in P50 animals (data not shown; all $t < 0.89$, $p > 0.396$). The density per section of 5αR1 expressing cells in the AMY was greater at P0 than P50 ($F = 16.94$, $p = 0.001$; Fig. 5a).

We saw no effect of sex ($F = 0.33$, $p = 0.48$), but did detect an interaction between sex and age ($F = 4.49$, $p = 0.048$) on the density per section. This value was increased in males at P0 compared to P50 ($t = 3.64$, $p = 0.005$, $\alpha = 0.0125$). Further, we detected a trend such that P50 females had a greater density per section than P50 males ($t = -2.78$, $p = 0.018$, $\alpha = 0.0125$). No other effects of age or sex were detected ($t < 2.33$, $p > 0.045$, $\alpha = 0.0125$). We also detected no significant effects on the density per section in Nissl stained tissue (data not shown; all $F < 3.83$, $p > 0.068$).

In the VMH, there were no effects of sex, age, or interactions in the number and density of 5αR2 expressing cells, and the volume of the region as defined by 5αR1 expression was equivalent across groups (all $F < 3.62$, $p > 0.07$; Table 3).

5αR2

In the POA, we found no effects of sex, age, or interactions in the number or density of 5αR2 expressing cells (all $F < 2.61$, $p > 0.122$; Table 1). However, the volume, of the region, as defined by 5αR2 mRNA was larger in P0 than P50 animals (data not shown; $F = 9.52$, $p = 0.006$). No other effects were detected on the volume of the region, as defined by 5αR2 expression (all $F < 1.41$, $p > 0.250$).

At P50, no effects of sex were detected on the number or density of cells, or the volume of the AMY ($t < 0.68$, $p = 0.508$; Table 3). Parallel results were detected on the average density per section of the cells for all groups; there were no effects of sex, age, or interactions on the density of 5αR2 expressing cells (all $F < 2.88$, $p > 0.106$).

No significant effects were detected in the VMH (all $F < 1.21$, $p > 0.285$; Table 3).

Discussion

The present experiment demonstrates that aromatase, 5αR1 and 5αR2 are expressed in the developing anole lizard forebrain and that expression of these enzymes changes between the day of hatching and 50 days later. The three enzymes are discussed individually below with context from other species.

Aromatase

Aromatase mRNA in developing green anoles was not different between the sexes in the POA, AMY, or VMH. This contrasts with adults of this species in which males have a greater number of aromatase expressing cells in the POA than females, and females have an increased density of aromatase positive cells in the AMY and VMH than males (Cohen and Wade, 2011). The fact that these sex differences were not detected in the present study suggests that the juvenile anoles at the ages that we sampled had not yet differentiated, at least in terms of aromatase expression. This pattern is largely consistent with other species. Although work from some vertebrates has demonstrated sex differences in brain aromatase activity during development (e.g. mice; Hutchison et al., 1995), studies in rats, frogs, and fish have indicated no changes in aromatase activity between developing males and females (Blaquez et al., 2008; Konkle and McCarthy, 2011; Urbatzka et al., 2007). In both rodents and birds (Japanese quail), neural aromatization of T during development is important for the organization of both adult male and female reproductive behaviors (Bakker et al., 2006; Bakker et al., 2004; Brock et al., 2011). Similarly, neural aromatase may also be important for anole development, although more work is necessary to determine the extent of the role of aromatase in reptilian brain maturation.

We did find substantial variability in aromatase mRNA expression in the POA among P50 males. This variability of expression and the fact that our Nissl analysis suggests that the morphology of the POA is not yet differentiated are consistent with the idea that these animals may be undergoing sexual differentiation of aromatase expression at 50 days after hatching. This idea parallels data from other structures critical for male reproductive displays, which begin to differentiate around two months after hatching (O'Bryant and Wade, 2001). While the full developmental trajectory has not been mapped in these animals, green anoles likely do not become sexually mature until at least six months after hatching (Crews, 1980). Puberty has not been well described in reptilian species, although data from alligators suggests that it is a lengthy process (Edwards et al., 2004). Thus, the present data suggest that aromatase expression in the AMY and VMH of P50 anoles has not yet begun to differentiate, while expression in the POA may be in early stages of the process.

5α-reductase

Much less information is available from the literature on the expression of the two forms of 5αR than on aromatase. In general, no sex differences in the developing brain have been detected in the expression of the two isozymes or overall 5αR activity in various species, including South African clawed frogs, Japanese quail, mice, and rats (Hutchison and Schumacher, 1986; Jacobson et al., 1997; Karolczak et al., 1998; Urbatzka et al., 2007). In the present experiment, both 5αR1 and 5αR2 are expressed in specific regions throughout the developing anole brain, including forebrain limbic areas that regulate the display of sexual behaviors in adulthood. Previous work in adult animals has shown that 5αR2 mRNA is expressed across the brain while 5αR1 mRNA is only detected in the brainstem (Cohen and

Wade, 2010a). These results suggest that forebrain expression of 5αR1 is decreased in adulthood and therefore might play a specific role in the development of these regions in anoles, and perhaps reptiles more broadly. This pattern differs from that in rodents in which 5αR1 is widely expressed in the brain in adults and 5αR2, with higher expression in females than males, is expressed in the forebrain during development - opposite of what we have found in lizards (Melcangi et al., 1998; Sanchez et al., 2006). Based on patterns of expression, it has been hypothesized that, in rodents, 5αR2 is important for masculinization and defeminization of the brain and 5αR1 is important for breaking down excess T from the brain (Poletti et al., 1998; Torres and Ortega, 2006). It is likely that these enzymes also play similar roles in anoles, although we suggest that, based on expression patterns in anoles, 5αR1 plays a role in development whereas 5αR2 may be more important for modulating T levels in the brain. Additional work is needed to determine the role of 5αR1 during development, but based on the present information it appears that 5αR1 and 2 may have been co-opted for different functions in lizards and rodents.

A number of sex and age differences in the green anole POA and AMY were detected in 5αR1 mRNA expression. For example, more cells expressed 5αR1 in the POA of P0 than in P50 animals. Because there is no difference in the total number of cells in the POA at these two ages, it appears that expression of 5αR1 decreases within existing cells as the animals get older. This decrease in 5αR1 with age is consistent with the lack of expression of 5αR1 in the adult POA (Cohen and Wade, 2010a). This pattern suggests that 5αR1 plays a role in the organization of the structure and/or function of the POA. In the AMY, the density of labeled cells per section also decreases between P0 and P50. Similar to the POA, the total number of cells in the AMY is consistent across this period, suggesting that expression decreases within cells. Additionally, we

detected sex differences such that P50 females had more cells expressing 5αR1 than males, supporting the idea that 5αR1 might play a role in female development in this area. DHT production in the AMY may be important for females, although it is difficult to speculate on a specific function. Perhaps more relevant is the idea that 5αR can also reduce progesterone to 5α-dihydroprogesterone (Lephart et al., 2001). In adult anoles, progesterone synergizes with E2 in females to increase receptivity, and works with T to increase male reproductive behaviors (Wu et al., 1985; Young et al., 1991). Although it is currently unknown whether progesterone plays a role in anole development, the present data suggest that more work needs to be done to examine this possibility.

Unlike 5αR1, 5αR2 expression in developing anoles was equivalent across the two ages and between the sexes. This is consistent with work from many other species (see above) and in adult anoles. For example, no sex differences in expression of 5αR2 exist in either the POA or VMH, although there is a female-biased difference in the adult AMY (Cohen and Wade, 2010a). Thus, similar to the aromatase discussion above, it is likely that anoles have not completely differentiated by 50 days after hatching and 5αR2 has not begun to differentiate in the AMY.

Conclusion

Based on the present data, it appears that, by 50 days after hatching, aromatase, 5αR1 and 5αR2 have not yet reached adult patterns of expression. We have found that aromatase and 5αR2 are distributed similarly across two ages during development in the green anole brain and largely do not differ between the sexes at those ages, although it appears that aromatase in the POA may be in the process of differentiation. 5αR1, on the other hand, seems to be more

important in the forebrain during development than adulthood because it appears to be absent in mature animals. Currently, not much is known about the distribution of the individual isozymes of 5αR across species, and our work raises some questions. For example, the timing of 5αR1 and 2 expression in lizards is opposite that of rodents, suggesting that these isozymes may perform opposite functions in both groups. More work needs to be done to determine the role of these enzymes in anoles, and the current work highlights the importance of comparative studies in understanding the role of testosterone-metabolizing enzymes in development.

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Table 7. Primers used to clone anole-specific aromatase, 5αR1, and 5αR2.

	Primers (5' to 3')		Probe length (bp)	NCBI Accession number
Aromatase	Forward:	GACATGCCGAAGCTGAA	181	XM_003225883
	Reverse:	TTGGGAAGAACTCAAGCCGA		
5αR1	Forward:	TGATGCTGCCGCTGAGCAA	658	XM_003220033.1
	Reverse:	TTCCTGTTGCGTGGATAGT		
5αR2	Forward:	CTTGGTTCCTGCAGGAGTT	576	XM_003215965.1
	Reverse:	GGTAGCTGCTGAATGTCCT		

Table 8. Relative intensity of aromatase, 5αR1, and 5αR2 expression in specific brain regions. Patterns were consistent between age and sex. Regions were based on an atlas of the green anole brain (Greenberg, 1982).

Brain Area	Aromatase	5αR1	5αR2
Anterior dorsal ventricular ridge	+++	++	+++
Bed nucleus of the stria terminalis	+	-	+
Dorsal cortex	++	++	+++
Nucleus accumbens	+	+	++
Oculomotor and trochlear nuclei	++	++	++
Preoptic area	++	++	++
Septum	+	+	+
Torus semicircularis	++	++	++
Ventromedial amygdala	+++	++	++
Ventromedial hypothalamus	+++	++	++

Table 9. Average total number and density ($x10^2$ cells/mm^3) of cells expressing aromatase (A), 5αR1 (B), and 5αR2 (C) in the three forebrain regions analyzed. Standard errors are in parentheses. Values that were not statistically significant are listed here and those associated with significant effects are depicted in the figures.

		POA	AMY	VMH
A. Aromatase	Number	Fig. 1	P50 only: 3,198 (228)	5,186 (343)
	Density	6,371 (190)	Both ages*: 5,535 (277)	3,949 (219)
B. 5αR1	Number	Fig. 2	Fig. 3	3,114 (118)
	Density	3,698 (144)	Fig. 3	2,748 (119)
C. 5αR2	Number	6,098 (307)	P50 only: 2,185 (157)	3,460 (205)
	Density	5,271 (216)	Both ages*: 3,388 (115)	3,174 (140)

* Density per section. The density of cells in the total area is similar (see text).

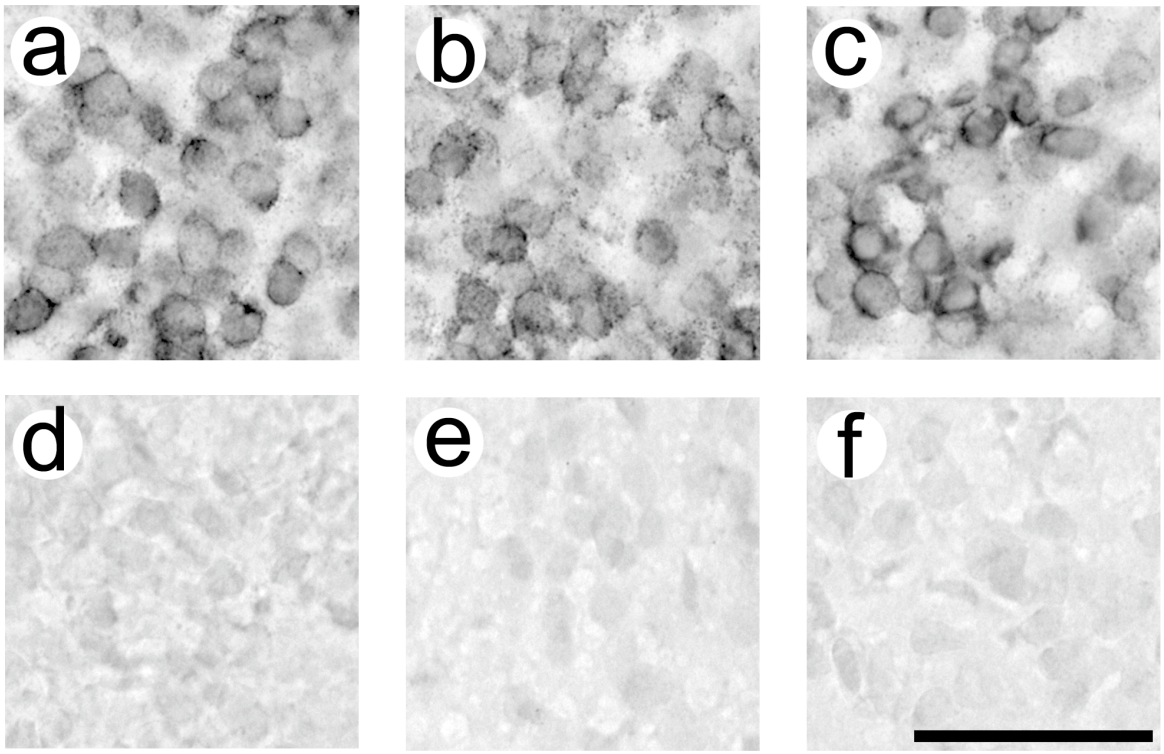

Figure 15. Validation of *in situ* hybridization procedures. Aromatase is depicted in (a) and (d), 5αR1 in (b) and (e), and 5αR2 in (c) and (f). Tissue treated with antisense probes (a-c) show dark cytoplasmic labeling, whereas tissue treated with sense probes (d-f) show no labeling. All pictures are from the VMH. Antisense and sense labeling for each gene is depicted from the same individual. P50 females are depicted in (a), (c), (d), and (f). A P50 male is depicted in (b) and (e). Scale bar = 50 μm.

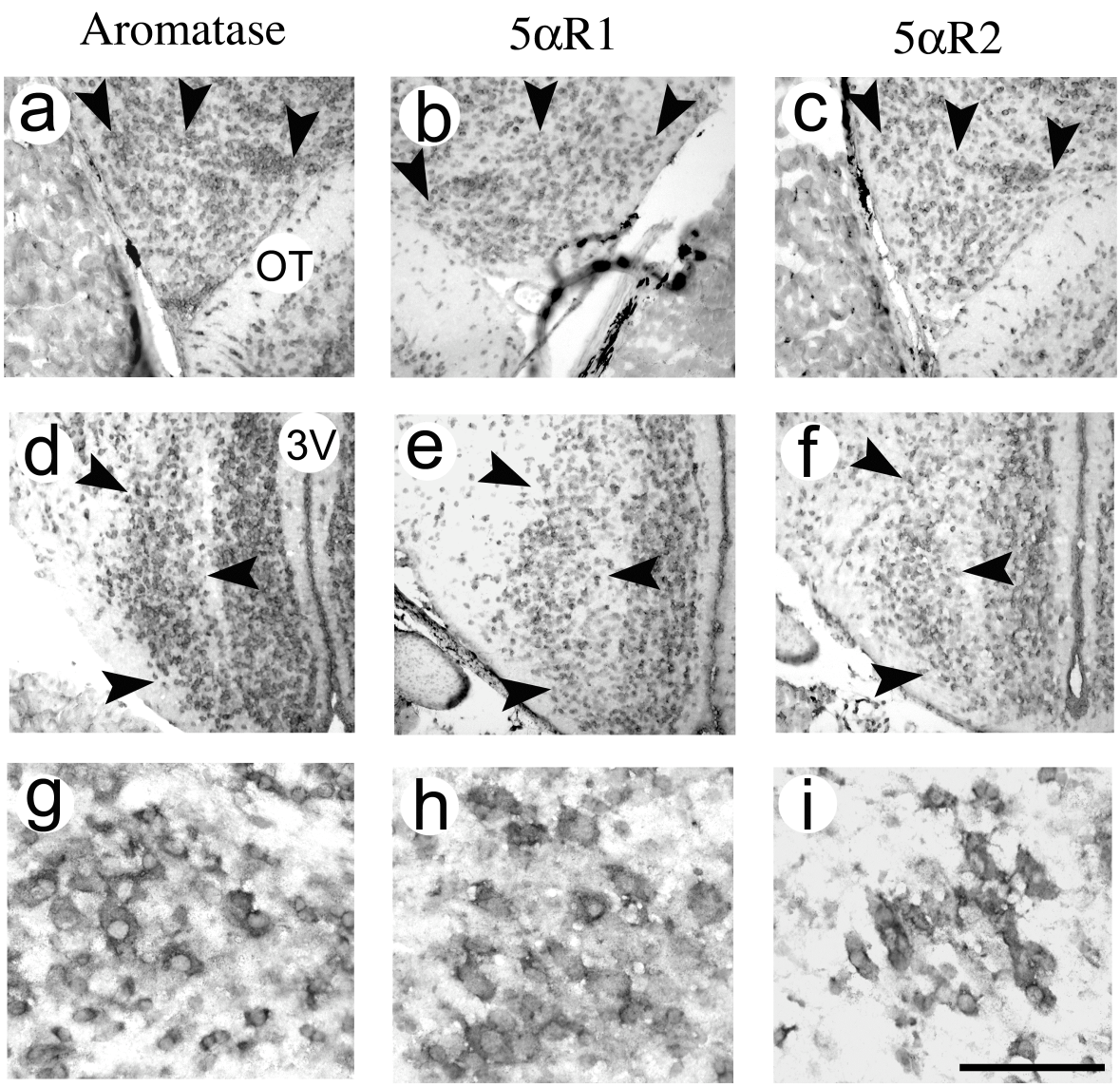

Figure 16. Distribution of aromatase (a, d, g), 5αR1 (b, e, h) and 5αR2 (c, f, i) among selected brain areas. The Amy is depicted in (a-c) and the VMH is depicted in (d-f). The glossopharyngeal portion of the nucleus ambiguous and the ventral motor nucleus of the facial nerve is depicted in (g-i). Arrows indicate the boundary of the brain areas. Pictures were taken from P50 males except (f) and (g), which were from P50 females. OT = optic tract, 3V = third ventricle. Scale bar = 100 μm in (a-f), 50 μm in (g-i).

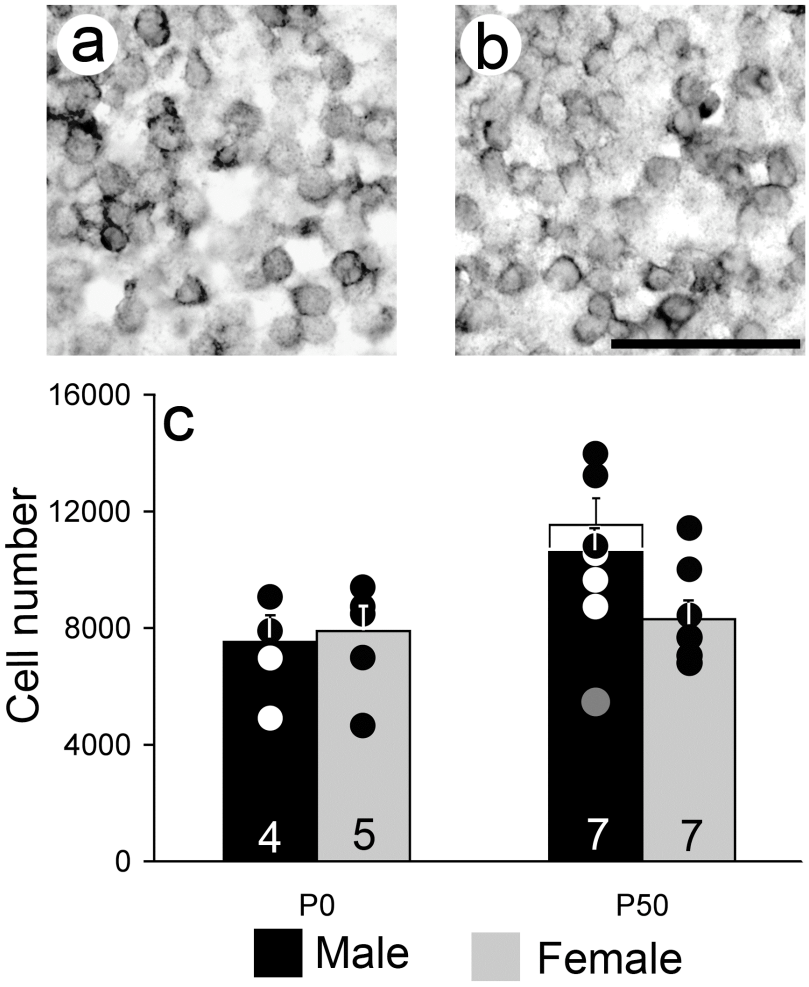

Figure 17. Aromatase mRNA expression in the POA. A male is depicted in (a) and a female in (b), both P50. The estimated total number of aromatase positive cells in the region is indicated in (c), mean plus standard error. If one animal from the P50 male group (indicated by a grey data point) is excluded from analysis (new mean and error indicated by the white bar and line immediately above), then P50 animals have a greater number of aromatase positive cells that P0 animals and, among P50 animals, males have a greater number of cells than females. Scale bar = 50 μm. Sample sizes are indicated in (c).

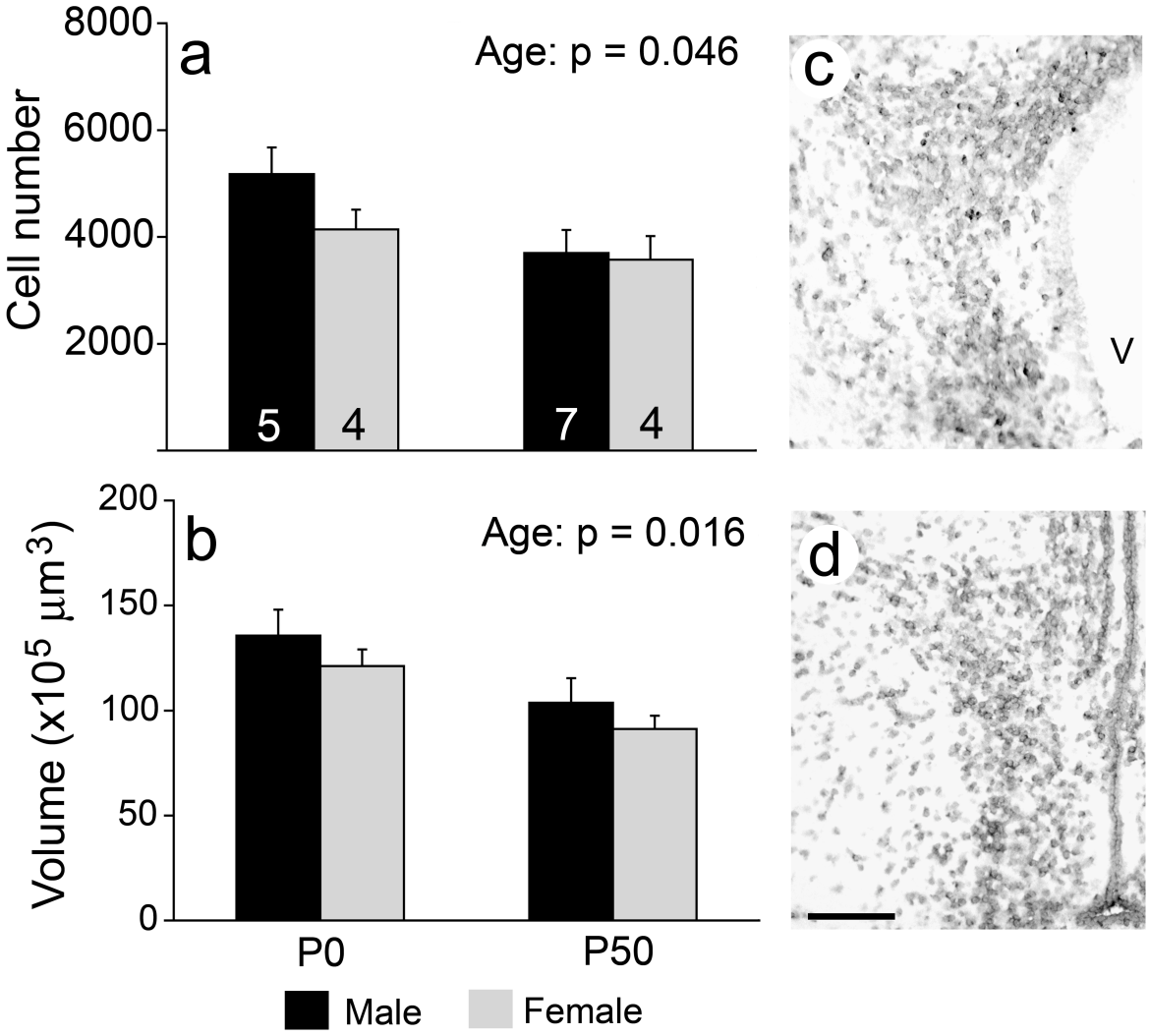

Figure 18. 5αR1 expression in the POA. Animals at P0 had a greater total number of cells (a) and volume of the region (b) than at P50. A P0 male is shown in (c) and a P50 male in (d). Scale bar = 100 μm. V = 3rd ventricle. Sample sizes are indicated in (a).

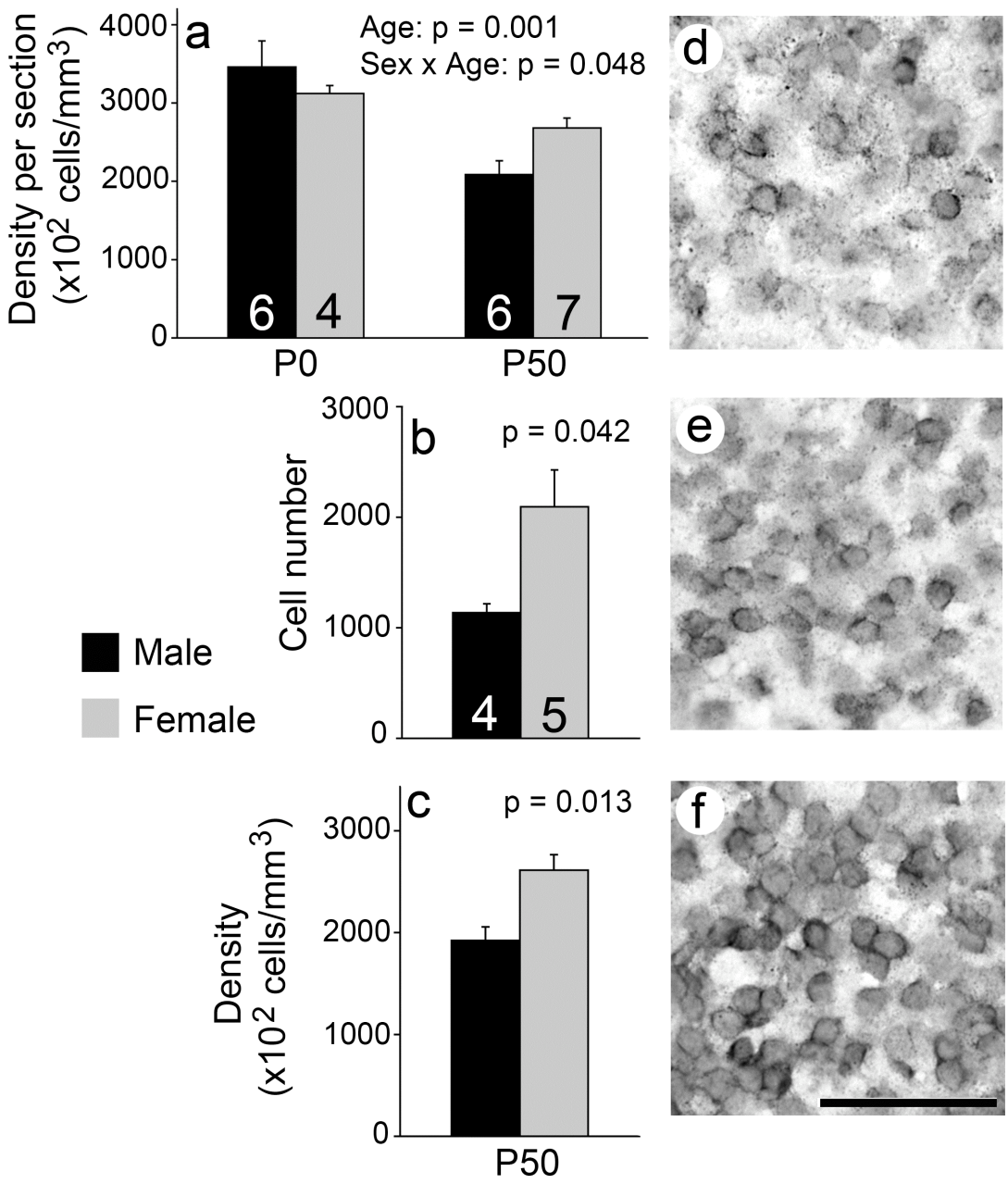

Figure 19. 5αR1 expression in the AMY. A main effect of age was detected on the density of cells – labeling was increased at P0 compared to P50; a sex x age interaction also existed (a). Among P50 animals, females had a greater number (b) and overall density (c) of 5αR1 positive cells than males. A P50 male is shown in (d), a P50 female in (e), and a P0 male in (f). Scale bar = 50 μm. Sample sizes are indicated in (a) and (b). (c) has the same sample sizes as (b).

Chapter 7: Discussion

In this dissertation I have tested the hypothesis that testosterone (T) regulates the activity and expression of aromatase and 5α-reductase (5αR) in green anole lizards using two categories of experiments: intact animals under naturally varying T levels and gonadectomized animals treated with T. In chapter 2, I treated animals with either a blank or T-capsule and measured aromatase and 5αR activity in the whole brain. I found that, in males, T increased 5αR activity regardless of season and increased aromatase activity in the breeding season (BS) only. In chapters 3 and 4, I cloned the aromatase, 5αR1 and 5αR2 genes and analyzed their mRNAs. I found that females had a greater density of aromatase expressing cells in the ventromedial amygdala (AMY) and ventromedial hypothalamus (VMH), whereas males had a greater number of aromatase positive cells in the preoptic area (POA). Females had a greater density of 5αR2 expressing cells in the AMY than males. In chapter 5, I examined aromatase and 5αR2 in blank and T-treated animals and found that T increased 5αR2 mRNA expression in the female VMH and decreased expression in the BS male VMH. Finally, in chapter 6, I examined these three enzymes during two different ages in development and found that the number of 5αR1 expressing cells in the POA decreased at 50 days after hatching (P50) as compared to the day of hatching (P0). The density of 5αR1 expression decreased in the AMY with age as well. Collectively, these results support the hypothesis that T may play a role in regulating the expression of these enzymes, although more work needs to be done to determine the full extent of that regulation, as well as additional factors that may contribute to it. Below, I discuss the main results for each type of enzyme (aromatase and 5αR) and draw interpretations from the data.

Androgen-metabolizing enzymes

Aromatase

I found a male biased sex difference in the total number of cells expressing aromatase in the POA and a female biased sex difference in the density of aromatase expressing cells in both the AMY and VMH (Chapter 3; Cohen and Wade, 2011). Increased aromatase expression in the male POA suggests a potential for more estradiol (E2) to be synthesized. The POA is associated with male reproductive behaviors in green anoles, as in other vertebrates (Balthazart and Surlemont, 1990; Hull and Dominguez, 2007; Wheeler and Crews, 1978); increased E2 production in the male POA is consistent with this hormone's facilitation of sexual motivation (Latham and Wade, 2010). These data are also consistent with those from other species (see Chapter 1), which show that many brain regions, including the POA, have higher aromatase activity and mRNA in males than in females. Although the number of cells in the anole AMY and VMH that express aromatase mRNA did not differ between the sexes, higher densities of aromatase positive cells in these regions in females may contribute to a more concentrated local production of E2. This idea is consistent with a role of aromatase in female receptivity in this species (Winkler and Wade, 1998). While it is clear that the VMH regulates female sexual behaviors (Emery and Moss, 1984; Kendrick et al., 1995; La Vaque and Rodgers, 1975), the AMY's role in female reproduction is less clear. This region is, however, important in sensory integration (Kostarczyk, 1986), which may allow the female anole to process male courtship signals.

There are a number of sex differences in anole aromatase expression, which may relate to the fact that males have higher levels of circulating plasma T than females (Lovern et al., 2001). If T is regulating aromatase expression, then it follows that males have more aromatase expression in the POA due to higher levels of T. However, this appears to not be the case in every brain region. For example, males and females have similar numbers of cells expressing aromatase in the VMH and AMY. Therefore, natural T levels are not playing a role in the number of cells expressing aromatase in these regions. The female-biased density of aromatase cells in the VMH and AMY is likely due to differences in the volume of the regions as defined by aromatase expression, which could be controlled by T in either adulthood or development, but more experimentation is necessary to determine this.

In contrast to the data from unmanipulated adults, I found no sex or age differences in aromatase expression during development, suggesting that T is not regulating expression at the ages I examined. However, variability in the POA suggested that aromatase expression in that area may be starting to differentiate by 50 days after hatching (Chapter 6; Cohen and Wade, submitted a). By about 35 days after hatching, circulating T is higher in male than in female juvenile anoles (Lovern et al., 2001) and the P50 time point was chosen to ensure a sex difference in circulating T. It is possible that higher levels of T are required to produce a more robust effect on neural aromatase expression so that sex differences would be detected in older juvenile anoles (where the sex difference in circulating T is presumably larger). T treatment during development does result in an increase in aromatase activity and the number of cells that contain the enzyme in other species, including mice and Japanese quail (Bardet et al., 2010; Hutchison et al., 1997; Schumacher and Hutchison, 1986). Thus, it is likely that increasing T

during development also plays a role in anole aromatase expression, although this probably happens at ages older than P50.

In adults, I showed that T increases aromatase activity selectively in the whole brain of BS males (Chapter 2; Cohen and Wade, 2010b), although there were no effects of T treatment on the number or density of cells expressing aromatase mRNA in the POA, AMY, or VMH (Chapter 5; Cohen and Wade, submitted b). Clearly, the data show that T does upregulate aromatase activity, but this is not necessarily translated into increases in cells expressing aromatase mRNA locally within the POA, AMY or VMH. Previous work on Japanese quail is consistent with this pattern, as aromatase activity in the hypothalamus + POA is higher in males than females without sex differences in the number of cells that express aromatase protein or mRNA in similar regions (POA and bed nucleus of the stria terminalis [BNST]; Voigt et al., 2007). A few possible reasons exist for our observation of higher activity but no changes in mRNA with T treatment. First, T simply may not regulate aromatase in the specific areas that were examined. We do not have activity data from the specific regions (see discussion of methodological issues below) and, thus, cannot claim that T is increasing aromatase activity in these areas. It is also possible that sexually dimorphic aromatase expression in the brain is established during development and adult T is not necessary to maintain this pattern of expression. If this were true, then I would expect the critical period for aromatase expression to occur after P50.

Alternatively, T may increase aromatase activity without changing the number of detectable cells expressing aromatase mRNA. That is, the hormone may up-regulate the amount per cell, which my procedure is not designed to reveal, and/or the sensitivity of the assay may not have been great enough to allow visualization of cells that express very low levels of

aromatase (see discussion of methodological issues below). It is also possible that the enzyme itself (either the amount of protein or its ability to react with substrate) may have been altered by hormone treatment without changes in mRNA. For example, increased translation of available mRNA, or post-translational modifications of the aromatase protein are undetectable in an *in situ* hybridization analysis. Additionally, recent evidence has suggested that the availability of cofactors for the reaction can affect aromatase activity (Pradhan et al., 2010). Calcium can also cause rapid changes in aromatase activity, an effect that differs between male and female Japanese quail (Konkle and Balthazart, 2011). Thus, many factors can lead to the modification of aromatase availability and activity, so the lack of an effect of T on mRNA as analyzed here does not necessarily mean that the hormone has no influence on the enzyme in the brain regions investigated.

5α-reductase

When examining the distribution of both isozymes of 5αR, I demonstrated that 5αR2 is expressed in many regions of the brain, including the forebrain, while 5αR1 is only expressed in the brainstem of adult lizards (Chapter 3; Cohen and Wade, 2010a). In juvenile anoles, I found that the two isozymes are expressed in the forebrain and that both the number of cells expressing 5αR1 in the POA and density in the AMY decrease between the day of and 50 days after hatching (Chapter 6; Cohen and Wade, submitted a). The patterns suggest that 5αR1 is expressed in the juvenile forebrain and decreases during development, whereas 5αR2 is expressed in the forebrain throughout life. The data are consistent with the idea that 5αR1 plays a role in development and, because 5αR1 decreases between P0 and P50, T may serve as a negative feedback signal in 5αR1 production. Based on its expression pattern, 5αR2 may be

important for either clearing excess T or maintaining local 5α-dihydrotestosterone (DHT) production in adults. The pattern of expression of the two isozymes in anoles is opposite that seen in rodents (Melcangi et al., 1998; Poletti et al., 1998), and suggests that these two isozymes may have been co-opted for the same but opposite functions in rodents and lizards.

In adult anoles, I found a sex difference in the density of 5αR2 expressing cells in the AMY, such that females had a denser population in this region than males (Chapter 4; Cohen and Wade, 2010a). Similar to aromatase, 5αR2 in the AMY may function in females to facilitate the processing of male courtship behavior. The breakdown of T in females may serve to clear excess T that may be detrimental and/or DHT itself may play a role in female receptive behaviors, although it is difficult to speculate on exactly what that role might be. 5αR is also known to act on progesterone, converting it to 5α-dihydroprogesterone (DHP; Lephart et al., 2001). Progesterone influences female reproductive behaviors across vertebrates (Fabre-Nys and Martin, 1991; Rubin and Barfield, 1980; Steel, 1981). In anoles, progesterone acts in synergy with E2 in females and T in males to facilitate sexual behaviors (McNicol and Crews, 1979; Wu et al., 1985; Young et al., 1991). My data on 5αR2 expression suggest the possibility that DHP may also play a role, or perhaps the metabolism of progesterone is important.

In adults, T increases whole brain 5αR activity, regardless of sex (Chapter 2; (Cohen and Wade, 2010b) and has specific effects on the density of 5αR2 expressing cells in the VMH. Among females, T treatment increases the density of 5αR2 cells in that region (Chapter 5; Cohen and Wade, submitted b). Additionally, I found that blank-treated BS males had a greater density of 5αR2 cells than those that were T-treated. These data suggest that while T potentially increases whole brain 5αR activity in males regardless of season, it has opposite effects on the mRNA expression of 5αR2 in the VMH in male and females. T treatment in females produces T

levels that are higher than what is normal for a female, which may be detrimental to normal function. High levels of T may induce increased levels of 5αR2 to breakdown this excess T and begin to metabolize the hormone completely. In males, the opposite may be occurring, such that the absence of T causes more 5αR2 to be produced in order to increase production of DHT. DHT production in males is important for the full expression of male sexual behaviors (Rosen and Wade, 2000) and perhaps the VMH plays a role in this. The VMH has been shown to increase immediate early gene expression (a measure of neural activation) in male hamsters after copulation (Kollack-Walker and Newman, 1995), suggesting a role of the VMH in male reproductive behaviors.

Brain areas important for sexual behaviors

In this dissertation, I chose to examine T–metabolizing enzyme expression in three specific brain areas that control reproductive behaviors: POA, AMY and VMH. As mentioned above, these three regions were chosen for a few important reasons. First, all three of these limbic regions are highly conserved in function across vertebrate species, and thus, we can examine how hormone action in these areas could play a role in facilitatating behaviors across different vertebrate groups (Goodson, 2005; Newman, 1999). The POA has been widely implicated in male sexual behavior, and the AMY is important for male sexual motivations. The VMH plays a critical role in female sexual behaviors. Thus, these three regions are critical for sexual behaviors. In anoles, as well as many other vertebrates, hormone action in these three areas are important for the expression of sexual behaviors (Crews and Morgentaler, 1979; Huddleston et al., 2006; Rubin and Barfield, 1980; Veney and Rissman, 2000b). In anoles

specifically, T is the primary hormone, although DHT is necessary for the full expression of male sexual behaviors and E2 facilitates them (Latham and Wade, 2010; Rosen and Wade, 2000; Wade, 2011). In female anoles, E2 is the primary hormone, although the progesterone increase during the ovarian cycle facilitates female receptivity (Jones et al., 1983; Wu et al., 1985). Aromatase and 5αR are important for male and female reproductive behaviors (Rosen and Wade, 2000; Winkler and Wade, 1998) and are present in the brain (Wade, 1997). Thus, it seems likely that areas associated with reproduction, such as the POA, AMY, and VMH, are good candidates for sites of T metabolism in the anole brain.

Additionally, the forebrain brain has been well mapped in green anoles, and the three areas I investigated have been characterized in terms of behavioral function, morphology, and hormone receptor expression (Beck et al., 2008; Beck and Wade, 2009c; Greenberg, 1982; Greenberg et al., 1984; Rosen et al., 2002; Wheeler and Crews, 1978). Thus, due to the wealth of information available on these areas in anoles, I chose to examine aromatase and 5αR in the POA, AMY, and VMH.

As Newman (1999) and Goodson (2005) suggested, however, other areas of the social network (Newman 1999) are important for the control of sexual behaviors, and T metabolism may likewise be important in these other regions. For example, the bed nucleus of the stria terminalis (BNST) plays a role in male sexual behaviors in hamsters (Been and Petrulis, 2010) and female behaviors in rats (Jenkins and Becker, 2001). Androgen receptor (AR) is present in the BNST of both anoles and eastern fence lizards (Moga et al., 2000; Rosen et al., 2002). Estrogen receptor (ER) β is also present in this region in anoles (Cohen et al. *in prep*). The presence of both AR and ER suggests the importance of hormone action in this area and it would not be surprising if T metabolizing enzymes were present as well. For example, in mice, both

aromatase and E2 production in the BNST during development is critical for masculinization of the region (Tsukahara et al. *in press*). Thus, it is entirely possible that T metabolizing enzymes in anoles may have a role in the BNST.

Another potential area of investigation includes the nucleus accumbens (Acc) and other regions in the reward circuit (Giuliano and Allard, 2001). Dopamine in the Acc is associated with sexual behavior in rats, and the area plays a role in partner-preference in male prairie voles (Aragona et al., 2003; Pfaus et al., 1990). Evidence in the lizard, *Psammodromus algirus*, suggests that the Acc is, at least morphologically and chemically, similar to the analogous region in mammals (Guirado et al., 1999). AR and ERα are both present in this region in anoles (Beck and Wade, 2009c; Rosen et al., 2002), which indicates hormone action and suggests that T metabolism may occur in this region. It seems likely that the Acc may have a role in sexual behavior in lizards, although no data are currently available on this possibility.

The brain areas associated with the social behavior network (Newman, 1999) are all extensively interconnected and it would not be surprising if all of these regions, as well as others in the reward system, were critical in the regulation of sexual behavior in anole lizards. Furthermore, aromatase and 5αR may exist in all of these regions associated with the social behavior network, which may have contributed to changes in the whole brain activity levels I detected that were not seen as mRNA changes in the POA, AMY, and VMH. Further examination is required to more fully elucidate the roles these regions have in sexual behavior and how T metabolism may play a role.

Methodological issues

In this dissertation, I used two techniques to examine aromatase and 5αR; an enzyme activity assay and DIG *in situ* hybridization. Although both of these techniques provided excellent information on the activity and patterns of mRNA expression of T metabolizing enzymes, there are limitations in the type of information that they can provide.

Currently, the only way to measure enzyme activity is to conduct a biochemical assay. The procedure I used in my dissertation measures how much product is made in a given time using a radioactive precursor (in this case, T). I was able to easily measure whole brain aromatase and 5αR activity using this technique. However, this assay is not able to detect enzyme activity in specific brain regions. Microdissections of the individual regions would be far too small to detect the product of the reaction. The only method to ascertain region-specific activity would be to dissect out the entire hypothalamus (and POA) and combine samples together as in Wade (1997). Because this would have required many more animals and potentially resulted in the loss of information due to sample pooling, I chose not to use this technique for the rest of my studies. It would be extremely informative to examine local enzyme activity directly in my subsequent experiments, but this experiment is unfortunately not feasible at this time.

DIG *in situ* hybridization allowed me to examine the number of cells in particular regions that were expressing aromatase, 5αR1 or 5αR2 mRNA. This technique is easy to use, results in clear labeling of discrete cells, and does not take as much time as other procedures using radioactivity. Although this technique allowed me to collect valuable data, it is also somewhat limited. DIG *in situ* hybridization amplifies the signal from the labeled mRNA probe using an antibody to DIG, resulting in cells that expressed the mRNA of interest to be positively labeled. Therefore, it is not possible to reliably quantify the amount of mRNA present per cell and I could

only detect changes in the number of cells expressing these enzymes. It is entirely possible that the amount of mRNA per cell may have differed among the various groups in these experiments. Radioactive *in situ* hybridization is another technique I could have used that determines the amount of mRNA present in a region, but is not optimal for identifying separate cells expressing the gene. Thus, both *in situ* techniques offer clear advantages and drawbacks. It would be useful to know both the number of cells that express the genes, as well as the amount of mRNA present in a specific region, but this is beyond the scope of this dissertation. Future work, as discussed below, should determine the amount of mRNA and protein of each gene present in the three regions.

Anoles are currently the only non-avian reptile in which the genome has been completely sequenced. Although the genome was completed while I worked on the experiments for this dissertation, I was able to use what was available at the time to determine the anole-specific sequences of aromatase, 5αR1 and 5αR2. Now that the genome has been completed, future work could more fully examine the regulation of testosterone-metabolizing enzymes by looking at promoter regions, DNA methylation, or other gene-specific regulatory mechanisms.

Remaining questions

In this dissertation, I have begun to test the hypothesis that T regulates aromatase and 5αR in the anole brain. I have shown that T likely has a role in aromatase and 5αR regulation in the anole brain, although the full extent of this role is not yet known. Future work will be necessary to determine the full extent of T regulation of its own metabolism in this species.

First, it will be important to determine the concentration of T metabolizing enzymes present in the POA, AMY, and VMH. As discussed above, limits to enzyme assays necessitate the use of other techniques to determine the concentration of these enzymes. Western blots using protein taken from microdissections of brain regions or immunohistochemistry on sections would allow one to determine protein expression. This is important because the total mRNA present does not necessarily equal the amount of protein expressed. However, both of these techniques require antibodies for each T metabolizing enzyme and anole-specific antibodies are not currently available. Thus, evaluation of mRNA remains the best tool at present to examine these enzymes in anole lizards. Quantitative PCR (qPCR) on RNA extracted from microdissections of the brain regions would determine the concentration of mRNA and whether that changes under varying levels of T. Alternatively, one could perform radioactive *in situ* hybridizations to determine the density of mRNA expressed in specific areas. Repeating the experiments from this dissertation using either qPCR or radioactive *in situ* hybridization would answer the question of whether the concentration of aromatase, 5αR1 and 5αR2 mRNAs are changing under varying conditions.

Another unanswered question is whether the presence of these enzymes in the POA, AMY and VMH are necessary in the control of reproductive behaviors. This question would be best answered by infusing fadrozole (an aromatase inhibitor) and/or finasteride (a 5αR inhibitor) directly into the POA, AMY, and VMH. After infusion of the inhibitors, any effects on the sexual behaviors of both male and female individuals would reveal the role of aromatase and 5αR in each brain region. Systemic injections of aromatase inhibitors have decreased female receptivity and 5αR inhibitors have decreased male sexual behaviors (Rosen and Wade, 2000; Winkler and Wade, 1998), but it is unclear at this time what role these enzymes play in specific

brain areas and directly inhibiting their action directly would help clarify their role in anole reproduction. With the available technologies, this technique is, unfortunately, not feasible at this time in anoles. It would require the implantation of in-dwelling cannulae and animals to carry pumps to deliver the drug. Anole lizards are far too small to carry the pumps that are currently available and their daily behaviors (climbing, jumping, etc.) increase the chance of dislodging the cannulae. If smaller pumps or different cannula designs become available, then this technique would offer excellent insight into the roles T metabolizing enzymes have in the POA, AMY, and VMH, and how that directly impacts behavior.

Another step for future research would be to more fully determine the regulation of these enzymes in juvenile anoles. First, examining mRNA expression of these enzymes at older ages would identify at which age differentiation of each enzyme occurs. Additionally, I would expect 5αR1 expression to continue to decrease in older ages. Another interesting experiment would be to experimentally manipulate T in juvenile anoles to determine if T is regulating expression of aromatase, 5αR1 and 5αR2. Manipulating T during development will allow one to determine whether T is organizing the expressions of T metabolizing enzymes, which, if true, would suggest that the dimorphic patterns in adulthood is determined during development and not necessarily influenced by adult hormones. These experiments would answer whether the increase of T in developing anoles is influencing T metabolizing enzyme expression.

Conclusions

Although these experiments have been an important first step, clearly more work is needed to fully test the hypothesis that T controls its own metabolism in green anole lizards

(Wade et al., 1995). In this dissertation, I have shown that there are sex and age differences in aromatase, 5αR1, and 5αR2 expression in adult and juvenile anoles. I have also demonstrated that T increases whole brain aromatase and 5αR activities, as well as region-specific 5αR2 expression. Although clearly T is not the only factor influencing aromatase, 5αR1, and 5αR2, some of the data I have collected do support the overall hypothesis for this dissertation. T may not directly increase the number of cells expressing these enzymes in every brain region, but with the techniques I used, I was unable to determine whether T affects the amount of mRNA expressed per cell and how that relates to protein action. Future work is necessary to determine the full extent of T regulation of these enzymes.

Reptilian models have been previously used to study steroid metabolizing enzymes (e.g. Belaid et al., 2001; Dias et al., 2009; Krohmer et al., 2002; Milnes et al., 2002; Wibbels and Crews, 1994), but this is the first work to examine the neural expression of all three T metabolizing enzymes in a reptile with genetic sex determination. More work has been done on aromatase than 5αR in reptiles, although the majority of that work has been done on sexual differentiation of the gonads in reptiles with temperature sex determination (Belaid et al., 2001; Milnes et al., 2002). Some work has been done on aromatase expression in garter snakes (ZZ/ZW system; Krohmer et al., 2002) and in parthenogenetic whiptail lizards (Dias et al., 2009). Anole lizards have genetic sex determination (XX/XY system) and the work I have presented in this dissertation has added much to the literature on T metabolism in reptiles.

The examination of T metabolism across multiple taxa is important to fully understand the roles aromatase, 5αR1, and 5αR2 have in sexual behavior and brain development, as well as how these roles might have evolved. Unlike avian and mammalian species, in the anole, T itself seems to be the more important hormone in regulating sexual behaviors (Crews and Morgentaler,

1979; Wade, 2011). Although T is most important, there are specific sex and seasonal differences in whole brain aromatase and 5αR activities, and both E2 and DHT have specific effects on sexual behavior in this species (Latham and Wade, 2010; Rosen and Wade, 2000; 2001). Thus, based on the smaller role for T metabolites in sexual behaviors, the roles of aromatase and 5αR in this species may not necessarily be the same as in mammals and birds. In fact, these enzymes may have been co-opted for reproduction much more strongly in birds and mammals than in anoles, and perhaps reptiles in general. T metabolism should be examined in more species to determine whether the apparent role of these enzymes is the same for each vertebrate group. Additionally, I found that 5αR1 and 2 were expressed in the exact opposite pattern in anoles and rodents, which suggests that different enzymes are used for similar functions in these species: maintenance in the adult brain (5αR1 in rodents and 5αR2 in anoles) and masculinization and/or defeminization (5αR2 in rodents and 5αR1 in anoles) of the developing brain. Overall, my experiments have highlighted the importance of comparative work and laid important groundwork for future research that will ascertain the role of these enzymes in anoles and the extent of their regulation by T.

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