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BRAIN MORPHOLOGY AND ESTROGEN RECEPTOR-
ALPHA EXPRESSION: A POTENTIAL LINK TO ESTRADIOL

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Laurel Amanda Beck

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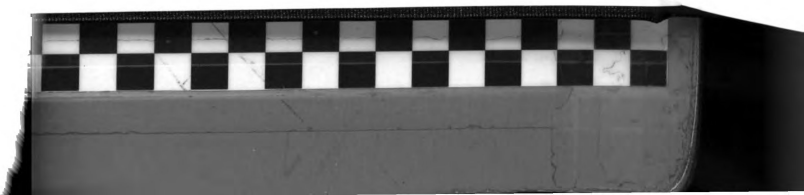

BRAIN MORPHOLOGY AND ESTROGEN RECEPTOR-ALPHA EXPRESSION: A POTENTIAL LINK TO ESTRADIOL

by

Laurel Amanda Beck

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ABSTRACT

BRAIN MORPHOLOGY AND ESTROGEN RECEPTOR-ALPHA EXPRESSION: A POTENTIAL LINK TO ESTRADIOL

By

Laurel Amanda Beck

Estradiol is a hormone that facilitates reproductive behaviors in both males and females in mammals and birds. However, this is not true in the green anole lizard, where estradiol only facilitates reproductive behaviors in females. Testosterone activates male sexual behaviors. However, estradiol synthesis in the male brain is actively regulated. To investigate the possible effects of estradiol on the anole reproductive forebrain, I examined potential sex, seasonal, and developmental differences in morphology and estrogen receptor alpha mRNA expression. I confirmed that these features differed between the sexes and seasons in unmanipulated adult anoles. Then, estradiol manipulates were performed to directly test the idea that estradiol might alter forebrain morphology of estrogen receptor alpha mRNA expression. Further, to examine potential organizational effect of estradiol, these features were examined in developing anoles during a time period where steroid hormones are fluctuating. While the effects of estradiol on forebrain morphology were minimal, estradiol regulates estrogen receptor alpha expression in a sex- and regionally-specific manner during adulthood.

DEDICATION

I would like to dedicate this dissertation to my grandfather, Donald Beck, who lived his life valuing hard work and determination above all else.

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Chapter 1: Introduction

Sex and seasonal differences in brain morphology exist in amphibians, birds, mammals, and reptiles. The reproductive system is ideal to examine potential effects of both sex and season on these structural differences, as there are frequently clear parallels to behavior. Males and females display different courtship and/or copulatory behaviors, and seasonally reproducing animals often present these reproductive behaviors only under breeding environmental conditions. Further, these behaviors are often modulated by steroid hormones, which differ between the sexes and seasons. These sex and seasonal differences are in naturally occurring ways through which one can understand the mechanisms regulating relationships between steroid hormones, structure (morphology), and function (behavior) in the brain.

Regulation of reproductive behaviors

Reproductive behaviors in many mammalian and avian species are facilitated by estradiol (E2) in both males and females (reviewed in Ball and Balthazart, 2002; Blaustein and Erskine, 2002, Hull et al., 2002). While males secrete primarily testosterone (T) from the testes, it is E2, locally aromatized from circulating T within the brain (Callard et al., 1978; Selmanoff et al., 1977; Steimer and Hutchison, 1980), which often facilitates male reproductive behaviors (Davis and Barfield, 1979; Nyby et al., 1992). Even though E2 is behaviorally relevant

for both males and females, the sites of action within the brain of E2 and subsequent behaviors differ between the sexes.

Parallels between reproductive behaviors and neural cytoarchitecture occur in at least three limbic forebrain nuclei: the preoptic area (POA), medial amygdala and ventromedial hypothalamus (VMH). Even though much of the work has been done in rodents to date, these relationships exist in a variety of species, including songbirds. In rodents, male copulatory behaviors such as mounting and intromission are facilitated by the POA, while sexual appetite is perhaps more influenced by the medial amygdala (reviewed in Meisel and Sachs, 1994). In female rodents, the VMH facilitates receptivity (e.g., Emery and Moss, 1984; La Vaque and Rodgers, 1975; Pfaff and Sakuma, 1979). By interactions with each of these regions, E2 facilitates the production of reproductive behaviors, which are dependent on sex. In rodents, for example, E2 administered to the male POA promotes copluatory behaviors (Christensen and Clemens, 1975), while E2 in the female VMH facilitates lordosis (Davis et al., 1979).

While these three regions are implicated in the control of reproductive behaviors, it is important to note that each of them also facilitate other behaviors. It is hypothesized that a combination of limbic regions, including the POA, amygdala, and VMH, are heavily interconnected and form a network that facilitates numerous social behaviors (Newman, 1999). Much like the large systems for memory and learning or speech and language, these regions interact to produce a wide variety of social behaviors. These behaviors include aggression, maternal behavior, social recognition, and feeding behavior, as well

as reproduction. Therefore, the sexual and seasonal dimorphisms in brain morphology in these regions may not relate solely to reproductive behaviors.

Sexual and seasonal dimorphisms in brain morphology

Sex differences in functional and behavioral response to E2 can be reflected in the gross morphology of the reproductive nuclei. In rodents, for example, both the POA and amygdala contain subregions (e.g.,, the sexually dimorphic nucleus of the preoptic area [SDN-POA] and posterodorsal medial amygdala [MePD]) which are larger in males than in females (Cooke et al., 1999, reviewed in Baum, 2002), due to increased soma size (Cooke et al., 1999), cell number (Gorski et al., 1980), and dendritic arborization (Greenough et al., 1977; Madeira et al., 1999) in males. While the function of the rodent VMH has been studied in detail, less information is available on the relationship between morphology and sexually dimorphic function of the region. However, some evidence suggests that the volume of the VMH in males is greater than in females, and the sex difference is eliminated by neonatal castration in rats (Matsumoto and Arai, 1983).

Some seasonally reproducing animals also exhibit differences in brain morphology between the breeding (BS) and non-breeding (NBS) seasons. For example, soma size in the medial amygdala of Siberian hamsters decreases when exposed to NBS environmental conditions (Cooke et al., 2001). In starlings, breeding animals exhibited increased volume of the medial preoptic nucleus compared to non-breeding animals (Riters et al., 2000).

Sex and seasonal differences in steroid hormone receptors

Not only does morphology of these three regions differ between the sexes and seasons, but neuronal protein expression is also plastic. For example, estrogen receptor-alpha (ERα) expression differs between the sexes and seasons. ERs are expressed in both males and females in brain nuclei involved in the production of reproductive behaviors in mammals (Lauber et al., 1991; Warembourg and Leroy, 2004; Wood and Newman, 1995), birds (Halldin et al., 2006; reviewed in Gahr, 2001), and reptiles (Crews et al., 2004), and in some instances, the extent of ER expression can differ between the sexes. For example, in the mammalian VMH, ERα expression is greater in females than in males (Lauber et al., 1991; Scott et al., 2000).

In the reproductive nuclei of some avian species, seasonal regulation of ERα is prevalent. Canaries, which sing only during the BS, express more ERα mRNA during the NBS than the BS in a sexually dimorphic song control nucleus (Fusani et al., 2000). Additionally, the spotted antbird shows an increase in ERα mRNA expression in the POA during the NBS compared to BS (Canoine et al., 2007). Furthermore, in Syrian hamsters, ERα expression increases following exposure to NBS conditions (short day length; Mangels et al., 1998).

E2-mediated sex and seasonal differences in adulthood

Steroid hormones frequently create or maintain structural differences in the brain. For example, soma size decreases in the medial amygdala of male rats following castration, yet larger soma size can be recovered following T

treatment (Cooke et al., 1999). Also, female rats treated with T in adulthood show a marked increase in soma size in this brain region, which suggests that T or one of its metabolites is involved in the maintenance of masculinization in adulthood (Cooke et al., 1999). Later work showed that E2 alone is able to partially restore a masculinized brain morphology to castrated adult male rats (Cooke et al., 2003), indicating that E2 plays a specific role in the maintenance of sexual dimorphism.

Work in seasonally reproducing animals indicates that seasonal fluctuations of steroid hormones may contribute to differences in morphology. The enhancements of forebrain morphology seen during the BS often coincide with elevated circulating levels of steroid hormones (reviewed in Tramontin and Brenowitz, 2000). Work in seasonally reproducing songbirds also points to a role for steroid hormones in morphological change in the forebrain. For example, HVC (used as a proper name; Reiner et al., 2004), a sexually dimorphic nucleus involved in song production, increases in volume during the BS in canaries (Nottebohm, 1980) and white-crowned sparrows (Brenowitz et al.,1998, Smith et al., 1997a), when circulating levels of E2 are higher. Also, HVC volume is modified by steroid hormone treatments in various songbirds (reviewed in Ball et al., 2004).

Not only does E2 influence morphology of the forebrain, but it may also regulate ERα expression levels. Seasonal fluctuations in ER expression coincide with changes in aromatase activity in male songbirds; aromatase is higher during the BS (Riters et al., 2004; Soma et al., 2003) and ERα expression is higher

during the NBS (Fusani et al., 2000), indicating that locally produced E2 may regulate ERα expression. Additionally in non-seasonal animals, E2 treatments can down-regulate ERα in a brain region-specific manner in female guinea pigs (Meredith et al., 1994) and female rats (Simerly and Young, 1991), and ovariectomy increases ERα expression (Hamada et al., 2005; Lauber et al., 1991; Liposits et al., 1990). The effects of E2 on ERα expression in males are less clear; it increases ERα in the VMH and decreases expression in the periventricular preoptic area of ferrets (Sisk and DonCarlos, 1995), but has no effect in the rat VMH (Lauber et al., 1991), indicating that the effects of E2 on ERα expression are dynamic and may be sexually dimorphic. Together these results indicate that E2 actively regulates expression of ERα, albeit in a brain region- and sex-dependent manner.

E2-mediated sex differences in development

In addition to structural and functional effects in adulthood, E2 also plays a role in morphological organization during development. Permanent structural and functional changes of these reproductive brain regions are created during critical periods in development (McCarthy, 1994; Resko and Roselli, 1997). For example, E2 masculinizes the SDN-POA in rodents, at least in part by decreasing apoptosis (reviewed in Morris et al., 2004). Circulating T, which in male rodents increases around birth (Weisz and Ward, 1980), is locally converted to E2 by aromatase, which is highly expressed throughout the preoptic area and hypothalamic nuclei (Roselli et al., 1985). Since developing female

rodents do not experience this rise in locally produced E2, they are also not protected from programmed cell death in the POA during development, leading to a greater number of cells in adulthood in the males. Additionally, ERα expression increases in the male rodent forebrain when circulating levels of T are relatively high (DonCarlos, 1996; Ivanova and Beyer, 2000). This indicates that E2 may act through ERα to masculinize of the developing male forebrain.

Implications across species and the value of reptiles in understanding neuroendocrinology

The vast literature on sex and seasonal differences and the role E2 plays in establishing or maintaining these differences is mainly derived from work in mammalian and avian species. Furthermore, the research has rarely examined the role of E2 in sex and seasonal differences within the same animals, simultaneously. While it is clear that the effects of E2 differ between the sexes and seasons in a variety of species, understanding how these two factors may interact to produce naturally occurring sexually and seasonally dimorphic behaviors is critical. Further, to fully understand the mechanisms of E2 within the brain, these ideas need to be examined in a wide variety of species, particularly in those which naturally differ between the sexes and seasons. Reptiles fill all of these needs. Studying neuroendocrine mechanisms of reproductive behavior in species which branched from a common ancestor can provide information on the conservation of function across many species, as well as provide insights as which features have evolved in mammals and birds and why these functions

were selected differently through evolution. In addition to filling this critical phylogentic gap in our understanding of relationships between structure and function in the brain, reptiles provide excellent models in which to study reproductive behaviors, as a number of them reproduce seasonally and show large sex differences in behavior. Further, hormone-brain-behavior relationships have been well established, particularly in green anole lizards.

Reproductive behaviors and hormonal implications in green anole lizards

Green anoles provide an exceptional model to study sex and seasonal differences within the same animals simultaneously; they display sex and seasonal differences in behavior, forebrain morphology, and protein expression. Male anoles will court females by extending the dewlap, a red throat fan, while performing a series of head bobs. If the female is receptive, identified by remaining stationary, head bobbing, and neck bending, the male will mount and intromitt one of two bilateral hemipenes into the cloaca of the female (reviewed in Lovern et al., 2004). Since anoles are seasonally breeding, these behaviors typically occur only during the BS, though treatment with steroid hormones during the NBS can elicit these behaviors, albeit to a lesser degree (e.g., Neal and Wade, 2007; Wu et al., 1985).

As in mammals, E2 facilitates receptive behaviors in female green anoles (Mason and Adkins, 1976; McNicol and Crews, 1979; Tokarz and Crews, 1980; Winkler and Wade, 1998). However, unlike rodents, male-specific behaviors in the green anole are produced by T. Treatment with E2 is unable to activate

masculine behaviors in anoles; aromatase inhibition does not diminish them (Crews et al., 1978, Winkler and Wade, 1998) and dihydrotestosterone produces suboptimal, if any, restoration of male reproductive behaviors (Crews et al., 1978; Rosen and Wade, 2000). Interestingly, forebrain aromatase activity is higher in males than females, and in males aromatase is higher in the BS than NBS (Rosen and Wade, 2001). These results suggest some sort of active regulation of local E2 levels, which would be consistent with the idea that the hormone has one or more specific functions, albeit not directly facilitate behavior. One hypothesis is that E2 serves more of a preparatory or priming function, by acting to masculinize morphology and/or alter steroid receptor expression to maximize the subsequent effect(s) of T.

In female green anoles, plasma E2 is highest prior to release of the egg from the ovary, dropping to lower levels after the egg is laid (Jones et al., 1983). It is assumed, though not known, that E2 levels are higher during the BS than NBS. Additionally, during the BS females consistently have at least one yolking follicle producing E2 (Jones et al., 1983), whereas the gonads are regressed during the NBS (reviewed in Lovern et al., 2004). These results suggest that baseline levels of E2, independent of specific ovulatory stage, would be higher in the BS compared to NBS. T is approximately two times higher in males and five times higher in females during the BS than the NBS (Lovern and Wade, 2001). Interestingly, the female's response to E2 also differs with the seasons; the female is more likely to display receptive behaviors following E2 treatment during the BS than NBS (Wu et al., 1985). This effect provides a tool for the

investigation of differential responsiveness to the hormone and a reason to test the hypothesis that brain morphology and/or ERα are regulated by E2, potentially to regulate other social behaviors associated with these limbic nuclei.

Sex and seasonal differences in green anole lizards in morphology and steroid receptor expression

Similar to in rodents, three regions have been implicated in reproductive behaviors in male and female anoles. Lesion studies indicate that the POA (Wheeler and Crews, 1978) and ventromedial amygdala (AMY; Greenberg et al., 1984) significantly decreases male anole reproductive behaviors. In females, lesions of the medial basal hypothalamus, which includes the VMH, result in diminished female reproductive behaviors (Farragher and Crews, 1979). Given the conservation of function across many species, it is likely that the VMH specifically is involved in female reproductive behaviors in the anole as well. Additionally, each of the three limbic regions is responsive to steroid hormones (Morrell et al., 1979; Martinez-Vargas et al., 1978; Neal and Wade, 2007). Only one study has examined steroid hormone receptors in the anoles, and discovered that androgen receptor expression is greater in females than in males, especially in the POA (Rosen et al., 2002).

Moreover, seasonal plasticity in forebrain morphology exists in the green anole. Intact breeding animals exhibit increased soma size (O'Bryant and Wade, 2002) compared to non-breeding animals in the POA and AMY. It is likely that these morphological changes with season are related to fluctuating levels of

steroid hormones, as soma size increased in males treated with T compared to blank-treated males, an effect that is enhanced in the breeding compared to NBS in the AMY (Neal and Wade, 2007). Thus, a clear morphological effect of a steroid hormone has been observed in these regions involved in male sexual behavior in anoles, but it is unclear if this effect is mediated by T directly or by the aromatization of T to E2.

Development in the green anole lizard

While information does not currently exist on the development of the forebrain itself, work in juvenile anoles details the development of anole social behaviors. One such social behavior is head bobbing, which males and females use in antagonistic encounters and males use to court females (reviewed in Lovern et al., 2004). In adulthood, head bobs can be characterized into one of three different categories (A, B, and C) dependent on the frequency, amplitude, and duration of the bob (described in Lovern and Jenssen, 2003). Juvenile anoles between 62 and 100 days after hatching respond to other anoles with head bobs, but this effect is significantly enhanced by T (Lovern et al., 2001) so much that it approaches the frequency of display rates documented in adult males (Jenssen et al., 2000). Juvenile anoles primarily generate the C type headbob, but as the animal ages it produces fewer C displays and more A and B head bobs, which are more common in adult males (Lovern and Jenssen, 2003). In both of these studies, however, no sex differences were evident in juveniles, though adult males perform head bobs significantly more than females (reviewed

in Lovern et al., 2004). Therefore, it is clear that further maturation occurs in regard to this display and that T can facilitate this display.

Role of E2 in male and female adult green anole lizards

At this stage, it is important to address in more detail what roles, if any, E2 has in the green anole. This question is important from an evoluationary perspective, among others. In many cases, mammals and birds use one steroid hormone, E2, to facilitate both male and female reproductive behaviors. However, this is not true in the green anole lizard, where T facilitates male behaviors and E2 facilitates female behaviors. The active regulation of E2 in the male brain suggests a role for it in males, albeit one that might not be directly related to reproductive behaviors. To being to evaluate the locations of ways in which E2 can act in the anole reproductive forebrain, I approached this topic by investigating potential sex, seasonal, and developmental differences in morphology and ERα mRNA expression, as these parameters can be influenced by E2 in adulthood in other species. However, before the idea that E2 facilitates morphology and transcription of its receptor in the forebrain was directly tested, it was important to confirm that these features differ between the sexes and seasons in unmanipulated adult animals. Then, following sex and/or seasonal differences in morphology and ERα mRNA expression, E2 manipulations were performed to directly test the hypothesis of hormonal regulation. To examine potential organizational effects of E2, developing anoles were also examined during a time period when steroid hormones are fluctuating.

Chapter Two

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Chapter Two: Sex and seasonal differences in morphology of limbic forebrain nuclei in the green anole lizard

Introduction

Parallels between reproductive behaviors and neural cytoarchitecture occur in at least three limbic forebrain nuclei: the preoptic area (POA), medial amygdala and ventromedial hypothalamus (VMH). While these relationships exist in diverse vertebrate species, much of the work has been done in rodents to date. In male rodents, the medial amygdala is involved in sexual appetite, whereas the preoptic area (POA) is more involved in facilitating copulatory behaviors, such as mounting and intromission (reviewed in Meisel and Sachs, 1994). Both of these structures contain subregions (the sexually dimorphic nucleus of the preoptic area [SDN-POA] and posterodorsal medial amygdala [MePD]) which are larger in males than in females (Cooke et al., 1999, reviewed in Baum, 2002), due to increased soma size (Cooke et al., 1999), cell number (Gorski et al., 1980), and/or dendritic arborization (Greenough et al., 1977; Madiera et al., 1999) in males. In female rodents, the VMH facilitates receptivity (e.g., Emery and Moss, 1984; La Vaque and Rodgers, 1975; Pfaff and Sakuma, 1979). While the function of this region has been studied in detail, less information is available on the relationship between morphology and sexually dimorphic function of the region. However, some evidence suggests that the volume of the VMH in males is greater than in females, and the sex difference is eliminated by neonatal castration (Matsumoto and Arai, 1983).

Steroid hormones are necessary for both the maintenance of the sex differences in areas regulating masculine reproductive displays (Bloch and Gorski, 1988; Cooke et al., 1999; Tobet et al., 1986) and the adult activation of the behaviors. For example, masculine morphology of the medial amygdala in rats (Cooke et al., 1999) and the medial preoptic nucleus in Japanese quail (Balthazart and Surlemont, 1990; Panzica et al., 1987) is maintained in adulthood by testosterone (T), as castration leads to a feminization and T treatment in females increases its volume. T and/or its metabolites facilitate reproductive behaviors in rodents by acting on the POA and MePD of males (Bonsall et al., 1992; Gréco et al., 1998; Huddleston et al., 2006; Vagell and McGinnis, 1997). Consistent with the roles of gonadal steroids, some seasonally reproducing animals also exhibit differences in brain morphology between the breeding (BS) and non-breeding seasons (NBS). For example, soma size in the medial amygdala of Siberian hamsters decreases when exposed to NBS environmental conditions (Cooke et al., 2001).

Relationships between brain morphology and behavior have also been documented in some reptiles, with limbic regions exhibiting sex differences, seasonal variations, and responsiveness to steroid hormones, as in mammalian species. For example, the anterior hypothalamus-POA (AH-POA) is involved in male reproductive behaviors in the garter snake (Friedman and Crews, 1985; Krohmer and Crews, 1987), whiptail lizard (Rozendaal and Crews, 1989), and leopard gecko (Edwards et al., 2004). It is larger in males than females, whereas the VMH is important for receptivity and is larger in female whiptail lizards (Crews

et al., 1990; Kendrick et al., 1995; Wade and Crews, 1992). Volumes of the VMH and POA in female garter snakes decrease during hibernation compared to both spring and fall conditions (Crews et al., 1993). In parallel, T treatment in whiptail lizards facilitates masculine reproductive behaviors and masculinizes morphology of the AH-POA and VMH in males (Wade et al., 1993).

The present study was designed to investigate potential effects of both sex and season on reproductive forebrain morphology in the green anole lizard. Facilitated by long days and warm temperatures, mating in the green anole occurs from approximately April through July (reviewed in Lovern et al., 2004). Circulating steroid hormone levels are higher in both males and females during the BS than NBS (Jones et al., 1983; Lovern and Wade, 2001). Gonadectomy greatly diminishes reproductive behaviors, and T and estradiol (E2) replacement, in males and females respectively, can restore or maintain these behaviors (Mason and Adkins, 1976; Neal and Wade, 2007; Tokarz and Crews, 1980; Winkler and Wade, 1998). Interestingly, the behavioral responsiveness of males and females to steroid hormones also differs with season; it is increased during the BS compared to the NBS (Neal and Wade, 2007; Wu et al., 1985).

As in other species, portions of the POA are involved in male anole sexual behaviors (Wheeler and Crews, 1978). In addition, a portion of the ventral amygdala (identified in green anoles as the ventromedial nucleus, and previously abbreviated as 'AMY'; Greenberg et al., 1984; O'Bryant and Wade, 2002) is important for male reproductive displays. This region has also been called the ventral posterior amygdala in other lizard species and appears homologous to

the posterior division of the medial amygdala and posteromedial cortical amygdala of rodents (Bruce and Neary, 1995; Lanuza et al., 1998).

In parallel, neuron soma size in both the POA and AMY of unmanipulated animals is larger during the BS than NBS, independent of sex (O'Bryant and Wade, 2002). Lesion studies of the anole VMH have not been conducted to confirm its role in receptivity in the anole, and sex and seasonal differences in its morphology have not been investigated. However, function of the region is highly conserved across species (Pfaff and Sakuma, 1979; Robarts and Baum, 2007; Takahashi and Lisk, 1985) and lesions to the basal hypothalamus in anoles alter reproductive behaviors (Farragher and Crews, 1979), suggesting a similar role. All three of these brain areas in anoles also express androgen receptors (AR) and estrogen receptors (ERα), indicating the potential for steroid hormones to act directly in these behaviorally relevant regions (Rosen et al., 2002; Martinez-Vargas et al., 1978; Beck and Wade, Chapter 3).

The present study was designed to evaluate sex and seasonal differences in the morphology of the three reproductive regions in more detail. It complements the previous study that investigated soma size and density in the POA and AMY (O'Bryant and Wade, 2002), as well as examines the VMH for morphological differences.

Methods

Animal Housing and Tissue Collection. Materials for this experiment are the same as those analyzed in O'Bryant and Wade (2002). Briefly, male and

female green anoles were purchased from Charles Sullivan Co. (Nashville, TN) in the spring and fall. They were housed in the lab in 29 gallon aquaria that contained one male and 5-7 females. Animals were captured from the field in the seasons in which their tissues were collected. All procedures adhered to guidelines of the Michigan State University Animal Use and Care Committee and the National Institutes of Health.

All animals (n = 10 of each sex during the BS; n = 8 of each sex during the NBS) were overdosed using Sodium Brevital (Eli Lilly, Co.). They were perfused with phosphate buffered saline followed by 10% phosphate buffered formalin. Gonadal condition was noted at time of perfusion. Breeding females had at least one yolking follicle, and breeding males had enlarged, vascularized testes. The gonads from all animals in the NBS were regressed. Brains were removed and incubated overnight in phosphate buffered formalin, then dehydrated in a series of alcohols and cleared in xylene prior to paraffin embedding. Brains were sectioned at 20µm, counterstained with thionin, and coverslipped with permount.

Stereological Analyses. All brains were analyzed under brightfield illumination using Stereo Investigator (MicroBrightfield Inc., Williston, VT) by an individual blind to experimental group. The volumes of the POA, AMY and VMH were estimated by tracing the borders of these regions throughout the rostrocaudal extent of each nucleus. Sections were separated by 80µm in the POA and VMH, and by 40µm in the AMY (because it is not as large in the rostrocaudal plane). Neurons were counted using the Optical Fractionator in Stereo Investigator, which selects a series of smaller boxes (measuring either

25μm^2 [POA and VMH] or 20μm^2 [AMY]) within a larger grid (ranging from 50μm^2 to 100μm^2, depending on brain region) placed over the boundary region defined by the user. Only cells with a classic neuronal morphology exhibiting clearly defined nuclei and nucleoli were counted. Stereo Investigator generated estimates for the volume and total cell number of each region. To ensure that the tissue was not under-sampled and that estimates are accurate, all animals used for statistical analysis had a Gundersen Coefficient of Error less than 0.1 for both cell count and volume values.

Separate two-way ANOVAs in each brain region were conducted to analyze the effects of sex and season on estimated brain region volume and total cell number. Due to histological artifact, not all brain regions could be analyzed in all animals; final sample sizes are indicated in Table 2-1.

Results

Brain region volume. Main effects of both sex and season were seen in the POA, such that its volume was significantly greater in the BS than NBS ($F=10.01$, $p=0.004$) and in males than females ($F=6.44$, $p=0.018$; Figure 2-1). Similarly, the VMH was larger in the BS than NBS ($F=5.36$, $p=0.031$; Figure 2-2), but a main effect of sex was not detected in this brain region ($F<0.01$, $p=0.999$). The AMY showed no main effects of season ($F=3.49$, $p=0.076$) or sex ($F=0.01$, $p=0.939$). No interactions between sex and season were detected in any of these limbic regions.

Estimates of neuron number. A main effect of season existed in the AMY, such that more neurons were detected in the NBS ($F=10.07$, $p<0.005$; Table 2-1, Figure 2-3). However, there was no main effect of sex in this region ($F=0.244$, $p=0.626$). Additionally, no main effects of sex or season were seen in either the POA (sex: $F=0.01$, $p=0.933$; season: $F=0.15$, $p=0.706$) or VMH (sex: $F=0.23$, $p=0.631$; season: $F=0.04$, $p=0.847$). As with brain region volume, no interactions were detected between sex and season in any of these limbic regions.

Discussion

General Conclusions

The present study newly reports seasonal and sexual dimorphisms in the green anole lizard brain. POA and VMH volume were increased during the BS across the two sexes, whereas this measure did not differ in the AMY. In contrast, a seasonal effect of neuron number was seen only in the AMY; individuals from the NBS had more cells than BS animals. While morphology of the VMH had not been previously investigated in the green anole, the POA and AMY data are consistent with earlier work. In the same tissue from the current study, O'Bryant and Wade (2002) found that soma size was larger in the BS than NBS in the POA, which fits nicely with increase in volume without a change in neuronal number. Further, T increases soma size in the POA and AMY (Neal and Wade, 2007; O'Byrant and Wade, 2002), indicating a role for steroid hormones in this morphological difference. In contrast, AMY neuron number was increased during the NBS, without a seasonal change in brain region volume. This result

suggests that an addition of neurons, along with the documented decrease in soma size during the NBS in the AMY (O'Bryant and Wade, 2002), result in no seasonal difference of brain region volume. Finally, we found a male-biased sex difference in volume in the POA independent of season. O'Bryant and Wade (2002) detected a trend for larger soma size in unmanipulated male anoles, yet the current study found no difference in neuronal number. Therefore, it is plausible that increased soma size contributes to the volumetric sex difference seen in the current study, but other factors such as dendritic arborization likely play a role as well.

Comparison to other species: brain region volume

Sex differences in POA volume similar to the one detected in anoles exist in this general region of the brain in diverse vertebrates, including the SDN-POA of rats (Gorski et al., 1978), sexually dimorphic nucleus of the preoptic area in sheep (oSDN; Roselli et al., 2004), the AH-POA of whiptail lizards (Crews et al., 1990), POA of tree lizards (Kabelik et al., 2006), and the medial preoptic nucleus in Japanese quail (Adkins-Regan and Watson, 1990). These male-biased sex differences parallel at least one function of the POA in anoles – facilitation of masculine reproductive behaviors (Greenberg et al., 1984; Wheeler and Crews, 1978; reviewed in Godwin and Crews, 2002).

The increase in volume of the POA during the BS compared to the NBS is also consistent with that function and parallels findings from other seasonally reproducing species. Some examples include the medial preoptic nucleus in

male starlings (Riters et al., 2000), the AH-POA in whiptail lizards (Wade and Crews, 1991a) and the POA in tree lizards (Kabelik et al., 2006), where the region is larger during the BS. Similarly, the increased VMH volume detected in the present study is consistent with findings from garter snakes (Crews et al., 1993) and similar to naked mole-rats in which the VMH is larger in reproductive compared to non-reproductive individuals (Holmes et al., 2007). In many of these instances, these types of increases in brain region volumes coincide with elevated circulating levels of steroid hormones (reviewed in Tramontin and Brenowitz, 2000). In male whiptail lizards, castration during the BS causes AH-POA and VMH volumes to become more feminine (Wade and Crews, 1991a), while T treatments reverse or prevent these effects (Wade et al., 1993), suggesting that this hormone mediates the seasonal changes in males. Work in seasonally-reproducing songbirds also points to a role for steroid hormones in morphological change in the forebrain. For example, HVC (used as a proper name; Reiner et al., 2004), a sexually dimorphic nucleus involved in song production, increases in volume during the BS in canaries (Nottebohm, 1980) and white-crowned sparrows (Brenowitz et al., 1998, Smith et al., 1997a). Also, HVC volume is modified by steroid hormone treatments (reviewed in Ball et al., 2004).

However, some seasonal differences in brain region volume may be influenced by other factors, such as photoperiod. For example, gonadectomized juncos (Dloniak and Deviche, 2001) and European starlings (Bentley et al., 1999) show increases in HVC volume when exposed to longer days. T treatments

enhance this photoperiodic effect, but during short days the effect of T is lessened (Smith et al., 1997b). Additionally, in tree lizards reproductive state does not fully predict seasonal changes in males and is unrelated to changes in females (Kabelik et al., 2006).

Increases in brain region volume can also be modulated by social cues and activity. For example, male hamsters exposed to short days and housed with a female maintain MePD volumes comparable to those of males in long days (Cooke et al., 2001), and singing behavior in canaries facilitates increases in HVC neuron recruitment that persists following castration and is correlated with amount of singing (Alvarez-Borda and Nottebohm, 2002). Thus, in the green anole, it is conceivable that in addition to T, reproductive behaviors and/or social cues from other individuals may be involved in seasonal changes in the volume of the POA and VMH.

Comparison to other species: estimates of neuronal number

In this study, estimated total neuronal number in the AMY was greater in the NBS than BS. It is possible that this seasonal difference is the result of a cyclical pattern of cell birth and/or death, which results in regular changes in neuron number due to routine loss of older and incorporation of new cells.

Adult neurogenesis occurs in many species, including various birds and lizards (Alvarez-Buylla, et al., 1988; Delgado-González et al., 2008; Smith et al., 1997b). Evidence exists that new neurons are incorporated into the amygdala in adult voles (Fowler et al., 2002), and in some non-human primates (Bernier et al.,

2002) and rats (Park et al., 2006) neurogenesis may occur locally within the amygdala. In birds, the incorporation of new neurons in song control nuclei is often correlated with increased levels of steroid hormones (Absil et al., 2003; Hidalgo et al., 1995; Kim et al., 2008; Rasika et al., 1994; for review, see Ball et al., 2004).

In contrast, in the present study AMY neuronal counts were increased by 55% during the NBS compared to BS in green anoles, at a time when steroid hormone levels are relatively low (Jenssen et al., 2001; Lovern et al., 2001). Changes of at least this magnitude are not uncommon. For example, in adult canaries the incorporation of newly-generated cells in HVC is maximal in October and March, and is 4- to 7-fold greater than the rate detected in January. Additionally, these peaks are preceded by periods of cell death (Kirn et al., 1994). Similarly, exposing adult female prairie voles to males for 2 days causes an almost 3-fold increase in newly generated cells in the amygdala compared to isolated animals (Fowler et al., 2002). Green anoles spend more time clustered in social groups in the NBS (reviewed in Lovern et al., 2004), thus it is plausible that social contact acts similarly in anoles to facilitate incorporation of newly generated neurons in the amygdala.

Specific mechanisms regulating the seasonal differences in cell number in green anoles are not clear, but at least two possibilities exist. First melatonin, which is likely produced in greater quantities during the NBS, may increase the number of neurons in the AMY. Positive effects of melatonin on neuronal survival have been documented in other regions of the brain in other species. For

example, neonatal pinealectomy, and subsequent decrease in melatonin, leads to decreased cell survival of Purkinje cells in the cerebellar cortex of chicks (Tunç et al., 2005). In adult rat hippocampus, pinealectomy causes cell loss in CA1 and CA3 while exogenous melatonin attenuates the response (De Butte and Pappas, 2007). While autoradiography in the anole did not detect strong melatonin binding in the AMY (Wiechmann and Wirsig-Wiechmann, 1994), it is quite possible that melatonin or other hormonal factors act elsewhere initially to influence cell survival and/or incorporation of newly generated neurons into the AMY. Second, growth factors such as brain-derived neurotropic factor (BDNF) promote cell survival in the mammalian and avian brain (Pencea et al., 2001, Scharfman et al., 2005). BDNF is expressed in the anole AMY (Michael Black, personal communication), although potential seasonal variation of BDNF is unknown. In contrast, it is also possible that increased production of E2 during the BS compared to the NBS in the forebrain of green anoles (Rosen and Wade, 2001) is toxic to neurons in the AMY. This hormone may increase expression of apoptotic pathway genes (Jaita et al., 2005) and/or facilitate activity of proapoptotic proteins (Fester et al., 2006). It will be important to look into these possibilities in the future; as we did not label newly generating or dying cells, at this point we do not know whether one or both of these contribute substantially to the increased number of neurons in the NBS in our lizards.

Summary and Future Directions

The present study provides novel evidence on sex and seasonal dimorphisms in the three limbic reproductive regions of green anoles. The results on brain region volume parallel those from a number of other species, and suggest that green anole lizards, like many mammals and birds, exhibit both sexual dimorphisms and seasonal plasticity in the reproductive limbic nuclei. T-induced increases in soma size contribute, at least in the POA and AMY, in the anole. However, it is currently unclear whether additional mechanisms, including those involving factors other than steroid hormones, are important for volumetric differences in the lizard forebrain. A potentially novel increase in neuron number in the AMY during the NBS was also discovered, and implies a role for factors other than the increase in steroid hormone that often facilitates incorporation of new cells. Uncovering critical mechanisms in the context of diverse vertebrate species will elucidate evolutionary trajectories for the natural regulation of adult plasticity on multiple levels. Understanding factors that are common, as well as those that are unique to particular vertebrate groups, will provide information on both naturally occurring biological principles and the extent of change that one might be able to induce from a biomedical perspective.

Table 2-1: Estimates of Neuronal Number

	Breeding Season		Non-Breeding Season	
	male	female	male	female
POA	27,956 ±1,436 (8)	25,165 ±1,463 (9)	24,640 ±1,329 (5)	27,129 ±2,723 (5)
AMY*	6,409 ±590 (7)	6,166 ±434 (7)	8,459 ±969 (5)	9,547 ±1488 (5)
VMH	19,229 ±1,436 (8)	17,896 ±2,006 (7)	18,409 ±1,129 (5)	18,034 ±1,627 (4)

Values reported represent mean (±SEM) of estimated cells. * = BS<NBS.

Figure 2-1. Sex and seasonal differences in volume of the POA.

Examples of the POA are shown in a breeding male (A), non-breeding male (B), and breeding female (C). Arrows in A indicate the boundaries of the POA. The graph (D) shows that males (black bars) had significantly larger volumes than did females (white bars; $p<0.01$) and that breeding animals had larger volumes compared to non-breeding animals (* = $p<0.02$). OC = optic chiasm, V = third ventricle. Scale bar = 100μm.

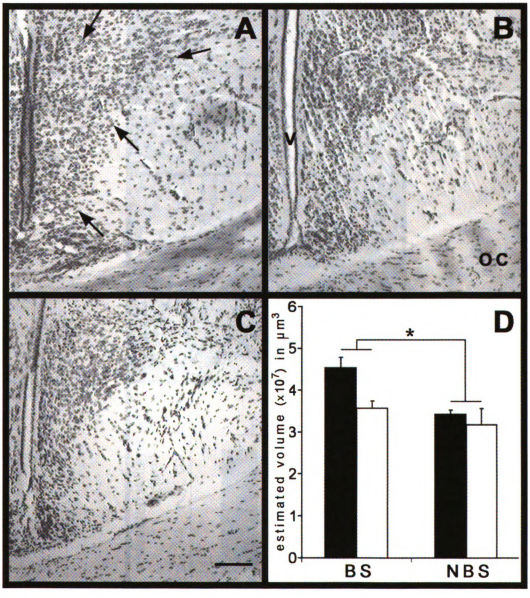


Figure 2-2. Seasonal difference in the volume of the VMH.

Breeding animals (A) had significantly larger volumes compared to non-breeding animals (B). Females are represented in A and B. The graph (C) shows that independent of sex (males = black bars; females = white bars), breeding animals had larger VMH volume than did non-breeding animals (* = $p<0.04$). Arrows in (A) delineate the boundary of the VMH. V = third ventricle. Scale bar = 100μm.

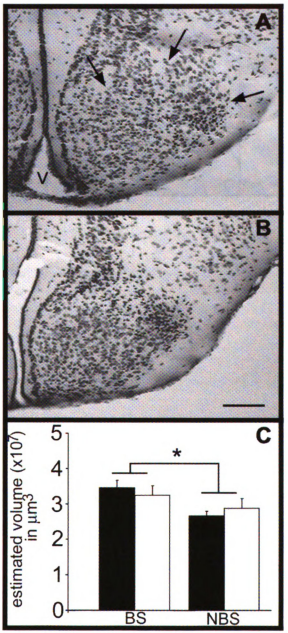
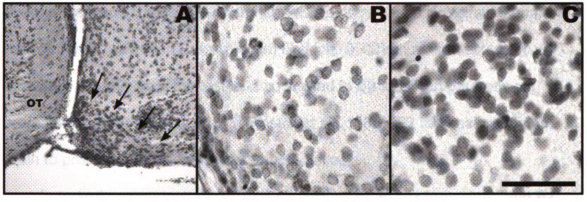


Figure 2-3. Seasonal difference in neuronal number in the ventromedial nucleus of the amygdala (AMY).

Arrows in (A) delineate the boundary of this brain region. In both sexes, the AMY contains significantly more neurons during the NBS (C) than BS (B; $p<0.005$). Females are represented in all panels. OT = optic tract; Scale bar = 250μm in (A) and 50μm in (B) and (C).



Chapter Three: Increased estrogen receptor α mRNA in the preoptic area and ventromedial hypothalamus of female green anole lizards

Introduction

Estradiol (E2) is a potent activator of both male and female sex behaviors in a variety of mammalian and avian species (reviewed in Ball and Balthazart, 2004; Meisel and Sachs, 1994). In females, E2 is released into circulation from the ovaries (reviewed in Blaustein and Erskine, 2002); in males testosterone (T) secreted primarily from the testes is converted to E2 locally in the brain via aromatization (Morali et al., 1977, Roselli et al., 1985). E2 then acts within the brain to promote sexual behaviors in both sexes.

In rodents, gonadectomy greatly diminishes male sex behaviors, and E2 administered either systemically (Dalterio et al., 1979; Wallis and Luttge, 1975) or locally within the forebrain (Davis and Barfield, 1979; Nyby et al., 1992) restores them. The same is true in male Japanese quail, in which E2 treatment following castration reinstates courtship and copulatory behaviors (Adkins et al., 1980). E2 is also critical to the activation of receptive behaviors in a variety of female vertebrates, including rodent species (Rubin and Barfield, 1983; reviewed in Blaustein and Erskine, 2002), whiptail lizards (Wade et al., 1991), and Japanese quail (Delville and Balthazart, 1987).

The preoptic area (POA) and medial amygdala are critical for the production of male-typical sexual behaviors, including courtship and copulation in rodents (reviewed in Meisel and Sachs, 1994), birds (medial preoptic nucleus and

nucleus taeniae, respectively; Balthazart and Surlemont, 1990; Watson and Adkins-Regan, 1989), and lizards (Kingston and Crews, 1994; Greenberg et al., 1984; Wheeler and Crews, 1978). In females, the ventromedial hypothalamus (VMH) is involved in the control of receptivity, including the extensively studied rodent lordosis (Emery and Moss, 1984; La Vaque and Rodgers, 1975; Pfaff and Sakuma, 1979). Estrogen is thought to bind to estrogen receptors (ER) located within these brain regions to facilitate reproductive behaviors.

ERs are expressed in the brains of both males and females in mammals (Lauber et al., 1991; Warembourg and Leroy, 2004; Wood and Newman, 1995), birds (Halldin et al., 2006; reviewed in Gahr, 2001), and reptiles (Crews et al., 2004); in some instances the extent of ER expression differs between the sexes. For example, in the mammalian VMH, ER expression is greater in females than males (Brown et al., 1996a; Lauber et al., 1991; Scott et al., 2000). Additionally, forebrain steroid hormone receptor expression can vary seasonally. For example, in the medial amygdala of Syrian hamsters (Mangels et al., 1998) and bed nucleus of the stria terminalis of California mice (Trainor et al., 2007) exposed to short-days, estrogen receptor-alpha (ERα) expression increases. Similarly, ERα decreases during the non-breeding season (NBS) in the song nucleus HVC (used as a proper name; Reiner et al., 1994) of canaries (Gahr and Metzdorf, 1997) and POA of spotted antbirds (Canoine et al., 2007). Seasonal fluctuations in circulating E2 may lead to these differences in receptor expression, as occurs with E2 treatment in some mammals (Hamada et al., 2005; Lauber et al., 1991; Meredith et al., 1994; Simerly and Young, 1991).

While much is known about E2 modulation of reproductive behavior in mammals and birds, less is known about the behavioral effects of E2 in the seasonal green anole lizard. Its role in females is clear; it activates receptivity (McNicol and Crews, 1979; Tokarz and Crews, 1980; Winkler and Wade, 1998), similar to other species (reviewed in Blaustein and Erskine, 2002). The strength of this behavioral response to E2 is reduced in the NBS compared to the breeding season (BS; Wu et al., 1985), which might be associated with differences in receptor expression. The role(s) that circulating and/or locally produced E2 may play in male green anoles is unclear. It has no direct effect on the activation of male sex behaviors (Winkler and Wade, 1998), yet brain aromatase activity is higher in males than females and, within males, it is higher during the summer BS than the winter NBS (Rosen and Wade, 2001). These results indicate that local synthesis of E2 from T can occur in the male brain in a pattern similar to other species (reviewed in Ball and Balthazart, 2004; Roselli et al., 2004), yet the function of this regulated E2 synthesis is unknown.

As in other species, the POA and amygdala (ventromedial portion, AMY; also known as ventral posterior amygdala, Bruce and Neary, 1995) are involved in the control of male courtship and copulatory behaviors in the green anole lizard (Greenberg et al., 1984; Wheeler and Crews, 1978). The VMH, as in mammals, controls receptivity in whiptail lizards (Kendrick et al., 1995; Wade and Crews, 1991b). It also likely regulates sexual behavior in female anoles. Lesions to the medial basal hypothalamus in green anoles, which encompasses the VMH, lead to a decrease in female receptivity (Farragher and Crews, 1979).

The following experiment was designed to assess ERα mRNA in the POA, AMY, and VMH in males and females during both BS and NBS. Expression of this receptor specifically has not been quantified in the adult green anole. However, autoradiography has detected binding of ^{3}H-estradiol in the forebrain (Martinez-Vargas et al., 1978; Morrell et al., 1979; see Discussion section).

Methods

Experimental Animals. Recently captured adult male and female green anoles (20 per sex, 10 from each season) during both the BS and NBS (in May and October, respectively) were used in this study (purchased from Charles Sullivan, Nashville, TN). After arrival in the lab, animals were group-housed, one male and 5-7 females per 29-gallon aquarium. Each of these contained peat moss, rocks, sticks, and water dishes. Fluorescent lights were present in the ceiling of the room, and full spectrum and heat lamps were provided above each cage. During the BS, laboratory conditions included a 14:10 light:dark cycle, with daytime temperatures ranging from 28-38°C (depending on distance from the heat lamp) and a nighttime temperature of 18°C. In the NBS, the lights were on a 10:14 cycle, with daytime temperatures ranging from 23-30°C and a nighttime temperature of 15°C. During both seasons, animals were fed calcium-dusted crickets or mealworms either three (BS) or two (NBS) times per week. Relative humidity was maintained at approximately 60-70%, and cages were misted daily with water during both seasons.

Animals were housed under laboratory conditions for one week after arrival in the lab prior to rapid decapitation. Reproductive status was noted at the time of decapitation. In the BS, all females had at least one yolking follicle, and males had enlarged, vascularized testes; the gonads of NBS animals were fully regressed. Brains were removed, flash frozen in cold methyl-butane, and stored at -80°C until sectioning. Brains were then sectioned into four alternate series of 20 µm frozen coronal sections, thaw-mounted onto SuperFrost Plus (Fisher Scientific, Hampton, NH) slides and stored with desiccant at -80°C until processing.

In situ hybridization. A green anole ERα cDNA (see Matthews and Zacharewski, 2000) was subcloned into pBlueScript. Using the MAXIscript In vitro Transcription Kit (Ambion, Austin, TX), ^{33}P-UTP was transcribed into sense (T3) and antisense (T7) probes. Excess nucleotides were removed using a G50 Sephadex bead column. Adjacent tissue sections from two series of slides (one for antisense, one for sense probes) were fixed in 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS; pH 7.4). Slides were rinsed in diethylpyrocarbonate (DEPC) water, treated with 0.25% acetic anhydride in triethanolamine buffer with 0.9% NaCl buffer (pH 8.0) and briefly rinsed in 0.1M PBS. Tissue was then dehydrated through a series of ethanols and air dried. The slides were then prehybridized in 1X hybridization buffer (4X SET, 1X Denhardts, 0.2% SDS, 250 µg/mL tRNA, and 25 µg/mL 5'-polyadenylic acid) and 50% formamide prior to an overnight incubation at 55°C with 1X hybridization buffer, 10% dextran sulfate, 40% formamide, and 5 X 10^6 cpm of sense or antisense

^{33}P-UTP-labeled probe. The next day, slides were rinsed in 4X SSC followed by 2X SSC then digested for 30 minutes with RNase A (20µg/ml). They were then rinsed in 2X SSC and 0.1X SSC prior to dehydration in ethanol, and air dried. The slides were exposed to Hyperfilm MP (Amersham Biosciences, Piscataway, NJ) with an intensifying screen (BioMax Transcreen LE; Eastman Kodak, Rochester, NY) for one week. Film was not analyzed; it served only as verification of sufficient signal. Slides were then exposed to NTB emulsion (Eastman Kodak, Rochester, NY) for 7 weeks, developed using D-19, fixed (Eastman Kodak, Rochester, NY), and lightly counterstained with 0.1% cresyl violet.

The relative intensity of labeling was first qualitatively assessed using an atlas of the green anole forebrain (Greenberg, 1982). Then, Scion Image (NIH image software) was used under darkfield illumination to quantify silver grains in the POA, AMY, and VMH (as in Beck and Wade, 2008). One section near the rostrocaudal center of each nucleus was selected for quantification (Figure 3-1) and the values summed for both hemispheres. Boxes were placed within the regions of interest (POA and AMY = 6829µm^2; VMH = 7531µm^2) in each hemisphere to quantify percentage of the area covered by silver grains. Using the density slice function to highlight silver grains, the area of covered pixels within the box was calculated. This value was divided by the box size to give the percent total area covered by silver grains. For each region, the value from a neighboring sense-treated section was subtracted from the antisense to correct for background labeling. Effects of sex and season on ERα mRNA expression

were determined by a two-way ANOVA for each brain region. All statistics were computed using StatView (SAS Institute, Cary, NC). Due to histological artifact, silver grains were not quantified in all regions for all animals. Final sample sizes are included in Figure 3-2.

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

Results

ERα expression. Receptor mRNA was observed in a variety of forebrain regions (Table 3-1), including the POA, AMY, and VMH. This general pattern was relatively consistent between the sexes and seasons. However, within the reproductive areas quantified, a main effect of sex was found in both the POA ($F=5.81$, $p=0.02$) and VMH ($F=5.36$, $p=0.03$); females expressed more ERα mRNA than males (Figures 3-1 and 3-2). Season did not affect the expression ($F<0.42$, $p>0.53$), nor did it interact with sex in these two brain areas (all $F<1.27$, $p>0.27$). Main effects were not detected in the AMY, although a trend for an interaction between sex and season ($F=3.62$; $p=0.06$) suggested that females might express higher levels of ERα mRNA in the NBS compared to BS (Figure 3-2).

Discussion

Localization of ERα mRNA. The present study documents ERα expression in a variety of regions in the green anole brain, including those

associated with the display of reproductive behaviors. Those with the highest levels include the VMH, mammillary nuclei, and septum. These results are similar to data from other reptiles. ER mRNA is expressed in many of the same regions in whiptail lizards, including the septum, amygdala, cortex, POA, suprachiasmatic nucleus, VMH, and toris semicircularis (Godwin and Crews, 1995; Wennstrom et al., 2003; Young et al., 1994). Additionally, ER mRNA has been documented in the septum and VMH of leopard geckos (Rhen and Crews, 2001).

Overall, this study also confirmed information determined by autoradiography of the green anole brain (Martinez-Vargas et al., 1978; Morrell et al., 1979), suggesting that much of the binding detected in these early studies reflected ERα expression. Relative levels of ERα mRNA appeared to differ in a few regions from previous results, however. For example, Morrell et al. (1979) and Martinez-Vargas et al. (1978) showed more extensive labeling in the POA and AMY than we detected. Presumably, all types of ERs would be labeled by the ^{3}H-E2 used in the earlier studies, not just ERα. Thus, it is possible that the increased labeling in the POA and AMY using autoradiography reflected a receptor other than ERα, such as ERβ. While ERβ has not been documented in anoles, it exists in these brain regions in a variety of species including rats (Laflamme et al., 1998), mice (Mitra et al., 2003), and Japanese quail (Foidart et al., 1999; nucleus taeniae, which is homologous to the mammalian amygdala, Reiner et al., 2004).

Alternatively (or in addition), the increased labeling in the previous studies on the green anole brain (Martinez-Vargas et al., 1978; Morrell et al., 1979) might

reflect procedural differences, as they examined lizards that were reproductively quiescent (both studies) and/or gonadectomized (Morrell et al., 1979), and sacrificed 1-3 hours after administration of a single dose of ^{3}H-hormone. ERα mRNA expression in the anole POA, AMY, and VMH suggests a potential role for E2 in each of these three regions, even if in some cases it is not directly related to the production of sexual behaviors.

Regional differences in label intensity and potential roles for E2. ERα mRNA expression in the VMH was up to 3 times greater than in the POA and AMY in both males and females. This pattern is consistent with E2-induced receptivity in females (see Introduction). The lower levels of ERα in the regions more important for masculine behavior may functionally result in decreased sensitivity to E2 compared to the VMH. However, detection of the mRNA in the POA and AMY, as well as substantial E2 binding (see above) suggests that action of this hormone in these two areas serves some purpose in the adult green anole. While it has yet to be determined, one possibility in males involves some sort of priming for androgenic activation of behavior. T treatments have a more profound effect on male behavior during the BS than NBS (e.g., Neal and Wade, 2007). It may be that this enhanced effect is due to previous exposure to androgens and/or E2 via local aromatization, as the levels rise prior to or early in the BS. It is also possible that E2 induces increases during the BS in the volume of the POA and VMH (Beck et al., 2008, Chapter 2) and soma size in the POA and AMY (O'Bryant and Wade, 2002). Such an effect of E2 would be similar to

what occurs in the medial amygdala of male rats; it maintains larger soma size (Cooke et al., 1999; Cooke et al., 2003).

Sex differences in ERα mRNA. Females expressed higher levels of ERα mRNA than did males in both the POA and VMH. These data agree with findings from other species, including voles (Cushing and Wynne-Edwards, 2006; Hnatczuk et al., 1994) and rats (Shughrue et al., 1992). The sexual dimorphisms in ERα mRNA expression in anoles may parallel the importance of E2 in facilitating their receptivity (Mason and Adkins, 1976; Tokarz and Crews, 1980; Wu et al., 1985). This is especially true in the VMH, but the POA may also be involved in female anole reproductive behaviors, as in rats (e.g., Bast et al., 1987). In parallel, inhibiting synthesis of functional ERα protein in the VMH, and perhaps elsewhere, abolishes female-specific sex behaviors in mice (Musatov et al., 2006; Rissman et al., 1997). Therefore, increased ERα mRNA expression may be less important for male than female anoles, as E2 does not activate their reproductive behaviors (Crews et al., 1978; Mason and Adkins, 1976; Winkler and Wade, 1998).

The decreased level of ERα mRNA expression in male green anoles may also relate to a difference in E2 availability; work in mammals indicates that E2 can reduce ER expression (Lauber et al., 1990; Lauber et al., 1991; Lisciotto and Morrell, 1993; Meredith et al., 1994; Sisk and DonCarlos, 1995). Higher local levels of E2 in the brains of males than females may result from more available T substrate and increased aromatase activity (Rosen and Wade, 2001), which could lead to ERα mRNA downregulation. However, the lack of seasonal

differences in ERα in the present study might be more consistent with a permanent organizational effect or adult regulation via a non-steroidal mechanism. These ideas will need to be investigated.

Sexual dimorphism of steroid hormone receptors is also is reflected in androgen receptor (AR) expression in the green anole. Females express higher levels of AR mRNA than males, especially in the POA (Rosen et al., 2002). The function of this increased expression in females in unknown. However, like males, they exhibit increases in brain region volume during or just prior to the breeding season (Beck et al., 2008, Chapter 2). At present, the mechanisms controlling these changes are not fully clear. It is possible that both androgens and E2 are involved to some degree. If so, enhanced AR synthesis may be required in females to increase sensitivity to the hormone, as circulating levels of testosterone are much lower in females than in males (reviewed in Lovern et al., 2004).

Cleary, more work must be done to elucidate the factors associated with the reproductive brain in green anole lizards. However, the present study provides an indication of likely locations of E2 action, specifically via ERα. Work currently underway will determine potential influences of E2 on morphology, ERα, and priming of male sexual behavior. In addition, future investigations regarding the function of and mechanisms associated with increased expression of steroid receptors in females, especially ERα in the POA and VMH, may lead to greater understanding the roles they play in the reproductive brain.

Table 3-1: Relative expression patterns of ERα mRNA in the anole brain*

brain region	relative expression
anterior dorsal ventricular ridge	++
bed nucleus of the hippocampal commissure	+
dorsolateral nucleus	+
lateral cortex	+
mammillary nucleus	+++
nucleus accumbens	+
nucleus paraventricularis magnocellularis	+
preoptic area	+
septum	+++
suprachiasmatic nucleus	++
torus semicircularis	+
ventromedial amygdala	+
ventromedial hypothalamus (lateral)	+++

*Based on a green anole forebrain atlas (Greenberg, 1982).

Figure 3-1. Photomicrographs in the preoptic area (A-C), ventromedial amygdala (D-F), and ventromedial hypothalamus (G-I).

Boxes in the brightfield images (left column; A, D, and G) are 150 μm x 150 μm, and indicate locations of the darkfield images in males (middle column; B, E, and H) and females (right column; C, F, and I). Sampling regions covered approximately one quarter of the area within these boxes/images. 3V = third ventricle, OT = optic tract. Scale bar in panel I represents 50 μm for all darkfield photographs.

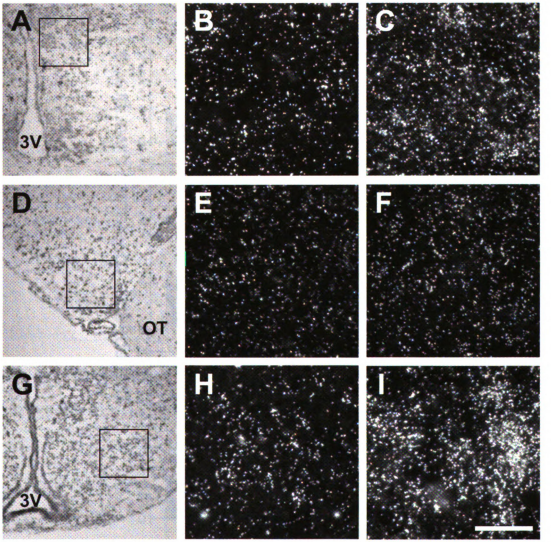
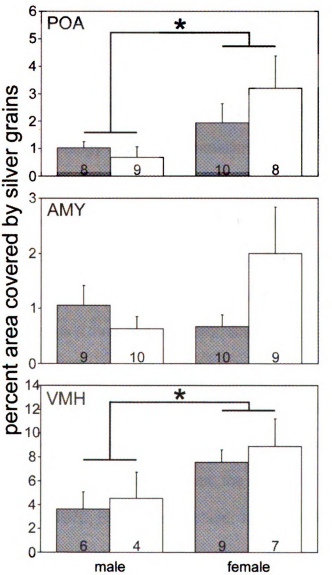


Figure 3-2. Relative expression levels of ERα mRNA in the green anole lizard.

In both the POA and VMH, females expressed higher levels of ERα mRNA than males. However, no seasonal differences were detected. Grey bars represent breeding animals; white bars represent non-breeding animals. Numbers within bars indicate sample sizes used in analyses. Note the different y-axes; levels in the VMH were substantially higher than the other two areas. * = $p<0.035$.



Chapter Four: Effects of estradiol, sex, and season on ERα mRNA expression and forebrain morphology in adult green anole lizards

Introduction

Estradiol (E2) has numerous effects on the brain including the facilitation of reproductive behaviors in adulthood. The hormone activates both male and female sexual displays in mammals and birds (e.g., Davis and Barfield, 1979; Davis et al., 1979; Balthazart et al., 1990). Critical brain regions include the preoptic area (POA), medial amygdala (MeA), and ventromedial hypothalamus (VMH). Masculine courtship and copulation are facilitated by the POA and/or MeA in rodents (Christensen et al., 1977; Powers et al., 1987), birds (nucleus taeniae, which shares homology with the mammalian amygdala [Reiner et al., 2004]; Watson and Adkins-Regan, 1989; Balthazart and Surlemont, 1990; Absil et al., 2002), and reptiles (Kingston and Crews, 1994). The VMH facilitates female-specific receptivity in a variety of species (e.g., Sakuma and Pfaff, 1979; Kendrick et al., 1995; Robarts and Baum, 2007; reviewed in Flanagan-Cato, 2000). In various mammals (Chen and Tu, 1992; Shughrue et al., 1997; Tobet et al., 1993; Scott et al., 2000; Cooke et al., 2003), birds (Balthazart et al., 1989; Canoine et al., 2007), and reptiles (Rhen and Crews, 2001; Wennstrom et al., 2003), these three regions express estrogen receptors. Additionally, direct application of E2 into these regions can facilitate the display of sexual behaviors (e.g., Davis and Barfield, 1979; Rubin and Barfield, 1983; Wade and Crews, 1991; Nyby et al., 1992).

Not only does adult E2 have behavioral effects within these regions, but it may alter the brain's responses by regulating expression of its receptor as well as morphology. For example, estrogen receptor alpha (ERα) expression in rats and guinea pigs is regulated in a sex- and region-specific manner by E2 (Lauber et al., 1991; Meredith et al., 1994). Lauber et al. (1991) found that E2 treatments down-regulate ERα mRNA expression in the VMH and arcuate nucleus, but not in the amygdala, and that this effect only exists in females. However, Sisk and DonCarlos (1995) documented both up- and down-regulation of ER in male ferret forebrain in a region-specific manner. E2 also affects morphology in adulthood; it increases volume of the posterodoral medial amygdala (MePD; Cooke et al., 2003) and dendritic spine density in the VMH (Calizo and Flanagan-Cato, 2000; Flanagan-Cato et al., 2001).

Activation of female reproductive behaviors in green anole lizards *(Anolis carolinensis)* is similar to rodents and birds; E2 directly facilitates the display of receptivity (Mason and Adkins, 1976, McNicol and Crews, 1979). In male anoles however, the primary facilitating hormone in reproductive behaviors is testosterone (T), not E2 (Crews et al., 1978; Winkler and Wade, 1998). As in other species, specific regions of the limbic forebrain are critical to the production of masculine and feminine anole reproductive behaviors. In male anoles, courtship and copulation are facilitated by the POA (Wheeler and Crews, 1978) and ventromedial amygdala (AMY, Greenberg et al., 1984; equivalent to the ventral posterior amygdala described in other lizards, Bruce and Neary, 1995). In females, it is likely that the ventromedial nucleus of the hypothalamus (VMH)

facilitates reproductive behaviors. Lesions of this particular area have not been conducted in green anoles, but destruction of the medial basal forebrain, which contains the VMH, disrupts female receptivity (Farragher and Crews, 1979) and the function of this region is highly conserved across species (e.g., Blaustein and Erskine, 2002).

Green anoles display reproductive behaviors during a spring and early summer breeding season (BS; reviewed in Lovern et al., 2004), when circulating levels of steroid hormones are high (Jones et al., 1983; Lovern et al., 2001). In parallel, the soma size of cells in the POA and AMY in intact animals is greater in the BS than the fall/winter non-breeding season (NBS; O'Bryant and Wade, 2002), and volumes of the POA and VMH are larger in the BS compared to the NBS (Beck et al., 2008, Chapter 2). In contrast, the AMY contains fewer cells in the BS than NBS (Beck et al., 2008, Chapter 2). Sex differences also exist in these regions associated with sexual behaviors in the green anole; the volume of the POA is larger in males than in females (Beck et al., 2008, Chapter 2). Females express higher levels of androgen receptor (AR) in the POA and ERα in both the POA and VMH than do males (Rosen et al., 2002; Beck and Wade, Chapter 3). However, whether or not these sex and seasonal differences are regulated by steroid hormones is unknown.

Interestingly, male green anoles exhibit increased neural aromatase activity compared to females, and among males, this activity is greater during the BS than the NBS (Rosen and Wade, 2001). Thus, there appears to be some active regulation of local E2 synthesis that parallels the display of masculine

sexual behaviors. However, as they are activated by T and not E2, the role of E2 produced in the male brain remains unclear. To begin to address these issues, reproductive forebrain morphology and ERα mRNA expression were examined in the POA, AMY and VMH of E2-treated and control males and females, during both the BS and NBS.

Methods

Experimental animals and hormone manipulations. Wild-caught green anoles (purchased from Charles Sullivan Company, Nashville, TN) were brought into the lab in each of the BS (May) and NBS (October; 20-22 per sex per season). Upon arrival, one male and 5-7 females were placed in a group cage containing a peat moss substrate, water dish, and sticks and rocks for perching. Fluorescent lights in the ceiling were used, along with full spectrum bulbs and incandescent heat lamps above each cage. Environmental conditions in the lab during the BS included a 14:10 light:dark cycle, with daytime temperatures ranging from 28-38°C, depending on distance from the heat lamp. At night, the temperature was set at 18°C. During the NBS, the light cycle was 10:14, daytime temperatures were 24-30°C, and at night it was 15°C. Relative humidity was maintained at 60-70%, and cages were sprayed with water daily. Animals were fed either three (BS) or two (NBS) times per week with calcium-dusted crickets or mealworms.

After one week to acclimate to laboratory conditions, animals were gonadectomized and implanted subcutaneously with estradiol benzoate (EB;

2mg in a 5mm slurry capsule, as in Neal and Wade, 2007) or a vehicle control capsule. This dose of E2 was used because it reliably induces receptivity in ovariectomized females (e.g., Neal and Wade, 2007). At the time of surgery, breeding condition was noted. BS males had large, vascularized testes, and females had at least one yolking follicle; NBS animals exhibited regressed gonads. All animals were rapidly decapitated one week following surgery. At this time, confirmation of the capsule's presence and completeness of gonadectomy was noted. Blood was collected from the head and neck in heparinized capillary tubes, and was centrifuged at 10,000 RPM for 10 minutes at 4°C. Plasma was stored at -80°C until radioimmunoassay. Brains were removed, flash frozen in cold methyl-butane, and stored at -80°C until sectioning. Four alternate series of 20 µm frozen coronal sections were collected onto SuperFrost Plus (Fisher Scientific, Hampton, NH) slides and stored with desiccant at -80°C until processed for Nissl staining or *in situ* hybridization. All procedures were carried out in accordance with NIH guidelines and the Michigan State University Animal Care and Use Committee.

Morphological analysis. One series of tissue (sections separated by 80 µm) was stained using thionin. It was analyzed under bright field illumination using Stereo Investigator (MicroBrightfield Inc., Williston, VT) by an individual blind to experimental group. The volumes of the POA, AMY, and VMH were estimated by tracing the borders of these regions throughout the rostrocaudal extent of each nucleus. Cells with typical neuronal morphology and a clearly defined nucleolus were counted using the Optical Fractionator in Stereo

Investigator. This method selects a series of smaller sampling sites (25 x 25 μm^2) within a larger grid (POA=100 x 100 μm^2, AMY=40 x 40 μm^2, VMH=80 x 80 μm^2) for counting within the defined borders. To ensure that the tissue was not under-sampled and that estimates of cell counts were accurate, a Gundersen Coefficient of Error near 0.1 was confirmed for both cell count and volume values.

Separate three-way ANOVAs in each brain region were conducted to analyze the effects of treatment, sex, and season on the estimates of brain region volume and total cell number using StatView (SAS Institute, Cary, NC). These analyses were broken down as appropriate when statistically significant interactions were detected (see below). Due to histological artifact, not all brain regions could be analyzed in all animals; final sample sizes are indicated in Figure 4-1A.

In situ *hybridization*. A green anole ERα cDNA (see Matthews and Zacharewski, 2000; Beck and Wade, 2008) subcloned into pBlueScript was used. ^{33}P-UTP was transcribed into an antisense probe with the MAXIscript In vitro Transcription Kit (Ambion, Austin, TX), which was then cleaned with a G50 Sephadex bead column. One series of slides was fixed in 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS; pH 7.4), then treated with 0.25% acetic anhydride in triethanolamine buffer with 0.9% NaCl buffer (pH 8.0) and dehydrated through a series of ethanols. Air dried slides were then prehybridized in 1X hybridization buffer (4X SET, 1X Denhardts, 0.2% SDS, 250 µg/mL tRNA, and 25 µg/mL 5'-polyadenylic acid) and 50% formamide for up to 24 hours.

Following prehybridization, slides were incubated overnight at 55°C with 5 X 10^6 cpm of probe in 1X hybridization buffer and 10% dextran sulfate in formamide. The next day slides were rinsed in SSC prior to 30 minute RNA digestion with RNase A (20µg/ml). They were then rinsed in SSC and ethanol-dehydrated before exposure to Hyperfilm MP (Amersham Biosciences, Piscataway, NJ) with an intensifying screen (BioMax Transcreen LE; Eastman Kodak, Rochester, NY) for one week. Film was not quantified; it served only as verification of successful hybridization. Slides were then exposed to NTB emulsion (Eastman Kodak, Rochester, NY) for 7 weeks, developed using D-19 and fixed (Eastman Kodak, Rochester, NY), prior to light counterstaining with cresyl violet.

Silver grains were analyzed under dark field illumination with Scion Image (NIH image) software by an individual without knowledge of experimental group. The density slice function was used to quantify the area covered by silver grains in boxes of known sizes in the left and right hemispheres of one section near the rostrocaudal center of each brain region (3842 μm^2 for AMY, 6830 μm^2 for POA and VMH). An area of the same size immediately outside of each region was also quantified to use for background correction. Silver grain assessments were each divided by the by the total area quantified to obtain the percentage of area covered. Numbers from the two sides of the brain were averaged, and the mean background level for each region was subtracted for each individual.

Using StatView (SAS Institute, Cary, NC), three-way ANOVAs tested for potential main effects and interactions of treatment, sex, and season individually for each brain region. Statistically significant interactions were further analyzed

using appropriate post hoc tests (see Results). One statistical outlier was detected in the POA (Dixon's test; Rohlf and Sokal, 1981); data from this individual are not reported. Final sample sizes are included in Figure 4-2.

Radioimmunoassay. Plasma samples from all animals were run in a single assay. They were first incubated with 1000 cpm of [3H]estradiol (1 μCi/μl; Perkin Elmer) at 4°C overnight to determine recovery efficiency. Estradiol was twice extracted from samples using diethyl ether, dried under nitrogen gas, reconstituted in PBS with 0.1% gelatin, and incubated at 4°C overnight. A standard curve of serially diluted E2 (ranging from 250 to 0.98 pg) was run in triplicate; samples were run in duplicate. Following overnight incubation with [^{3}H]estradiol and E2 antiserum (NEG307H; Biogenesis, Kingston, NH), the samples and standard curve were incubated at 4°C with dextran-coated charcoal for 15 minutes to remove unbound E2. The tubes were then centrifuged at 3000 RPM for 25 minutes, and the supernatant decanted and counted with 3.5 ml UltimaGold scintillation fluid (Perkin Elmer, Waltham, MA). Values were corrected for initial plasma volume and percent recovery. The intra-assay coefficient of variation was 6%. This E2 assay was previously validated in our lab (Lovern and Wade, 2003). In addition, prior to running the present samples, parallelism and accurate detection of known concentrations were demonstrated (data not shown).

A three-way ANOVA on log-transformed values was used to assess the effects treatment, sex, and season (StatView, SAS Institute, Cary, NC). Values for all but one individual from the blank-treated group fell below the sensitivity of

the assay. To be conservative, those values were set at the minimum detectable value (7.81 pg/tube). Similarly, values for some E2-treated animals were above the range of the curve. These were set at the maximum detectable value (250 pg/tube).

Results

Brain region volume. In the 3-way ANOVA, a main effect of sex was detected in each of the three brain regions ($F \geq 4.310$, $p \leq 0.042$; Figures 4-1 and 4-3); they were larger in males than females. An interaction between sex and season also existed in the POA ($F=6.401$, $p=0.014$). Collapsing across treatment, which had no effect, planned comparisons revealed that this brain region was larger in males than females in both seasons (BS: $t=5.893$, $p<0.0001$; NBS: $t=2.268$, $p=0.0302$). However, an effect of season was detected in females only, such that volume was greater during the NBS than BS ($t=3.043$, $p=0.005$). In addition to the main effect of sex, an interaction between sex and treatment was detected in the AMY ($F=4.362$, $p=0.0413$). Volumes were on average larger in males than females, but the sex difference is more pronounced in E2- ($t=5.301$, $p<0.0001$) than blank-treated animals ($t=2.493$, $p=0.018$). Main effects of season and treatment did not exist in the VMH, nor did interactions occur in that brain region ($F<1.825$, $p>0.182$).

Cell number. In both the AMY and VMH, a main effect of sex existed; more cells were present in these areas in females than males ($F>4.934$, $p<0.031$; FIgure 4-1A and 4-1B). A main effect of season also existed in the AMY

(F=4.121, p=0.0471); cell number was increased in the NBS compared to BS. An interaction between sex and season was detected in the VMH (F=8.822, p=0.004), which stemmed from an increased number of cells in females compared to males in the NBS (t=3.511, p<0.002), but not BS. In parallel, within females only, cell number was greater in the NBS than BS (t=2.387, p=0.0237) No main effects or interactions were detected on the estimates of total cell number in the POA (all F<3.617, p>0.062).

ERα mRNA. A three way interaction (F=4.827, p=0.032; Figure 4-2) was detected in the POA. Breaking this down, a 2-way ANOVA within males revealed a significant interaction between treatment and season (F=4.404, p=0.044); an effect of treatment existed in the BS only (t=2.402, p=0.0297), such that ERα expression was diminished with E2-treatment compared to the control manipulation. In the VMH, a main effect of sex existed; females expressed more ERα mRNA than males (F=10.33, p=0.002; Figures 4-2 and 4-4). A treatment by sex interaction (F=4.189, p=0.046) was also detected. Collapsed across seasons, which had no effect, a sex difference existed among E2-treated, but not control, animals; treated females expressed more ERα mRNA than males in the VMH (t=3.959, p=0.0004). Within males, E2-treated animals expressed less ERα mRNA than blank- treated individuals (t=2.382, p=0.0235). No main effects or interactions were detected in the AMY (F<2.40, p>0.127; Figure 4-2).

Radioimmunoassay. A main effect of treatment existed, such that treated animals had higher plasma levels of E2 than did blank controls (F=620.4, p<0.0001). Hormone treatment produced a range of 50-80 ng/ml (up to 65ng/ml

excluding the values that were too high to accurately detect from the standard curve). These values are ~25-40 times greater those reported in BS females (Jones et al., 1983). To our knowledge, plasma E2 levels have not been documented in unmanipulated males.

Discussion

Forebrain morphology summary. The POA, AMY and VMH were each larger in males than females. In addition, within females only, POA volume was larger in the NBS than BS. In the AMY, E2 magnified the sex difference in volume. Estimates of total cell number were greater in females than males in both the AMY and VMH, and in the VMH this sexual dimorphism was driven by data from the NBS. Also, within females, more VMH cells were detected in the NBS than BS. The same seasonal difference was detected in the AMY, and did not interact with sex.

Sex differences in morphology. The sex difference in POA volume detected in the present study across seasons replicates data collected in unmanipulated animals (Beck et al., 2008, Chapter 2). The results therefore suggest that the sexual dimorphism is present across diverse endocrine conditions. As it is likely not solely maintained by adult steroid hormones, it is possible that it is permanently established during development, as in a number of other species. For example, sexual differentiation of the POA in rats (Döhler et al., 1984), ferrets (Tobet et al., 1986) and sheep (Roselli et al., 2007), occurs during early perinatal development, and is often irreversible in adulthood (e.g.,

Gorski et al., 1978). However, if the POA of green anoles permanently differentiates prior to adulthood, it occurs somewhat later, as no sex differences are evident in the 10 days surrounding hatching in these lizards (Beck and Wade, Chapter 5).

In the current study, gonadectomy exposed a male-biased sex difference in volume of the AMY and VMH, which was not detected in intact animals (Beck et al., 2008, Chapter 2). These data parallel the larger soma sizes detected in gonadectomized males than females in the AMY during the NBS, regardless of whether the individuals were treated with T (O'Bryant and Wade, 2002). Further, E2 enhanced the sexual dimorphism in AMY volume. While not inducing a direct activational effect, adult E2 thus seems to facilitate change in one or more of the characteristics that contribute to overall AMY volume other than total cell number, which might include soma size or dendritic arborization. While the latter has not been investigated, T treatment does increase soma size in the AMY of males, and the same dose is more effective in the BS than NBS (Neal and Wade, 2007). In addition, the specificity of this effect indicates that while levels of E2 were supraphysiological, they did not induce widespread toxicity.

While it is not necessarily common for gonadectomy to reveal sex differences in morphology, steroid hormones can affect the volume of brain regions in adulthood. For example, the MePD is larger in male than female rats naturally, yet treatment with T can enlarge the female MePD to male levels (Cooke et al., 1999). Perhaps the levels of T in female anoles (~100pg/ml in the NBS and ~750pg/ml in the BS; Lovern et al., 2001) are sufficient to maximally

increase brain region volume, thus masking an underlying sex difference in AMY size. The same may be true for the VMH, in which a sex difference was detected in the present study, but not in gonad-intact individuals (Beck et al., 2008, Chapter 2).

Combined with the male-biased difference in the volumes of the AMY and VMH, a greater number of cells in females in these regions suggest that they may be smaller and/or more densely packed in females than males. Additionally, the female-biased sex difference in AMY and VMH cell number detected in the present study suggests that neuroprotection may be naturally heightened in females. Consistent with this idea, cultured female astrocytes from mice are more resistant to cell death in an E2-free media than cells from males or aromatase-null cells, indicating that genetically determined factors can promote cell survival in females (Liu et al., 2008). Similarly, evidence in prairie voles suggests that hippocampal cell survival in females is heightened during the NBS (Galea and McEwen, 1999).

Seasonal effects on morphology. The present study replicated a seasonal effect on AMY cell number seen in unmanipulated animals; a greater total number of cells exist during the NBS than BS (Beck et al., 2008, Chapter 2). These results suggest that some feature(s) of non-breeding environmental conditions facilitate(s) the addition to or survival of cells in this region. It may be related to the display of social behaviors. Female voles held in isolation have significantly fewer newly proliferated cells in the amygdala than those exposed to males (Fowler et al., 2002). Green anoles cohabitate without territories in the

NBS (reviewed in Lovern et al., 2004), so it is possible that new cells are incorporated into this nucleus in both sexes following social interactions during the NBS. Alternatively, perhaps cells incorporated into the AMY during or prior to the NBS are inhibitory and somehow diminish the potential for the display of sexual behaviors. A direct role of this area in the production of receptivity has not been documented in the female green anole, but it is important for reproductive behavior in males (Greenberg et al., 1984). Data from the VMH are a little more complicated to interpret, as a seasonal difference in cell number was detected in gonadectomized animals, some of which received E2-treatment, but not in intact green anoles (Beck et al., 2008, Chapter 2). Clearly more work must be done to determine the functional relevance of the increased number of cells in the AMY and VMH during the NBS, as well as whether they are mediated by differences in cell birth, incorporation or survival.

Regardless of their function(s), the fact that these differences exist in the absence of gonads and are unaffected by E2-treatment suggests that the seasonal effect on cell number in unmanipulated animals may not be maintained by steroid hormones. Non-steroidal cues, such as melatonin, could facilitate the seasonal differences. This hormone can increase the rate of cell survival (Tunç et al., 2005; De Butte and Pappas, 2007).

In unmanipulated adult green anoles, volumes of the POA and VMH (but not AMY) are greater during the BS across the two sexes (Beck et al., 2008, Chapter 2). However, mechanisms regulating the seasonal change in volume are not clear, as increased volume during the BS was not detected in the POA or

VMH in the current analysis. The seasonal difference detected in intact animals could therefore involve gonadal secretions during adulthood other than E2, perhaps T, or possibly non-gonadal factors as suggested above. Circulating levels of T are significantly higher during the BS in both males and females (Lovern et al., 2001). Experiments involving T manipulations in gonadectomized adult green anoles have documented that T increases soma size in the POA and AMY, and in the AMY it does so to a greater extent in the BS than NBS (Neal and Wade, 2007). Both of these effects, the stimulation by T and greater sensitivity in the BS, are mirrored in behavioral responses (Neal and Wade, 2007).

ERα mRNA. Expression in the VMH was 2-3 times higher than in the POA and AMY (see Figure 4-2). However, dramatic and specific effects of E2 treatment were seen on ERα expression in both the POA and VMH, such that treatment down-regulated receptor mRNA in males only. Further, in the POA, the effects of E2 treatment only existed during the BS. In the VMH, females expressed greater levels of ERα mRNA than males.

Autoradiography assessing binding of 3H-estradiol and the one study on ERα mRNA in adult green anoles (evaluating sex and season in unmanipulated animals) indicate that ER is present in all three brain regions investigated in the present study (Martinez-Vargas et al., 1978; Morrell et al., 1979), and that the mRNA is higher in the VMH than the other two areas (Beck and Wade, Chapter 3). Further, under these conditions, expression of ERα mRNA is sexually

dimorphic in the POA and VMH; levels are higher in females than in males, but equivalent in the BS and NBS (Beck and Wade, Chapter 3).

The detection of a main effect of sex in ERα in just the VMH in the current study, but in both the POA and VMH in intact animals (Beck and Wade, Chapter 3) suggests that the sex difference in the POA might require the presence of some gonadal secretion other than E2, whereas the dimorphism in the VMH does not. It is possible the female anoles constitutively express higher levels of ER than males, as in the VMH of rats (Lauber et al., 1991). Alternatively, baseline expression of ER in female anoles may be modulated by the noradrenergic system as in guinea pigs (Tetel and Blaustein, 1991). Or, social interactions may influence female ER expression, as exposure to a reproductively active animal increases ER expression in the VMH of parthenogenetic whiptail lizards (Hartman and Crews, 1996), and rats mated to satiety show increased levels of ERα in the POA (Phillips-Farfán et al., 2007). However, since the females in the current study were group-housed with a gonadectomized or E2-treated male, it is unlikely that ERα mRNA expression seen here reflects mating behaviors specifically. It is possible that the naturally occurring sex differences of ERα in the VMH and POA of anoles (Beck and Wade, Chapter 3) are due to down-regulation by increased E2 produced by greater aromatase activity in the male compared to female brain (Rosen and Wade, 2001). One idea is that combined effects of androgens and E2 are necessary for complete down-regulation of ERα in the male POA, as in the VMH

of female rats (Brown et al., 1996). This may account for the lack of sex difference in the current study following only E2 manipulations.

Unlike in females, E2 treatments significantly decreased ERα mRNA in the POA and VMH of males. Extensive data exists on the regulation of ER by steroid hormones, though the results are not completely consistent. For example, up-regulation of ER in response to E2 exists in the POA and mediobasal hypothalamus in ewes (Bittman and Blaustein, 1990), the medial VMH in male ferrets (Sisk and DonCarlos, 1995), and VMH of female whiptail lizards (Godwin and Crews, 1995; Young et al., 1995). In contrast, down-regulation of ERα by E2 exists in the VMH of female and POA of male rats (Lauber et al., 1991; Lisciotto and Morrell, 1993; Brown et al., 1996), the POA, amygdala, and ventral hypothalamus of female guinea pigs (Meredith et al., 1994), and periventricular POA of male ferrets (Sisk and DonCarlos, 1995). However, in the one study that examined the effects of E2 on ER expression in both sexes (Lauber et al., 1991) only females responded to E2 treatment with a down-regulation of ER in the VMH; no response was evident in males. Additionally, in Sisk and DonCarlos (1995), E2 either up- or down-regulated ER, depending on brain region. Further, in Brown et al. (1996), down-regulation of ER by E2 was enhanced in the presence of dihydrotestosterone (DHT), a metabolite of T. While collectively these results are not simple to interpret, it is clear that E2 can either increase or decrease ER expression, and does so in a sex- and regional-dependent manner. Down-regulation by E2 in the current study suggests active brain-region specific modulation of ERα mRNA in males.

Conclusions. E2 alone had no significant effects of forebrain morphology in the POA and VMH, yet gonadectomy revealed previously undetected sex and seasonal differences. The one interaction detected between treatment and sex in AMY volume is consistent with the idea that some specific priming by E2 may occur in a brain region relevant for male reproductive behaviors. It is clear that a combination of gonadal hormones, likely T, and other factors, perhaps including sex chromosome genes, modulate forebrain anatomy. However, the effects of E2 on ERα mRNA expression in the male POA and VMH indicate an active regulation of the receptor by its hormone in a sex- and region-specific manner. This modulation may be a mechanism by which males suppress female-like behaviors, particularly in the VMH.

Figure 4-1A. Effects of treatment, season, and sex on brain region volume and cell number in anole reproductive nuclei.

Statistically significant effects are identified within each graph. Volumes of all three regions was larger in males (black bars) than in females (grey bars); in the AMY and VMH, females had more cells than males. Also, NBS animals had more cells than BS animals in the AMY. Significant interactions indicate that POA volume is lowest in females during the NBS, the sex difference in the AMY is enhanced by E2-treatment, and that VMH cell number is highest in NBS females. Sample sizes are identified within the bars.

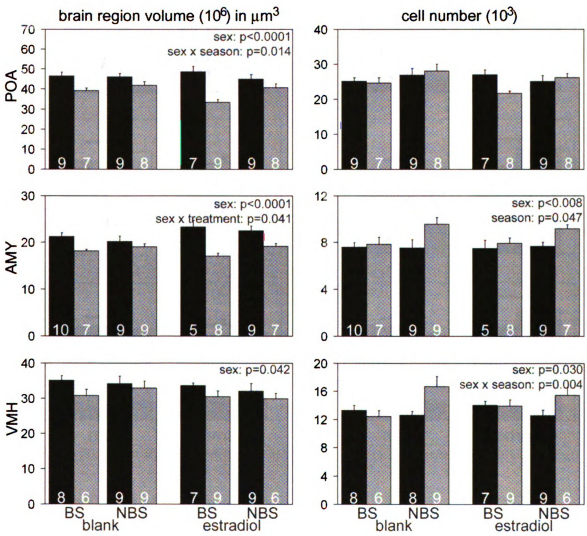


Figure 4-1B. Effects of treatment, season, and sex on brain region volume and cell number in anole reproductive nuclei.

The same data from Figure 4-1A are represented here, but collapsed across groups in which no significant effects were detected. Asterisks indicate significant main effects of sex, while letters distinguish differences between either BS (black bars) and NBS (dark grey bars) or blank treated (patterned bars) and estradiol treated (white bars).

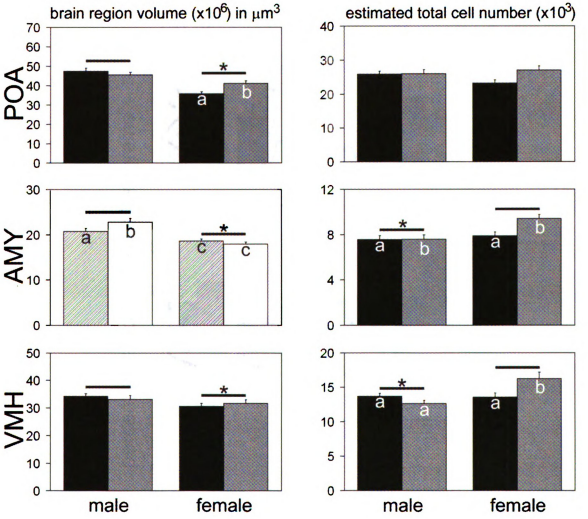


Figure 4-2. Estrogen receptor-alpha expression in the preoptic area, ventromedial amygdala, and ventromedial hypothalamus.

As in previous studies, expression was highest in the VMH (note differences in Y-axes). Black bars = males; grey bars = females. Sample sizes are indicated within each bar. Asterisk in top graph indicates that BS males had significantly less ERα mRNA than other groups. Letters in bottom graph indicate a female-biased sex difference and down-regulation by E2 in males only.

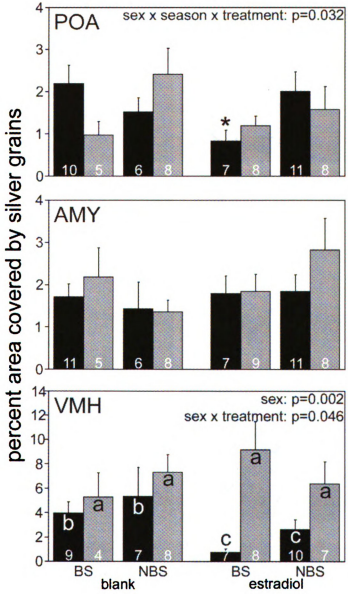


Figure 4-3. Brightfield images of the preoptic area (A and B), ventromedial amygdala (C and D) and ventromedial hypothalamus (E and F).

Each of these regions is larger in males (A, C, E) than in females (B, D, F). All animals shown are from the breeding season and were E2-treated. Arrows delineate the boundaries of the nucleus. 3V = third ventricle; BST = bed nucleus of the stria terminalis; OT = optic tract. Scale bar = 200μm.

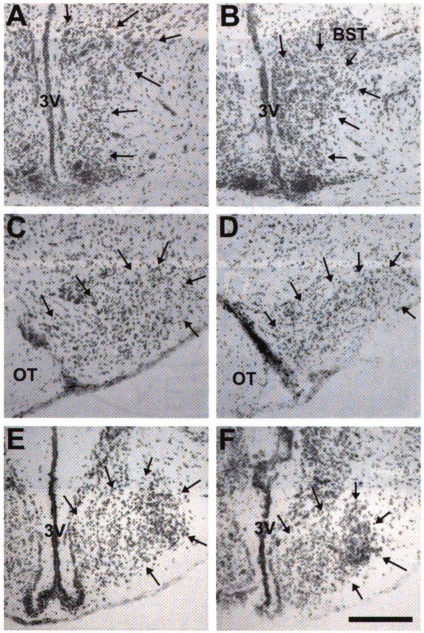
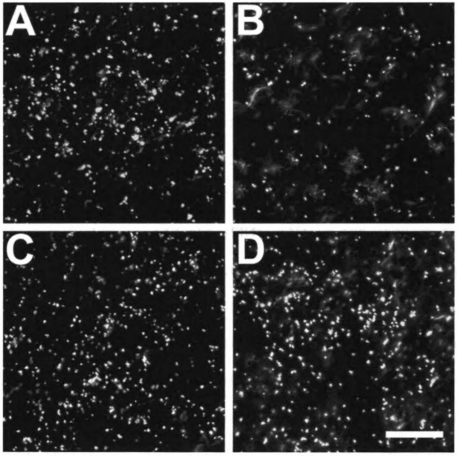


Figure 4-4. Darkfield images of silver grains representing ERα mRNA in the lateral ventromedial hypothalamus.

This is where labeling was heaviest and quantification was conducted in the VMH (near the three right-most arrows in figure 3). All images are from the non-breeding season (males = A and B; females = C and D). Estradiol (B and D) decreased ERα mRNA compared to the control treatment (A and C) in males only. Scale bar = 25 μm.



Chapter Five: Morphology and estrogen receptor alpha mRNA expression in the developing green anole forebrain

Introduction

Sexual dimorphisms in the adult forebrain exist in a variety of vertebrate species. They frequently parallel dimorphisms in behavioral displays, particularly in reproductive circuitry, in regions such as the preoptic area (POA), amygdala, and ventromedial hypothalamus (VMH). Much of this research has been conducted in rodents. In males of numerous species, portions of the amygdala are involved in the regulation of sexual appetite and sub-regions of the POA facilitate the display of copulatory behaviors, such as mounting and intromission (reviewed in Baum, 2002; Meisel and Sachs, 1994). These areas are often larger in males than in females (reviewed in Morris et al., 2004). In females, the VMH facilitates receptivity (Emery and Moss, 1984; La Vaque and Rodgers, 1975; Pfaff and Sakuma, 1979), and some evidence suggests that in rats it may be larger in females than in males (e.g., Matsumoto and Arai, 1983). Available results are similar in reptiles. For example, the POA regulates masculine sexual behaviors (Kingston and Crews, 1994), whereas the VMH is involved in female receptivity (Kendrick et al., 1995; Wade and Crews, 1991b) in whiptail lizards. These regions are sexually dimorphic; the POA is larger in males, and the VMH is larger in females (Crews et al., 1990; Wade and Crews, 1992). Steroid hormones, including estradiol (E2) and testosterone (T), facilitate these behaviors in adulthood (for reviews see Baum, 2002; Blaustein and Erskine,

2002; Moore and Lindzey, 1992). All three regions express steroid hormone receptors, some in a sexually dimorphic pattern that parallels behavior (see Chen and Tu, 1992; Tobet et al., 1993; Scott et al., 2000).

Sex differences in morphology are often established during development, guided by steroid hormones (Handa et al., 1985; reviewed in McCarthy, 1994; Resko and Roselli, 1997). For example, the sexually dimorphic nucleus of the POA (SDN-POA) in rats differentiates perinatally (Davis et al., 1996a). Circulating T, which in males increases around birth (Weisz and Ward, 1980), is locally converted to E2 by aromatase (Callard et al., 1978; Roselli et al., 1985; Selmanoff et al., 1977; Steimer and Hutchison, 1980). This rise in locally produced E2 leads to masculinization of the SDN-POA, in part by inhibiting cell death (reviewed in Morris et al., 2004). In the rat posterodorsal medial amygdala (MePD), postnatal E2 exposure masculinizes morphology (Nishizuka and Arai, 1981), and work in hamsters suggests that this sex difference may be also be influenced by pubertal steroid hormone exposure (Schulz et al., 2004; De Lorme and Sisk, unpub. observ.).

While the role of E2 in both the organization and activation of reproductive forebrain nuclei has been extensively studied in mammals, its function remains somewhat unclear in the green anole lizard, *Anolis carolinensis*. Unlike rodents, in which E2 facilitates masculine reproductive behaviors (Davis and Barfield, 1979; Nyby et al., 1992), it is T that is the primary activating hormone in male green anoles (Crews et al., 1978; Mason and Adkins, 1976; Winkler and Wade, 1998). Estradiol, however, does facilitate female reproductive behaviors in green

anole lizards (Mason and Adkins, 1976; Tokarz and Crews, 1980), as in rodents (Davis et al., 1979). Similar to other species, three forebrain regions are particularly associated with anole reproductive behaviors in the green anole. Lesion studies indicate that the POA (Wheeler and Crews, 1978) and ventromedial amygdala (AMY, Greenberg et al., 1984; also known as ventral posterior amygdala, Bruce and Neary, 1995) significantly decrease male reproductive behaviors. In females, lesions of the medial basal hypothalamus, which includes the VMH, result in diminished female reproductive behaviors (Farragher and Crews, 1979). Steroid hormone receptors are expressed in adulthood in these three regions in green anoles (Martinez-Vargas et al., 1978; Morrell et al., 1979; Rosen et al., 2002). However, little is known about the ontogeny of these regions in this species, including whether steroid hormones organize their structure or function.

In this study, we take a first step toward understanding the mechanisms regulating sexual differentiation of the POA, AMY and VMH in green anoles by examining morphology and characterizing ERα mRNA in these regions in late embryonic and early post-hatching development. We targeted our analysis to shortly after embryos appear to start producing steroids, which occurs by day 24 of incubation (Lovern and Wade, 2003), or about 10 days prior to hatching (which occurs approximately 34 days after oviposition under our laboratory conditions, Lovern et al., 2004)

Methods

Experimental animals. Eggs were collected from cages every day and individually incubated at 27.5°C in a plastic cup with moist vermiculite (1:1 vermiculite:water by mass). Tissue was collected at embryonic (E) days 26 and 31, as well as the day of hatching (P0) and five days after hatching (P5). A small hole was punctured and the egg shell cut to facilitate embryo removal. Embryos were separated from the yolk prior to decapitation. Hatchlings were also rapidly decapitated. To preserve morphology, whole heads and torsos were flash frozen in cold methyl-butane and stored at -80°C until sectioning. Torsos were sectioned at 20 µm and stained with hematoxylin and eosin to determine gonadal sex. Heads were cut into four alternate series at 10 µm. After sectioning, the head tissue was stored with desiccant at -80°C until Nissl staining or *in situ* hybridization (see below). Final sample sizes are included in Figure 5-1 and Tables 5-1 and 5-2.

Morphological analysis. Slides were warmed to room temperature for 20-30 minutes prior to staining. Following a rinse in water, the tissue was stained with thionin, differentiated in 70% ethanol with acetic acid, and dehydrated in a series of ethanols. After soaking in xylene twice for five minutes, the tissue was coverslipped. All brains were analyzed under brightfield illumination. Using Stereo Investigator software (MicroBrightfield Inc., Williston, VT), the volumes of the POA, AMY, and VMH were estimated by tracing the borders of these regions every 40 µm throughout the rostrocaudal extent of each nucleus on both sides of the brain. To determine cell density, a box of 2500 μm^2 was placed over each

region (POA, AMY or VMH) and cells located within that box with a clearly defined nucleolus and classic neuronal morphology were counted. This procedure was carried out in three sections, in each of the rostral, central, and caudal areas of the nucleus in both the left and right hemispheres, following an atlas of the *Anolis* forebrain (Greenberg, 1982). The perimeter of 30 cells (15 per hemisphere) were traced using Neurolucida software (MicroBrightfield Inc., Williston, VT), and the area determined via Branch Structure Analysis in Neurolucida Explorer (MicroBrightfield Inc., Williston, VT). For both the cell density and soma size analyses, individual values per animal, 6 and 30 respectively, were averaged prior to statistical analysis. Separate two-way ANOVAs (Statview; SAS Institute, Cary, NC) for volume, cell density, and soma size were conducted for each brain region, comparing between sex and across ages. If significant interactions were found, one-way ANOVAs were used followed by appropriate pairwise comparisons.

In situ hybridization. A green anole ERα cDNA (Matthews and Zacharewski, 2000; Beck and Wade, 2008) subcloned into pBlueScript was used. ^{33}P-UTP was transcribed into antisense probes using MAXIscript In vitro Transcription Kit (Ambion, Austin, TX). Unincorporated ^{33}P-UTP was removed using a G50 Sephadex bead column. Tissue sections were fixed in 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS; pH=7.4). Slides were rinsed in diethylpyrocarbonate (DEPC) treated water prior to treatment with 0.25% acetic anhydride in triethanolamine buffer. They were then briefly rinsed in 0.1M PBS, dehydrated through a series of ethanols and air dried. Slides were

prehybridized for up to 24 hours at 55°C in 1X hybridization buffer and 50% formamide prior to an overnight incubation at the same temperature with 5 X 10^6 cpm of antisense ^{33}P-UTP-labeled probe. The next day, the slides were rinsed in SSC and digested for 30 minutes with RNase A (20µg/ml). The slides were rinsed in 2X SSC and 0.1X SSC prior to dehydration. Once dry, the slides were exposed to Hyperfilm MP (Amersham Biosciences, Piscataway, NJ) with an intensifying screen (BioMax Transcreen LE; Eastman Kodak, Rochester, NY) for 1 week to confirm signal strength. Slides were then exposed to NTB emulsion (Eastman Kodak, Rochester, NY) for 7 weeks, developed using D-19 and fixed (Eastman Kodak, Rochester, NY), then lightly counterstained with cresyl violet.

Analysis of silver grains was conducted on both sides of the brain in one equivalent section from each animal. Centrally located sections within the AMY, POA, and VMH were selected for quantification, using the Greenberg (1982) atlas. Within the POA and AMY sections, sampling sites were located near the middle of the nucleus. However, the lateral portion of the VMH was analyzed, as ERα is concentrated in this region in rodents (e.g., Shughrue et al., 1997) and whiptail lizards (Wennstrom et al., 2003), and labeling appeared heavier there in our sections as well. Under darkfield illumination, the density slice function in Scion Image (NIH image software) was used to quantify the area covered by silver grains in a 2500 µm^2 box placed over each region. This value was divided by the box size to give the percent of the area covered by silver grains. Values for the two hemispheres were averaged within individuals prior to statistical

analysis. Two-way ANOVAs (Statview; SAS Institute, Cary, NC) in each brain region were used to compare ERα mRNA between sexes and across ages.

Results

Brain region volume. A significant effect of age was detected in the POA. It decreased in volume between E26 and P0 ($F=27.22$, $p<0.001$; Figs. 5-1 and 5-2). An interaction between sex and age was also detected ($F=3.04$, $p=0.043$; Fig. 1). POA volume diminished in both females ($F=22.14$, $p<0.0001$) and males ($F=10.29$, $p=0.0004$). However, in females, the volume at E26 was greater than all other ages (each Scheffe's test $p<0.001$), which did not differ from each other. In males, POA volume was equivalent at E26 and E31, and at P0 and P5. Differences existed between E26 and the two post-hatching ages (both Scheffe's test $p<0.008$) and between E31 and P5 (Scheffe's test $p=0.042$). Planned comparisons within each age group revealed significantly larger POA volumes in males than in females at E31 ($t=2.382$, $p=0.044$) and P0 ($t=2.607$, $p=0.035$). No main effect of sex was detected in the POA ($F=1.092$, $p=0.304$). In the AMY, no effects of sex or age were detected for volume, and no interactions existed (all $F\leq1.483$, $p\geq0.264$; Fig. 5-1). In the VMH, a trend for the volume to decrease with age was detected ($F=2.766$, $p=0.058$), but a sex difference and an interaction between sex and age were not evident ($F\leq1.578$, $p\geq0.213$; Fig. 5-1).

Cell density. Significant increases in cell density were seen with age in both the POA ($F=20.31$, $p<0.001$; Fig. 5-1) and AMY ($F=4.92$, $p=0.006$; Figs. 5-1 and 5-2). In the POA, cell density was greater at P0 and P5 than E26 and E31

(all Scheffe's $p \leq 0.014$). In the AMY, cell density was decreased in E26 compared to P5 animals (Scheffe's $p=0.038$). Main effects of sex were not detected in these two brain regions (all $F<0.301$, $p>0.586$). An interaction between sex and age existed in the AMY ($F=4.04$, $p=0.015$), but not the POA ($F=0.383$, $p=0.766$). One-way ANOVAs within each sex in the AMY revealed that cell density in males was similar across the ages examined ($F=0.947$, $p=0.438$), whereas in females it increased over time ($F=9.466$, $p<0.001$), such that E26 differed from both P0 and P5 (Scheffe's $p \leq 0.003$). No main effects or interactions were detected in the VMH (all $F \leq 1.933$, $p \geq 0.174$).

Soma size. In all three brain areas, soma size significantly decreased with age (all $F>17.82$; $p<0.001$), but no main effects of sex were detected (all $F<1.105$, $p>0.301$; Table 5-1). Cells at E26 and E31 had larger cross sectional areas than did those at P0 and P5 (all Scheffe's test $p \leq 0.003$; Table 5-1 and Fig. 5-2).

In situ *hybridization*. All three brain regions expressed ERα mRNA, though it was approximately 3-4 times greater in the VMH than POA and AMY (Table 5-2; Figs. 5-3 and 5-4). Main effects of sex and age were not detected in the POA or AMY (all $F \leq 1.993$, $p \geq 0.133$). However, ER mRNA expression differed across the ages in the VMH ($F=5.52$, $p<0.003$). Higher levels were detected at P0 than E31 or P5 in this region (Scheffe's $p<0.05$; Fig. 5-4). A main effect of sex was not detected in the VMH ($F=1.223$, $p=0.276$), nor were significant interactions detected in any of the three regions (all $F \leq 0.682$, $p \geq 0.569$).

Discussion

A number of effects were detected in the present study, most of which involved changes in morphology of the POA and AMY as animals aged. In addition, ERα expression was substantially greater in the VMH that then other two areas, peaking around the day of hatching. These results are discussed in detail below, but overall they are consistent with at least two ideas. First, while diminishing soma size in late embryonic and early post-hatching green anoles may be relatively non-specific, the density of cells increases more selectively in areas involved in the control of masculine behaviors. Second, ERα expression is both relatively high and dynamic in the VMH, which is involved in the regulation of feminine sexual behaviors. Thus it is possible that masculinization and feminization of the brain are occurring during this developmental timeframe, and E2 may be playing a role in feminization.

Forebrain morphology. Volume of the POA decreased between E26 and P5, and females exhibited a more rapid decline with age than did males. A decrease in soma size and an increase in cell density also occurred in this brain region. Together, these results suggest that neurons migrate closer together as their somas shrink. However, it is also possible that some neurons die during this period, as is the case in rodents (reviewed in Morris et al., 2004), and/or that synaptic pruning is occurring, as seen in rats during development (Dreher et al., 1985; reviewed in Luo and O'Leary, 2005). The differing rates of decline in overall POA size of males and females and transient sex difference in volume at E31 and P0 suggest that early dendritic pruning might be increased in females

compared to males. Alternatively, males may have more spines than females around the time of hatching. A similar situation occurs in the POA of newborn male rats, which reflects sex differences in E2-induced synthesis of prostaglandin-E2 (PGE_2) and subsequently the dendritic spine protein spinophilin (Amateau and McCarthy, 2002). The role of steroid hormones and other key factors will need to be investigated in the developing green anole.

Given the stable and low level of ERα mRNA expression in the POA over these time points, E2 seems an unlikely candidate for mediating the morphological changes in that brain region. However, androgens may be involved, as T can increase brain region volume in adult tree lizards (Kabelik et al., 2008) and hamsters (Cooke et al., 1999). While androgen receptor (AR) expression in the developing anole forebrain has not yet been investigated, it is expressed in the POA of both sexes of the adult anole (Rosen et al., 2002).

In the AMY, cell density increases with age only in females, while soma size decreases in both sexes during this time period. It is therefore likely that these two types of morphological change are unrelated. As the volume remains stable in both sexes, the data are consistent with the idea that new cells are added only to the female AMY during this time. This is the case in developing rats, in which terminal division of sexually dimorphic vasopressin cells in the centromedial amygdala between E12 and E13 is greater in females than in males (al-Shamma and De Vries, 1996).

If females do have increased cell addition in this brain region, which is important for the regulation of masculine sexual behaviors, perhaps they are

inhibitory. This type of situation exists in the anteroventral periventricular nucleus of the hypothalamus (AVPV), a region associated with estrous cyclicity (reviewed in Simerly and Swanson, 1987). In that case, inhibitory neurons projecting from the bed nucleus of the stria terminalis, exist in male, but not female, rats (Polston et al., 2004). As sex differences in neuronal number are not present in the adult anole AMY (Beck et al., 2008, Chapter 2), if such a scenario is true, the function is likely related to a developmental process.

In the VMH, a trend for decreased brain region volume coincides with significant decreases in soma size but no change in cell density. Thus, it seems likely that the diminishing cell sizes contribute to the trend for a change in volume. In fact, soma size decreases with age in all three areas examined. This effect can be a marker for pre-apoptotic cells (Bortner and Cidlowski, 1999; reviewed in Bortner and Cidlowski, 2002). As cell death is common in the maturation process, it is feasible that the decline in soma size reflects some cells preparing for programmed cell death.

The mechanisms regulating the decreases in soma size occurring between E31 and P0 in all three brain regions are presently unclear. However, a number of examples exist in which T can modulate cell size. For example, in adulthood, T leads to an increase in neuron soma size in the amygdala in various species including rats (Cooke et al., 1999), hamsters (Cooke et al., 2002), and green anoles (Neal and Wade, 2007), as well as in the medial preoptic nucleus of Japanese quails (Panzica et al., 1991). A similar effect occurs in development. For example, female zebra finches treated with T after hatching exhibit increased

soma size in the robust nucleus of the arcopallium, a sexually dimorphic song control nucleus (Grisham and Arnold, 1995). In green anoles, yolk T decreases between E24 and E32 (Lovern and Wade, 2003), just prior to the decline in soma size in the present study. However, embryonic T increases between E16 and E24, and then remains stable (Lovern and Wade, 2003). Thus, it is possible that the decrease in yolk T affects the soma size, but it seems more likely that a direct influence of another endogenous factor in the developing brain is critical.

ERα mRNA expression. The current study confirmed that the embryonic and late post hatching anole forebrain expresses ERα mRNA. Levels were detectable in the POA and AMY, but substantially higher in the VMH. Adult expression of ER in these brain areas occurs in other lizard species, including whiptails (Crews et al., 2004; Young et al., 1995) and leopard geckos (Rhen and Crews, 2001). Some evidence in whiptail lizards suggests that ER is also expressed at least in the VMH during development (Wennstrom et al., 2003). Increased ERα mRNA in the VMH compared to the POA and AMY of anoles during early development is consistent with the role that E2 plays in adult behaviors. The latter two regions are involved in male-specific reproductive behaviors, which are not activated by E2 (Mason and Adkins, 1976; Winkler and Wade, 1998), and the VMH likely regulates E2-facilitated receptivity (Mason and Adkins, 1976; Wade and Crews, 1991b). Given that the current results mirror those seen in adulthood, in which ERα mRNA expression is highest in the VMH (Beck and Wade, Chapter 3), it seems likely that this region exhibits increased sensitivity to E2 throughout life in the anoles. If so, the functions prior to sexual

maturity are currently unknown, but might include neuroprotection, as E2 can support neuronal survival (Fan et al., 2008) and up-regulate brain-derived neurotrophic factor mRNA expression (Zhou et al., 2005).

In adulthood, adult female anoles express significantly higher levels of ERα mRNA in the VMH than do males (Beck and Wade, Chapter 3). However, in the current study, no sex differences were evident during anole development. It is unclear when the sex difference emerges, but perhaps it is influenced by physiological changes occurring with the onset of sexual maturity. While density of cells increases with time in the POA of both sexes and the AMY of females, ERα mRNA expression remains static. Therefore, it is possible that individual cells within the POA and AMY may express less ERα mRNA with increased age. A decrease in ERα mRNA per neuron may result in decreased sensitivity to E2, which may in turn contribute to cell death, as E2 can reduce apoptosis (Wright and Smolen, 1987).

Conclusions. Morphology of the anole forebrain undergoes substantial changes during late embryogenesis and in hatchlings. During this time period, regions important in both masculine and feminine reproduction express ERα mRNA, although levels in the VMH are much higher than in the POA and AMY. E2 may therefore influence development of the structure and/or function of these areas in the green anole, particularly the VMH. The nature of these hormonal effects requires further investigation, and the recent sequencing of the green anole genome (http://www.broad.mit.edu/models/anole/) will greatly enhance those efforts.

Table 5-1: Soma size in reproductive nuclei in both sexes during development

		E26[a]	E31[a]	P0[b]	P5[b]
POA	male	47.91 ±2.22 (5)	42.90 ±1.13 (6)	33.75 ±1.92 (6)	29.24 ±2.28 (6)
	female	46.57 ±2.62 (5)	41.94 ±2.06 (5)	33.81 ±1.75 (6)	30.31 ±2.44 (6)
AMY	male	42.17 ±2.56 (6)	49.67 ±3.10 (6)	30.03 ±3.17 (4)	31.08 ±3.07 (5)
	female	42.38 ±1.82 (4)	42.44 ±3.17 (5)	30.78 ±2.05 (6)	29.11 ±2.39 (6)
VMH	male	46.47 ±2.22 (6)	43.37 ±1.28 (6)	31.27 ±3.19 (6)	28.54 ±3.04 (4)
	female	47.53 ±0.80 (5)	40.49 ±3.89 (4)	31.21 ±1.10 (6)	28.19 ±2.17 (4)

Values reported represent mean (± SEM) of the cross sectional area of soma (in squared microns); sample sizes are included in parentheses. Across all regions, soma size was significantly greater in E26 and E31 animals than P0 and P5 animals (a>b).

Table 5-2: Percent area covered by silver grains in reproductive nuclei across ages in both sexes

		E26	E31	P0	P5
POA	male	4.40 ±0.52 (7)	4.28 ±0.56 (6)	3.03 ±0.92 (5)	3.44 ±0.42 (6)
	female	4.29 ±0.71 (5)	2.89 ±0.80 (4)	2.822 ±0.62 (4)	3.60 ±0.25 (6)
AMY	male	3.14 ±0.27 (7)	2.29 ±0.48 (5)	2.10 ±0.27 (4)	3.47 ±1.07 (6)
	female	2.98 ±0.73 (6)	2.14 ±0.30 (5)	2.33 ±0.35 (5)	2.29 ±0.47 (6)
VMH	male	11.21 ±1.13^{a,b} (6)	9.22 ±0.66^{a} (6)	21.48 ±4.07^{b} (6)	12.00 ±3.00^{a} (6)
	female	13.06 ±1.88^{a,b} (6)	7.00 ±0.84^{a} (5)	16.11 ±4.84^{b} (6)	9.05 ±1.34^{a} (5)

Values reported represent mean (± SEM) of the percent area covered by silver grains (2500 μm^2); sample sizes are included in parentheses. Different letters indicate significant differences in percent area covered by silver grains.

Figure 5-1. Brain region volume and cell density (counts per box) across ages in both sexes.

Different letters above bars (in black) denote significant differences across ages. Sex differences within each age group, determined by planned comparisons, are indicated by asterisks above the bars. Effects of age within each sex, determined by one-way ANOVA, are indicated by different letters within bars (in white). Sample sizes are indicated at the bottom of the black (male) and grey (female) bars. POA = preoptic area; AMY = ventromedial amygdala; VMH = ventromedial hypothalamus.

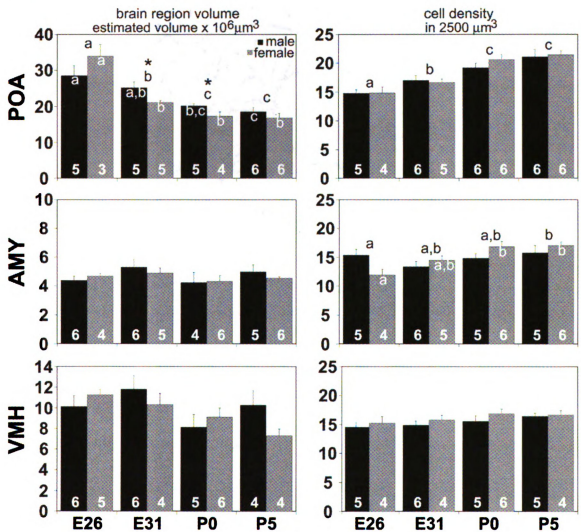


Figure 5-2. Photomicrographs depicting differences in brain region volume, cell density, and soma size across age groups.

Embryonic day (E) 26 females are represented in panels A, C, and E and post-hatching day (P) 5 females are represented in B, D, and F. Brain region volume significantly decreased over time in the POA (A and B). In females only, cell density increased with age in the AMY (C and D). Soma size decreased with age in all brain regions; the VMH is represented here (E and F). In A and B, "3V" indicates the third ventricle and arrows define the boundaries of the POA. Scale bar = 250 μm for A and B, 73.3 μm for C and D, 50 μm for E and F.

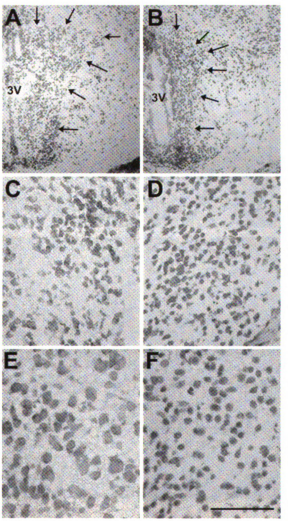


Figure 5-3. Estrogen receptor α mRNA expression in the POA and AMY. Borders of the brain regions (POA, A and B; AMY C and D) of embryonic day (E) 31 males are denoted with arrows in panels A and C. Thin line in C marks the ventral edge of the brain. Darkfield images (B and D) are enlargements of the boxes delineated in the adjacent brightfield panels. Neither POA nor AMY expresses high levels of ERα mRNA in this species at this age. 3V = third ventricle, OC = optic chiasm, OT = optic tract, SCN = suprachiasmatic nucleus. Box measures 100 x 100 μm^2.

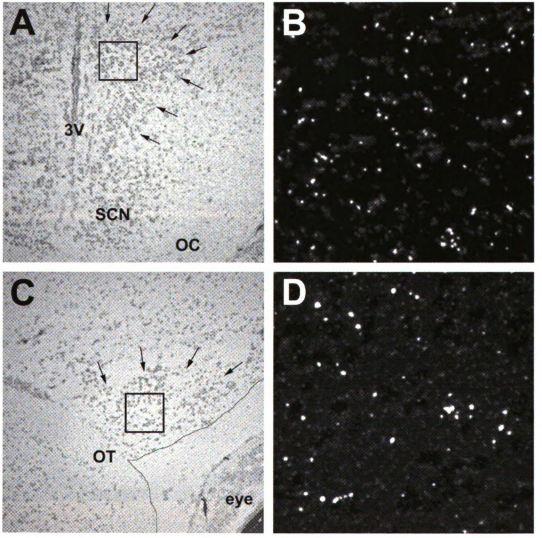
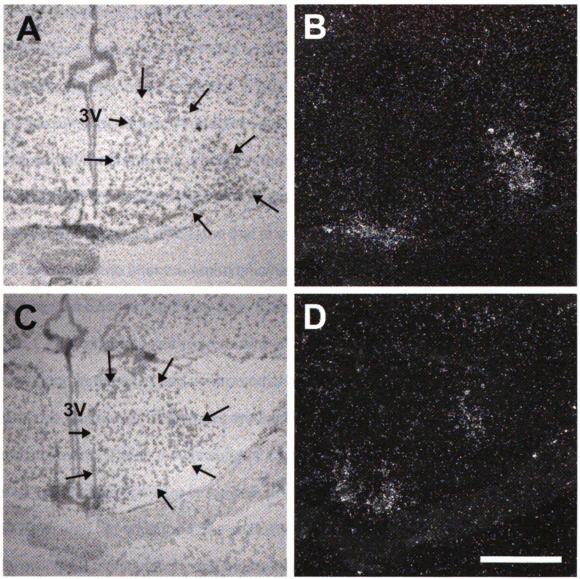


Figure 5-4. Estrogen receptor α mRNA expression in the VMH.

Males at P0 (A and B) and P5 (C and D) show the difference between ages. Arrows delineate the border of the brain region. Expression in the lateral portion of the VMH was consistently greatest (depicted in the darkfield images, B and D). Analyses were conducted within those areas in all animals. 3V = third ventricle. Scale bar = 100 μm.



Chapter Six: Discussion of Results

The preceding set of experiments investigated the potential influence of estradiol (E2) on forebrain morphology and estrogen receptor-alpha (ERα) mRNA expression in green anole lizards from sex, seasonal, and developmental perspectives.

The effects of E2 on morphological sex differences are unclear. A sex difference in preoptic area (POA) volume is likely established prior to adulthood, as gonadectomy or E2-treatment in adulthood does not affect it. The potential effects of testosterone (T) on POA volume have not been investigated, but since this sexual dimorphism is present in the absence of T following gonadectomy, it is likely that a non-gonadal source maintains this sex difference. Perhaps, it is a permanent structural difference that is established during development. If so, this occurs sometime after the embryonic/hatchling period, because no sex differences exist during these ages (Chapter 5; Figure 6-1).

Similarly, females express higher levels of ERα mRNA than males in adulthood in the POA and ventromedial hypothalamus (VMH) during both seasons, yet no sex differences are evident during the early period of anole development. These results suggest that the sex difference in ERα mRNA expression develops later in the animal's life, similarly to the sex difference in POA volume. In adulthood, the sex difference in ERα is maintained in the VMH following E2 manipulation, and males display decreased ERα following treatment in this region (Figure 6-2). However, this sex difference seen in the POA of

unmanipulated animals disappears following E2 manipulation, yet down-regulation of ERα mRNA by E2 in males exists during the BS, similar to the VMH.

While seasonal differences exist in adult POA and VMH volume and in ventromedial amygdala (AMY) cell number in unmanipulated adult anoles, it is clear that E2 alone does not influence the changes between seasons; treating gonadectomized animals with this hormone has no effect on these differences (Figure 6-3). Further, even though ERα mRNA does not differ naturally across seasons, E2 down-regulates expression of this receptor in the male POA and VMH, indicating that it may be modulated in part by steroid hormones in a sex- and regional-dependent manner.

Forebrain morphology in green anoles

Information obtained through the above experiments merges with existing knowledge on forebrain morphology in the green anole with respect to sex, season, and T manipulation studies. While the parameters measured within these studies were not completely consistent, some conclusions can be drawn based on a synthesis of the available information.

Preoptic area. Volume of the POA is larger in intact males than in females and both the volume of and soma sizes in this region are larger during the breeding season (BS) in unmanipulated animals (Chapter 2; O'Bryant and Wade, 2002). However, no differences in total neuron number or cell density exist between the sexes or the seasons (Chapter 2; O'Bryant and Wade, 2002). Following gonadectomy, a seasonal difference in POA volume is not observed

(Chapter 4), soma size is larger in males than in females (O'Bryant and Wade, 2002), and the sex difference in POA volume is maintained (Chapter 4). T treatment increases soma size independent of season (Neal and Wade, 2007), yet E2 treatment has no documented effects on morphology (Chapter 4), though soma size was not quantified.

Therefore, it is likely that seasonal difference in POA volume stems from increases in soma size facilitated by T, but not E2. Furthermore, the male-biased sex difference in POA volume is likely due to increased soma size compared to increased dendritic arborization or cell number, and that maintenance is not dependent on gonadal hormones, as this sex difference in maintained following gonadectomy. However, the sex difference in POA volume and soma size may be permanently established during juvenile development, as sex differences in these parameters did not exist prior to or just after hatching (Chapter 5).

Ventromedial amygdala. In the AMY, no sex or seasonal effects exist in volume (Chapter 2), yet non-breeding season (NBS) animals have more cells than BS animals do (Chapters 2 and 4) and soma size is increased during the BS compared to the NBS (O'Bryant and Wade, 2002). T treatment increases soma size, and it does so to a greater degree during the BS than the NBS (Neal and Wade, 2007), while gonadectomy reveals a male-biased sex difference in AMY volume, which is even greater in E2 treated animals (Chapter 4). Additionally, gonadectomy reveals a female-biased sexual dimorphism in the estimated total number of cells (Chapter 4) without affecting the seasonal difference seen in unmanipulated animals (Chapters 2 and 4). This indicates that the seasonal

difference in cell number is likely not facilitated by steroid hormones, but rather a non-gonadal mechanism dependent on season.

In intact anoles, no seasonal differences in brain region volume were observed, however this result might reflect an increase in cells during the NBS (Chapter 2) and larger soma size during the BS (O'Bryant and Wade, 2002). Together, these seasonal changes in cell number and soma size could account for no net change in overall brain region volume. Additionally, the enhanced sex difference in AMY volume following E2 treatment could perhaps be explained via an increase in soma size. The effects of E2 on soma size have not been documented in green anoles, but E2 can influence soma size in other animals (e.g., Cooke et al., 2003). It is possible the effects of T treatment on soma size (Neal and Wade, 2007) are attributable to E2, not androgenic action. T could locally be metabolized into E2, and it may be E2 that directly affects soma size, as in other species (e.g., Cooke et al., 2003). If this scenario were true, male AMY neurons are likely more influenced by E2 than females.

A female-biased sex difference in AMY cell number after gonadectomy may reflect an innate difference in cell number between the sexes, but one that is normally masked by gonadal hormones. This parallels data on some size, where gonadectomy reveals that larger somas exist in males than in females (O'Bryant and Wade, 2002). Further, since cell number is unaffected by E2 treatment, it is possible that T obscures an inherent sex difference in cell number, by either promoting cell survival in males or reducing cell survival in females. Therefore, it

is feasible that baseline sex differences exist in the AMY, but are naturally eradicated by circulating gonadal hormones, likely T.

Ventromedial hypothalamus. Unlike the POA and AMY the only work on morphology of the VMH conducted in green anoles is the two studies in this dissertation (Chapters 2 and 4). VMH volume is larger in the BS than the NBS, and no sex difference is detected in this measurement in unmanipulated animals (Chapter 2). Gonadectomy eliminates the naturally occurring seasonal difference and reveals a male-biased sex difference in volume, neither of which appear to be affected by E2 treatment (Chapter 4). Furthermore, gonadectomized females possess more cells than males and more cells during the NBS than the BS. These results suggest that seasonal variations in VMH volume are not facilitated by exclusively E2, though it is possible that T and/or E2 facilitate this difference, as gonadectomy removes this seasonal effect on volume. Additionally, the appearance of male-biased sexual dimorphism in VMH volume may be due to T as well; T might increase soma size in the female VMH, as in the POA and AMY (Neal and Wade, 2007), which could increase brain region volume.

While T may influence soma size and overall brain region volume, it is unclear how T or E2 may be involved in the genesis, survival or incorporation of cells in the anole. E2 is often implicated with increased cell genesis and survival (e.g., Galea and McEwen, 1999; reviewed in Galea, 2008), but that does not explain the sex and seasonal effects on cell number following gonadectomy in the anoles. If E2 supports cell survival in the anoles, E2 treated animals would likely have more cells than vehicle treated animals, therefore it is likely some

other mechanism by which cell survival is enhanced. Similar to the AMY, it is possible that non-steroidal cues dictate this baseline sex difference in cell number.

Estrogen receptor in green anoles

ERα mRNA is expressed in the POA, AMY, and VMH of the green anole lizard, among other areas. These results are consistent with autoradiography studies, which detected binding in these three regions (Martinez-Vargas et al., 1978; Morrell et al., 1979). Additionally, the VMH expresses up to 3 times more message than the POA or AMY (Chapter 3), which is consistent with data from late embryonic and early post-hatching animals (Chapter 5). Adult females express higher levels of ER mRNA than males in the POA and VMH (Chapter 3) and E2 treatment down-regulates ERα mRNA expression in the male POA and VMH (Chapter 4). Furthermore, gonadectomy and gonadectomy + E2 treatment eliminate the sex difference in the POA that exists in unmanipulated animals, yet does not alter the sex difference in the VMH (Chapter 4).

These data suggest that the VMH, functionally relevant to female reproductive behavior, may be naturally more sensitive to E2 than other regions, particularly in females, as it expresses the highest level of ERα mRNA out of all regions quantified. Decreased expression of ERα in unmanipulated males compared to females may be related to increased local E2 production by aromatase in males (Rosen and Wade, 2001) and this natural down-regulation

may be reflected in the female-biased sex difference in the POA and VMH (Chapter 3). This idea is discussed in greater detail below.

Comparison of anoles with other species

Morphological plasticity in adulthood. While brain morphology is largely organized during development in many species, evidence suggests that aspects of forebrain morphology can be altered in adulthood. Morphological plasticity in the adult can be facilitated by a host of factors; including those related to gonadal hormones, season, and social exposure.

One example of steroid hormone regulation of morphology exists in the posterodorsal medial amygdala (MePD) of rodents. This region is larger in male rats than in females (Hines et al., 1992) and this sexual dimorphism is maintained by steroid hormones in adulthood. MePD phenotype is sex-reversed by adult gonadectomy in male and adult T-treated female rats (Cooke et al., 1999; Cooke et al., 2003), and T treatments, but not dihydrotestosterone (DHT), maintain dendritic morphology in the MePD of Syrian hamsters (Gomez and Newman, 1991). This indicates that either E2 or T alters MePD morphology. Further, E2 treatments can also increase dendritic spine density in the VMH (Calizo and Flanagan-Cato, 2000). Similarly in the sexually dimorphic song control nuclei of canaries, androgen treatments increase the volume of the HVC (used as a common name, Reiner et al., 2004) and the robust nucleus of the acropallium (RA; Brown and Bottjer, 1993) in females. Steroid hormones may function similarly in anoles to alter morphology in adulthood. T clearly increases

soma size in the anole POA and AMY (Neal and Wade, 2007), and thus, likely affects brain region volume as well. While quantification of volume of brain regions in green anoles has not been conducted in T-treated animals, the fact that seasonal differences in the POA and VMH volume were abolished with gonadectomy and not rescued by E2 (Chapters 2 and 4) is consistent with the idea that T facilitates this seasonal difference. Additionally, E2 has no effect on morphological sex differences with one exception – a male-biased sex difference in AMY volume. This effect of E2 might be attributed to an increase in soma size, similar to that seen in T manipulated anoles, or possibly enhanced dendritic morphology, as seen in hamsters (Gomez and Newman, 1991). With the removal of gonads in adult green anoles (Chapter 4), many previously undetected sex and seasonal differences emerged (Figure 6-4). While the function of this is unclear, it is possible that gonadal hormones act to create equality of function between the seasons and sexes, an idea which is discussed in greater detail below.

Seasonal cues also mediate structural changes in the brain. In red-winged black-birds (Kirn et al., 1989) and Nuttal's white crowned sparrows (Brenowitz et al., 1998), for example, song control nuclei (HVC, RA, and Area X) exhibit a marked increase under BS compared to NBS environmental conditions. While the size of a brain region is typically larger during longer days (BS), data on the role of season on cell number are conflicting. Song sparrows housed under BS conditions show increases in neuron number in HVC (Smith et al., 1997a), yet NBS conditions enhance cell proliferation and/or survival in the hippocampus of

golden hamsters (Huang et al., 1998) and female voles (Galea and McEwen, 1999). These effects of season, however, are also accompanied by changes in circulating steroid hormones. This makes it difficult to determine the exact contribution of photoperiod on seasonal changes. Still, mounting evidence suggests photoperiod contributes to morphological changes independently of steroid hormones; these effects have been detailed in songbirds. HVC volume is significantly greater in castrated sparrows (Bernard et al., 1997) and starlings (Bentley et al., 1999) exposed to a long-day photoperiod than those in a short-day photoperiod. Melatonin, which is secreted in greater quantities under short-day conditions, attenuates the response in HVC volume induced by increased photoperiod (Bentley et al., 1999). While steroid hormones and photoperiod-regulated cues often work in tandem, gonad-independent modulation of morphology can occur. This appears to be true in the anoles, where morphological modifications between seasons exist independent of steroid hormones. Since the seasonal effect in AMY cell number seen in unmanipulated animals persisted despite gonadectomy or E2 treatment (Chapter 4), it is possible that gonad-independent mechanisms, like photoperiod or melatonin, modulate cell proliferation/survival in the anoles, as in songbirds.

In addition to hormonal and seasonal regulation, forebrain morphology may also be influenced by social cues. For example, female European starlings that prefer BS male song contain larger HVC volumes than females who preferred NBS song (Riters and Teague, 2003). In prairie voles, newly proliferated cells were greater in the amygdala of females exposed to males than

those housed in isolation (Fowler et al., 2002), indicating a role of social context on neurogenesis. This may also account for seasonal differences in neuron number seen in the anole AMY, as neither gonadectomy nor E2 treatments affected this naturally occurring seasonal difference.

Not only do the combined effects of hormonal and seasonal cues influence morphology, but gonadal steroids together with social context can alter the forebrain. One example of this exists in the song system of canaries. The magnitude of the effect of T treatments on male canaries on HVC and RA volume and singing rate is altered by social context; males sing more and have a larger HVC and RA when following behavior testing with other males rather than females (Boseret et al., 2006). Furthermore, these changes in volume are due to increased cell migration and survival in the HVC (Balthazart et al., 2008). This may provide an explanation for more cells in gonadectomized female anoles in the AMY and VMH. T treatment within a socially relevant context may promote cell survival in male anoles, but without androgens fewer cells survive, resulting in a female-biased sex difference in cell number.

While the effects of these factors have not yet been fully investigated in anole, a large body of evidence suggests adult forebrain plasticity can be mediated by hormonal, seasonal, and social cues in a variety of species.

Development of sexual dimorphisms. Robust effects of steroid hormones, especially E2, exist in the developing forebrain across many mammalian and avian species. A classical example of steroid hormone influence on sexual differentiation is in the rat POA. Near the time of birth in males, circulating T

levels increase (Weisz and Ward, 1980), and are aromatized in the brain to E2 (Selmanoff et al., 1977; Callard et al., 1978; Steimer and Hutchison, 1980; Roselli et al., 1985). Increased local E2 concentrations lead to the masculinization of the SDN-POA, possibly by inhibiting cell death in males (reviewed in Morris et al., 2004). These effects of steroid hormones occur during perinatal development and are relatively permanent (e.g., Handa et al., 1985). However, sexual differentiation is not exclusive to this developmental time point.

In rodents, the anteroventral periventricular nucleus (AVPv), a region highly involved in estrous cyclicity, is larger in females than in males (Davis et al., 1996b). However, this sex difference is not present in neonates; it appears near puberty (Davis et al., 1996b), and is likely facilitated by initial production of gonadal hormones. Even though the region is not sexually dimorphic in pups, it is clear that the perinatal hormonal environment guides the differentiation of this region during puberty as orchiectomy prior to, but not after, postnatal day 5 results in a feminine phenotype in adulthood.

A similar mechanism of masculinization may exist in the green anole. It is clear the animal undergoes further morphological maturation with age, as the POA volumetric sex difference seen in adulthood is absent during development. Therefore, it is likely that the anole forebrain undergoes a suite of morphological changes later in development, yet prior to adulthood. If the anole POA develops similarly to the rodent AVPv, this maturation likely occurs at the onset of the following BS as in some mammals (Ebling and Foster, 1989) which, similar to puberty, is accompanied by marked changes in circulating hormones.

It is possible that the sexual differentiation of the POA in the anole forebrain may be influenced by hormones during development, but that those differences may not emerge until later, as demonstrated in the rodent AVPv. However, it is unclear at this time if E2 influences late embryonic and early post-hatching development in the POA of the green anole lizard, given that ERα mRNA expression was low and static.

However, a direct relationship between ERα mRNA expression and morphological changes exist only in the VMH. ERα mRNA expression peaks near hatching in this region, concurrent with a decrease in soma size. It is possible that E2 may directly regulate the development of the VMH during this time, though it likely does not directly influence the morphological development of the POA and AMY.

Since all regions are not fully developed by post-hatching day 5, perhaps other, non-steroidal mechanisms drive maturation prior to adulthood.

Regulation of estrogen receptor expression. Regulation of ER by E2 exists in diverse taxa and the magnitude and direction of this regulation is dependent on the species, sex, and brain region investigated.

In whiptail lizards, E2 treatments up-regulate ER in the female VMH; yet do not affect expression in the female POA or any brain area in males (Godwin and Crews, 1995; Young et al., 1995). Up-regulation of ER in response to E2 also exists in the the medial VMH in male ferrets (Sisk and DonCarlos, 1995) and POA and mediobasal hypothalamus in ewes (Bittman and Blaustein, 1990).

In contrast, down-regulation of ERα by E2 occurs in the VMH of female and POA of male rats (Brown et al., 1996b; Lauber et al., 1991; Lisciotto and Morrell, 1993), the POA, amygdala, and VMH of female guinea pigs (Meredith et al., 1994), and periventricular POA of male ferrets (Sisk and DonCarlos, 1995). However, in the one study that examined the effects of E2 on ER expression in both sexes (Lauber et al., 1991) only females responded to E2 treatment with a down-regulation of ER; no response was evident in males. Yet in Sisk and DonCarlos (1995), E2 had both up- and down-regulatory effects, dependent on brain region.

While these results are contradictory, it is clear that E2 can either increase or decrease ER expression, albeit in a sex- and regionally-dependent manner. E2 treatments reduce ERα mRNA expression in the POA and VMH of male anoles (Chapter 4). However, in the POA, a main effect of sex disappears with gonadectomy and/or E2 treatment indicating that E2 alone can not completely down-regulate ERα expression. Long-term T exposure in female whiptail lizards depresses the E2-inducable up-regulation of ERα mRNA expression in the VMH (Crews et al., 2004), and down-regulation is enhanced in the presence of DHT, a metabolite of T, in female rats (Brown et al., 1996b). Considering the information from other animal models, it seems possible that high levels of locally produced E2 in combination with DHT (Rosen and Wade, 2001) down-regulate ER mRNA expression in the male POA, and possibly VMH as well. Combined, E2 and DHT could create a complete down-regulation, resulting in a female-biased sex difference in ERα mRNA expression in the POA seen in unmanipulated animals.

While much research details the effect of steroid hormones on ER expression, other factors may be involved in regulation, such as social interactions and/or genetics. However, at this time, it is unclear if ERα is regulated by similar non-steroidal mechanisms in anoles. Reproductive interactions increase ER expression in the VMH of parthenogenetic whiptail lizards (Hartman and Crews, 1996), and male rats mated to satiety show increased levels of ERα in the POA (Phillips-Farfán et al., 2007), indicating social modulation of ER expression. Therefore, it is possible that agonistic behaviors during the NBS and sexual encounters during the BS keep ERα levels constant in females.

Additionally, ERα mRNA expression is regulated by epigenetic mechanisms, such as methylation of DNA. This targeted DNA methylation greatly reduces gene expression by interfering directly with either the protein machinery required for transcription or by recruiting histone deacetylaces, which further compact DNA (reviewed in Wilson et al., 2008). Methylation of CgP regions in the 5' ERα promoter is often detected in ERα-negative breast cancer cells, yet ERα expression increases following demethylation (Ferguson et al., 1995). Evidence of methylation regulation of ERα exists in the brain as well, as a function of maternal care; adult rats that did not experience high levels of licking and grooming during development exhibited significantly lower levels of ERα and increased CgP methylation of the Erα1b promoter region in the POA (Champagne et al., 2006). This may be a mechanism by which ER mRNA is regulated in male anoles, without steroid hormones. Increased DNA methylation

in males, while not documented in the anole, could contribute to the sex differences in ERα expression.

Evolutionary conservation of morphology and gene expression

Sexually dimorphic morphology across species. The POA is a region highly associated with male-specific reproductive behaviors across numerous taxa (reviewed in Meisel and Sachs, 1994). This is also true in the male green anole, where POA lesions severely inhibit sexual behaviors (Wheeler and Crews, 1978). Not only is the function similar across a variety of species, but a male-biased sex difference in volume/area exists here in mammals (e.g., Gorski et al., 1980), Japanese quail (Balthazart and Surlemont, 1990), and the green anole lizard (Chapter 2). Not only is this region sexually dimorphic, but this dimorphism in green anoles persists despite various endocrine environments in adulthood, indicating that the morphology of this region is permanently established, as in other animals (e.g., Gorski et al., 1978). It is possible that this clear structure-function relationship is highly conserved and may have been established in a common ancestor of mammals, birds, and lizards.

However, conservation of amygdalar and VMH morphology are less consistent among species. While a relationship exists between the structure and function of these brain areas and reproductive behaviors, this relationship is not always reflected by sexually dimorphic forebrain morphology. For example, the size of the MePD is larger in male than female rats (Cooke et al., 1999) and the VMH may be larger in female than in male rats (Matsumoto and Arai, 1983). In

other lizards, such as the whiptail, the VMH is larger in females than in males (Crews et al., 1990). However, similar sex differences in size were not documented in AMY or VMH of the green anole, similar to the nucleus taeniae (homologous to amygdala) in Japanese quail (Adkins-Regan and Watson, 1990). While it is clear that the sex difference in the POA is highly conserved, sexually dimorphic morphology of the amygdala and VMH are more variable across species, possibly indicating that the functions of the amygdala and VMH also vary between species, while POA function is relatively consistent.

Conservation of ERα localization in the lateral VMH. While the role of E2 differs between males and females in anoles, and the role of E2 in reproductive behaviors differs between lizards and mammals and birds, the localization of ERα mRNA to the outer edge of the VMH appears to be highly conserved across sexes and species (reviewed in Blaustein and Erskine, 2002). Typically, this localization of ER is associated with feminine reproductive behaviors (reviewed in Blaustein and Erskine, 2002). However, localization of ERα is still rather prevalent in male anoles, even though expression in higher in females (Chapters 3 and 4). Similar expression patterns are seen in male and female rats as well (e.g., Weiland et al., 1997).

Together, this information indicates that lateral distribution of ERα in the VMH may be evolutionarily conserved. Furthermore, it is likely that this collection of ER expression may have effects in the brain independent of feminine sexual behaviors. Perhaps it is additional ER in females that facilitates reproductive behaviors, yet baseline expression levels facilitate other behaviors. While the

VMH is highly associated with reproductive behaviors, a role for the VMH in other behaviors, such as feeding, has also been implicated (reviewed in King, 2006). Relationships between E2, restricted diet, and general arousal exist; ovariectomized females treated with E2 and a restricted diet displayed a significantly decreased arousal state compared to groups treated with only estradiol or restricted diet (Shelley et al., 2007). Therefore, it is entirely possible that ERs in the VMH are involved in a basic homeostatic mechanism, such as feeding behavior, which is conserved across many species.

Functional consequences of morphological differences

It is clear that structure-function relationships exist in the morphological and protein expression patterns in sexually dimorphic nuclei in many species. However, the question still remains: what is the functional relevance of morphological differences in regions known to facilitate reproductive behaviors?

It is possible that a larger brain region contains more neurons and glia, and these cells may have larger soma size, and in the case of neurons, larger dendritic trees or more numerous dendritic spines. Enhancements in any or all of these characteristics would increase both cytoplasmic and cell surface area located within the brain region, adding more sites for potential connectivity with other neurons or cells. Larger surface areas provide an individual neuron with more area available for synapses and larger somata may be able to produce or maintain additional receptors. In the medial POA, for example, not only is a volumetric sex different evident (e.g., Gorski et al., 1978), but males also display

increased dendritic length (Madeira et al., 1999), spine density (Amateau and McCarthy, 2004), and receive more serotinergic input in males than in females (Simerly et al., 1984). Similarly, these same mechanisms may be at work in seasonally reproducing songbirds, where exposure to BS conditions induces plasticity in the forebrain, increasing brain region volume in song control nuclei (e.g., Brenowitz et al., 1998; Kirn et al., 1989). It is not simply that the region is larger in males or during the BS, but that gross volumetric difference may represent a larger network of connectivity a network that might cumulatively facilitate sexually and seasonally dimorphic behaviors. Yet, it is possible that sex and seasonal differences do not reflect differences in function.

In the current experiments, two regions (AMY and VMH) that do not display sex differences in either volume or number naturally did following gonadectomy. Further, the patterns of sex differences were perhaps paradoxical; females have more cells, yet the brain region volume is larger in males. This supports the idea that naturally occurring sex differences may exist only to create behavioral equality between the male and female brain (De Vries, 2004). Particularly in these regions, which facilitate a host of social behaviors, functional equality between the sexes and seasons may be critical. This idea of functional equality was originally put forth as it relates to the sexually dimorphic vasopressin system of biparental prairie voles (reviewed in De Vries, 2004). With the exception of nursing, males display the same parental behaviors as do females. Following pregnancy and parturition, females naturally experience a hormonal shift that facilitates maternal care. However, males never experience these

physiological changes, yet care for their young to a similar degree. It is hypothesized that a male-biased sex difference in vasopressin acts to facilitate parental behaviors in males; vasopressin may provide a physiological compensation in the absence of hormonal changes associated with pregnancy and parturition (De Vries, 2004). A variation on that theme may be at work in the anoles. Here, sex differences occur in the absence of gonads. Perhaps T increases soma size in female anoles to create a larger volume and promotes cell survival in males, as in rodents (reviewed in Galea et al., 2008). It is possible that gonadal steroids act to enhance and/or inhibit certain morphological characteristics within a particular sex to create equality between the sexes in these multi-function brain regions.

Similarly, seasonal differences occur in brain region volume of the song control nuclei even in songbirds that sing year round, though the communicative goal of song production differs between the seasons (reviewed in Catchpole and Slater, 1995). One such example of this is in the European starling, where T and BS conditions elicit song control nuclei to be volumetrically larger and have lower levels of noradrenergic innervation than under NBS conditions (Riters et al., 2002). Perhaps this increase in NBS noradrenergic innervation of song control nuclei facilitates singing behavior, even when circulating T is low and photoperiod is short. A similar compensatory mechanism may exist in anoles as well. Chapters 2 and 4 detailed seasonal effects of cell number in the AMY and VMH, where NBS animals had more cells than did BS animals. Perhaps increased cell numbers are necessary to facilitate behaviors that are less seasonally

dependent, such as eating (VMH) and coping with fear (AMY), which is likely relevant in either season. While these behaviors have not been linked to these regions in the anole, the VMH and amygdala facilitate these behaviors in various other species (reviewed in King, 2006; Toufexis, 2007).

Why might E2 down-regulate its receptor?

Numerous studies have documented regulation of ERα by E2. However, the functional relevance of this is unclear. A down-regulation of ERα by E2 may essentially create a scenario where that particular cell becomes less sensitive to further E2 stimulation. Modulating sensitivity of a cell to E2 activity may be necessary, as ERs can often act via genomic and rapid non-genomic pathways to alter protein expression (Lonard et al., 2004; reviewed in Klinge, 2001), as well as activate second-messenger G-coupled protein receptor pathways and increase neuronal excitability (Kow et al., 2005; Kow et al., 2006).

In females, ERα is necessary for the production of receptive behaviors (Musatov et al., 2006; Rissman et al., 1997). Further, many of the intracellular changes resulting from ERα activation are associated with increased reproductive displays, such as lordosis (Cohen and Pfaff, 1992; Etgen, 1987). It is proposed that these intracellular changes occur in response to the E2 spike during the estrus cycle, in efforts to coordinate female receptivity with ovulation (e.g., Etgen et al., 2001). Even though E2 is necessary for the production of these behaviors, it may still be valuable to regulate the degree and timing of these intracellular responses due to ERα activation. Additionally, E2 mediated

down-regulation ERα once the desired effect is initiated may reduce the risk for deleterious effects of E2 on cell survival (Fester et al., 2006; Jaita et al., 2005).

However, in green anoles E2 appears to facilitate ERα down-regulation in males only, and only in a brain-region specific manner, similar to studies in rodents (see above). It could be that males have higher local levels in the brain than females due to aromatase activity, even though females are producing E2 from the ovaries during the BS (Jones et al., 1983). Given the close relationship between ERα and female reproductive behaviors in many species (e.g., Musatov et al., 2006), it is possible that the reduction of message in males serves to suppress feminine behaviors, especially considering that local E2 production is considerably higher in males than in females (Rosen and Wade, 2001). Therefore, E2 levels may be higher in the male brain than female brain, even with circulating levels of E2 from the ovaries.

However, it is important to note that E2-dependent ERα mRNA regulation documented in the male anole may not necessarily mean a change in protein expression. While mRNA labeling can certainly localize cells that express ERα, it is the protein that directly influences gene transcription and intracellular properties. Very recently, an antibody specific to the anole was developed, however the studies in this dissertation were planned and in progress prior to its development. However, similar studies charting the expression and possible regulation of ERα protein by E2 would help complete this course of research.

Conclusions

Initially, I had suggested that E2 in the male green anole has functions independent of reproductive behaviors, which differs from the role of E2 in male mammals and birds. To examine potential effects of E2 in the male brain, I examined forebrain morphology and ERα mRNA expression.

I had thought that E2 might masculinize male forebrain morphology both in development and adulthood and that perhaps these changes facilitate the display of T-induced sexual behaviors, even though E2 does not directly activate them. In the light of the current data, it is unlikely that E2 facilitates morphological changes in the male brain during adulthood, as the effects of E2 treatment on morphology were limited to the enhancement of a sex difference in the AMY; a difference which is not detected in unmanipulated animals. However, this effect of E2 on anole AMY volume occurs without a concurrent difference in ERα mRNA expression, indicating that perhaps other estrogen receptors are involved. These results differ from those seen in rats, where E2 can increase soma size and brain region volume in the MePD (Cooke et al., 2003), indicating an evolutionary difference in amygdala morphological maintenance in adulthood.

At this time it is difficult to conclude the role of E2 in development without manipulation studies, as parallel changes in ERα mRNA and morphology only existed in the VMH during development. However, it is unlikely that E2 guides the developing POA and AMY, though E2-mediated morphological maturation may exist during a different developmental time. The development of these regions in the anole, particularly the POA, appears to greatly differ from sexually

differentiation of the brain in rodents, where the male-biased sex difference in the POA is established near the time of birth (e.g., Gorski et al., 1978). This suggests that, while the POA is sexually dimorphic in adult anoles, the mechanism by which this region differentiates may differ from those documented in some mammalian species.

I also suggested that E2 may modulate a cellular response, such as reducing ERα mRNA expression, which could be related to seasonally and sexually dependent functions of these forebrain regions in the anole, perhaps including changes and differences in reproductive and social behaviors. ERα mRNA is expressed highest in the female VMH and subsequently, it is down-regulated in this region, as well as the POA, in males. Together, these data suggest that the differential behavioral response to E2 between males and females may be a function of ERα expression. If this idea is true, then perhaps the POA is also involved in female-specific behaviors.

The function of E2 in the male brain remains unclear, though modulation of the ERα mRNA is clearly involved and this mechanism may be evolutionarily conserved. Investigating potential functions of ERα in the male brain as well as downstream effects may be able to provide more complete answers as to the role of ERα in male anoles.

Figure 6-1. Sex differences in the preoptic area are likely permanently established in development. In intact adult animals, a sex difference in POA volume exists (A), such that males (grey) are larger than females (white). Following adult gonadectomy and/or estradiol treatment, this sex difference remains (B). However, this region is sexually monomorphic in late embryonic/early posthatching development (C), indicating that this region sexually differentiates later in the animal's life.

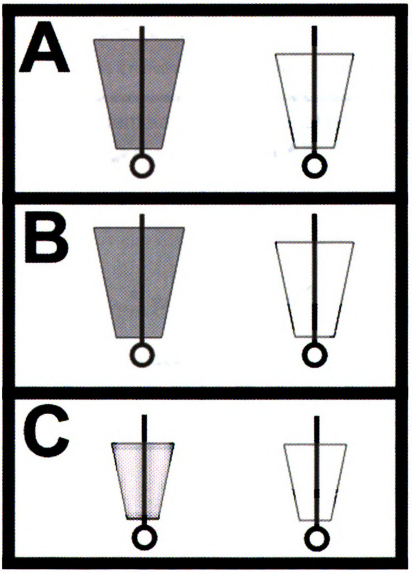


Figure 6-2. Effects of age, sex, and estradiol treatment on estrogen receptor alpha mRNA expression in the green anole. In adult intact animals, the ERα mRNA expression is highest in the VMH, compared to the POA and AMY. Additionally, females (grey dots; right column) express more ERα mRNA than males (black dots; left column) in the POA and VMH (A). Following gonadectomy or E2 treatment (B), ERα mRNA is down-regulated in the male POA and VMH, though expression in females remains unchanged (not shown). In developing anoles, expression patterns mirror those seen in adulthood, and a peak of ERα mRNA occurs near the day of hatching (P0) in the VMH in both sexes (white dots; C), while it remains unchanged with age in the POA and AMY.

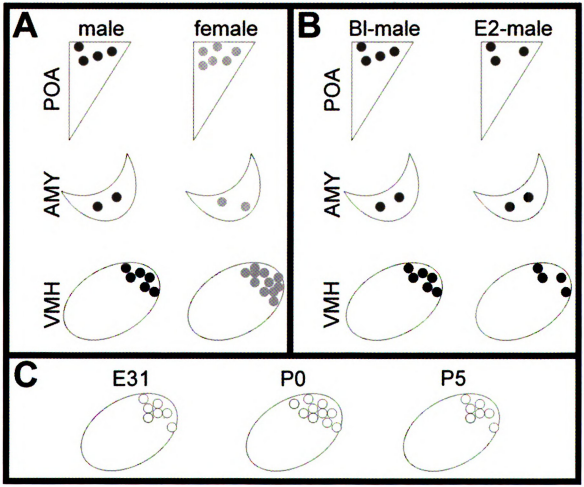


Figure 6-3. Seasonal effects on volume may be influenced by testosterone while seasonal differences in cell number are maintained by a non-gonadal cue. Under unmanipulated conditions (A) breeding (grey) animals have larger POA and VMH volumes and POA and AMY soma sizes than non-breeding animals (white), and NBS animals have more cells than BS animals in the AMY. Following gonadectomy and/or estradiol treatment (B), the seasonal effects on volume disappear while the seasonal difference in cell number is maintained. These results suggest that seasonal effects on volume are gonad-hormone dependent while differences in cell number are not.

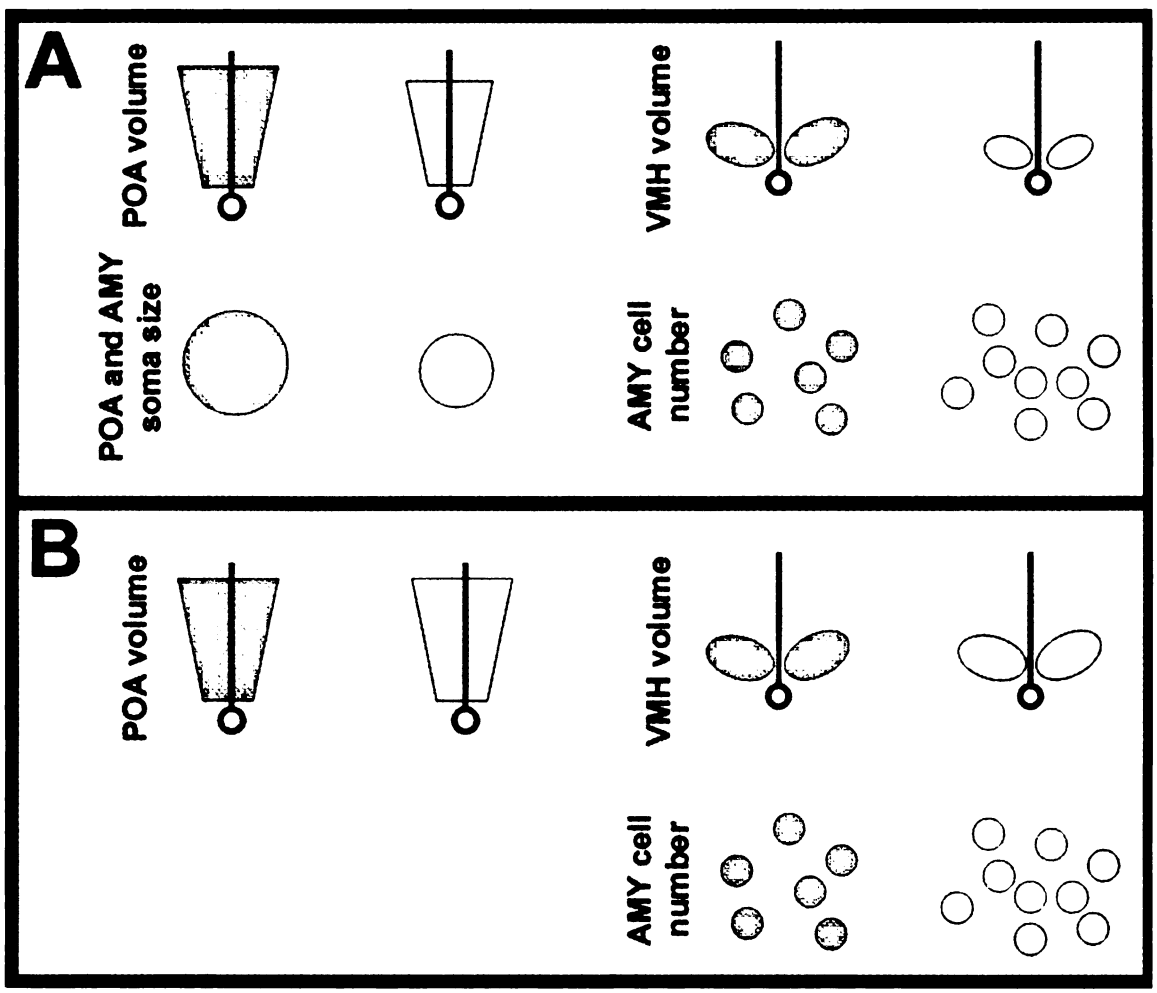
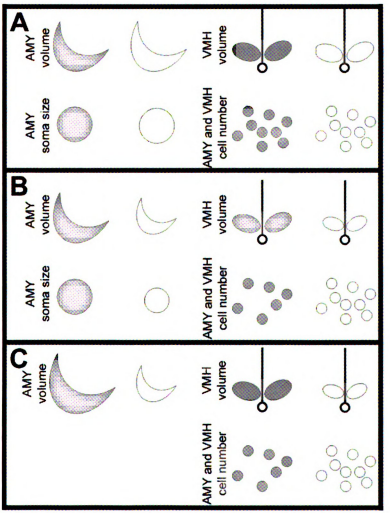


Figure 6-4. Steroid hormones mask inherent sex differences in forebrain morphology. Gonadally intact anoles are sexually monomorphic in AMY and VMH volume and cell number, and soma size is equal between males (grey) and females (white) in the AMY (A). However, gonadectomy reveals sex differences in volume (larger in males), soma size (larger in males), and cell number (more in females; B). Further, if the animal is treated with E2, the volumetric sex difference is more pronounced in the AMY (C), though E2 had no significant effects on VMH volume or cell number in either region. These results indicate that gonadal secretions mask underlying sex differences in the green anole.



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