MOLECULAR MECHANISMS OF SEASONAL RESPONSIVENESS TO TESTOSTERONE IN THE GREEN ANOLE LIZARD

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ABSTRACT

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Green anole lizards are seasonal breeders, with male sexual behavior primarily regulated by an annual increase in testosterone (T). However, T is much more effective at activating behavioral, morphological and biochemical changes associated with reproduction in the breeding season (BS) compared to the nonbreeding season (NBS). The goal of this research is to identify the molecular mechanisms that govern this seasonal responsiveness to T in male anoles. In the experiments conducted for this dissertation, I investigate the roles of androgen receptor (AR) and two of its coactivators, steroid receptor coactivator-1 (SRC-1) and CREB binding protein (CBP), in the brain regions and copulatory tissues responsible for reproduction, as well as their potential regulation by T.

I show that AR protein expression in the copulatory organs (hemipenises) is more abundant in the BS and also increased by T-treatment, the latter to a greater extent in the BS than the NBS. Additionally in the hemipenis, transcriptional activity appears generally diminished in the NBS. Changes in this copulatory organ may limit physical ability to engage in sexual behavior in the NBS. AR mRNA and protein expression in the brain are not likely playing a role in seasonality, as there are no regionally specific changes in AR in gonadally intact animals, but expression is consistent with a role in regulating some differences between the sexes in the display of reproductive behavior.

SRC-1 appears to be regulated by circulating T in a regionally specific manner, as demonstrated by male-biased sex differences within the preoptic area (POA) and ventromedial hypothalamus (VMH), and it is regulated by T in the POA and ventromedial amygdala (AMY). Additionally, SRC-1 protein is negatively regulated by T in the hemipenis. SRC-1 may also have an endogenous seasonal rhythm of expression in the VMH, but this brain region is largely implicated in the control of female receptivity. While this coactivator may facilitate some aspects of male sexual behavior in the BS, it is not likely a candidate for facilitating the seasonal responsiveness to T in male anoles.

CBP protein expression is increased in the BS compared to NBS in hemipenis tissue, where it may serve to coactivate the increased AR protein during this season. CBP does not, however, differ across season or hormone-manipulation in the brain. The only difference in CBP expression within the anole brain is a female-biased increase in the AMY. This female-biased sex difference in the AMY also exists in AR, for both the number of AR+ cells and volume, as well as a female-biased increase in the density of SRC-1+ cells in this region as well. While the specific roles these molecules play remain to be identified, the data suggest that the AMY may be an important region for steroid hormone receptor and coactivator action, especially in females.

Collectively, the present body of work represents an initial effort in determining the molecular mechanisms of seasonal responsiveness to T in the green anole. While it is clear that AR, SRC-1, and CBP are contributing to the suite of reproductive behaviors and associated changes in the anole, these molecules are not likely facilitating a seasonal responsiveness to T in the brain. They may, however, be involved in regulating the function of copulatory tissues across season. This possibility should be further investigated.

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KEY TO ABBREVIATIONS

Testosterone	T
Dihydrotestosterone	DHT
Breeding season	BS
Non-breeding season	NBS
Androgen receptor	AR
Retractor penis magnus	RPM
Preoptic area	POA
Ventromedial hypothalamus	VMH
Amygdala	AMY
Androgen response element	ARE
cAMP response element binding protein	CREB
CREB binding protein	CBP
Steroid receptor coactivator-1	SRC-1
Histone acetyltransferase	HAT
Testosterone proprionate	TP
Blank	Bl
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH
Ribosomal protein s18	RPs18
Polyvinylidene fluoride	PVFD
Phosphate-buffered saline	PBS
Enhanced chemiluminescence	ECL
Saline-sodium citrate	SSC

INTRODUCTION

Steroid hormones, testosterone (T) in particular, are necessary for the displays of courtship and copulation associated with reproduction in various vertebrate species (Tennent et al., 1980, Steel, 1981, Fabre-Nys and Martin, 1991, Ball and Balthazart, 2002, Hull and Dominguez, 2007, Fusani, 2008). An annual rise in T facilitates seasonal breeding in many species of birds and reptiles. The metabolites of T, estradiol (converted by the aromatase enzyme) and dihydrotestosterone (DHT) (converted by 5α-reductase), are critical for the display of sexual behaviors in a variety of mammalian and avian species (Ball and Balthazart, 2002).

Relatively little information is available about the downstream molecular mechanisms associated with steroid hormone effects on neural structure and function (McCarthy et al., 2012). The discovery of the first steroid hormone receptor coactivator, which mediates nearly all processes involved in steroid-related gene expression, was only in 1995. From that time the discovery of over 350 coregulator proteins have been made (York and O'Malley, 2010). We are just beginning to gain some understanding of the myriad of mechanisms that must coordinate to regulate gene expression, and consequently, behavior.

Hormonal Regulation in Anoles

Green anoles are exceptionally amenable to laboratory study of molecular interactions with steroid hormones. The genome of this species was recently sequenced and is available online (Alfoldi et al., 2011), which facilitates the study of genetic mechanisms, and their reproductive behavior depends on steroid hormones. These animals

are primarily found in the southeastern United States, and their breeding season (BS) in the field lasts from approximately April through July. At the end of the BS, anoles will enter into the non-breeding season (NBS), in which gonads of both sexes will regress and reproductive behaviors are no longer displayed (Wade, 2005, 2011, 2012). In males, a seasonal rise in circulating testosterone (T) activates the display of sexual behavior. Although the metabolites of T are important for courtship and copulation in other species, T is the most potent facilitator of male sexual behavior in the green anole (reviewed in Wade, 2011). DHT alone is not sufficient, and while estradiol enhances motivation, its administration to castrated males does not result in the display of male sexual behavior (Winkler and Wade, 1998, Latham and Wade, 2010). In addition, inhibiting aromatase (the enzyme that catalyzes conversion of T to estradiol) does not inhibit male sexual behaviors in this species (Winkler and Wade, 1998, Rosen and Wade, 2000). Thus, T most likely acts by binding to androgen receptors (AR). The changes in T levels in adult males across the year are dramatic and predictable, and as it is the primary activator of male sexual behavior, circulating T is therefore one likely mechanism contributing to seasonal changes in behavior.

Absolute levels of T suggest that other factors are probably also involved, however. For example, plasma T of male adult Sprague-Dawley rats averages 2-3 ng/mL (Mock et al., 1975), and seasonally breeding animals such as coyotes and Syrian hamsters have plasma T levels that average 3.31ng/mL and less than 0.5 ng/mL in the BS and NBS, respectively (Minter and DeLiberto, 2008, Piekarski et al., 2010). Even in NBS conditions, male green anoles have higher plasma T concentrations than breeding male rodents, averaging 11ng/mL, while BS males average 20ng/mL (Lovern et al., 2001). Therefore, in addition to

lower T in the NBS, there are likely other mechanisms influencing seasonal changes in reproductive structures and behaviors.

Green anoles exhibit a robust suite of courtship and copulatory behaviors, which are easily observable, and are relatively unchanging from the field to the lab. Males display a red throat fan, called a dewlap, and perform a series of head bobs to court females. Males will mount receptive females and intromit one of two bilateral hemipenises. These muscles are each controlled in part by a retractor penis magnus (RPM) muscle, which pulls the hemipenis back into the cloaca following copulation. These behaviors are controlled by three sexually dimorphic systems within the animal, the first including limbic forebrain regions, such as preoptic area (POA), ventromedial hypothalamus (VMH), and amygdala (AMY). The second includes brainstem motoneurons that innervate the ceratohyoid muscle in the throat that controls the dewlap, essentially controlling courtship displays. Finally, motoneurons in the caudal spinal cord innervate the copulatory neuromuscular systems, which include the RPM (Wade, 2005, 2011, 2012). Throughout this dissertation I focus on the forebrain and peripheral structures associated with copulation in this species.

Seasonal Sensitivity to T

Despite the fact that T activates male sexual behavior in the green anole, it has surprisingly limited effects in the NBS when exogenously elevated. T has differential effects in the BS and NBS on behavioral, morphological, and biochemical functions related to reproduction. The same dose of T administered to castrated males produces larger increases in male courtship displays and rates of copulation in the BS compared to NBS (Neal and Wade, 2007). The volume of two of the limbic forebrain regions that control

sexual behavior and motivation, POA and VMH, are larger in the BS than NBS in both males and females (Beck et al., 2008). Treating castrated males with T increases neuronal soma size in the POA and AMY, and the magnitude of effect is greater in the BS than NBS in the AMY. Copulatory tissue size is also seasonally sensitive to T in castrated males, with T increasing the size of RPM fibers and hemipenis cross-sectional area to a greater degree in the BS than NBS (Holmes and Wade, 2004, Neal and Wade, 2007). T upregulates whole brain aromatase activity in a sex and seasonal specific manner as well. Aromatase activity is selectively upregulated by T in the BS but not the NBS in male anoles only (Cohen and Wade, 2010). Each of these differences between seasons might contribute to the increased ability of T to activate the display of sexual behaviors in the BS. The mechanism by which seasonal sensitivity to androgens is occurring at various levels of reproduction is still unclear. One possibility I investigated involves differing levels of AR expression across seasons.

Androgen Receptor

AR is described in mammals as a 110 kDa nuclear receptor activated by steroid hormone ligands. It has four functional domains: the NH_2 terminal domain associated with transactivation, the DNA-binding domain, hinge region, and ligand-binding domain. In order for T to have its effect in specific tissues, it must bind to AR located either in the cytoplasm or the nucleus. Upon binding, the steroid-receptor complex will dissociate from chaperones such as heat shock proteins, and the receptor will dimerize and be translocated to the nucleus. Once in the nucleus, the receptor complex is free to interact with DNA and both recruit coactivators and inhibit repressor molecules. The complex binds to

corresponding androgen response elements (ARE's) and increases the transcription of target genes, including itself (Grad et al., 1999, Gelmann, 2002, Heinlein and Chang, 2002, Stanisic et al., 2010, Coffey and Robson, 2012). AR can also be regulated by negative feedback, as demonstrated in rat with an increase in mRNA transcription upon castration, and subsequent decrease in transcription by androgen replacement (Tan et al., 1988, Quarmby et al., 1990). The effect that androgens have on transcriptional rates of AR can be tissue and cell specific (Gonzalez-Cadavid et al., 1993, Kerr et al., 1995, Drengler et al., 1996, Wiren et al., 1997, Grad et al., 1999)

AR is conserved across species, with the ligand binding domain of the green anole AR sharing 97% homology with the human sequence (Wade, 2011). Previous work from our lab indicates that peripheral copulatory muscle shows increased percentage of AR+ nuclei in the presence of T treatment, and this occurs in both BS and NBS. AR does not, however, differ across seasons in immunoreactivity (ICC) in this tissue in gonadally intact males (Holmes and Wade, 2005). Other techniques quantifying mRNA and protein would allow for a more detailed analysis of peripheral tissue across seasons. AR mRNA and immunoreactivity have been studied in the brain in this species, but only in the BS. The distribution of AR is comparable across the two sexes, although females express more than males when several forebrain regions are considered collectively (Rosen et al., 2002).

Androgen Receptor Coactivators

Another potential mechanism for conferring seasonal sensitivity to T is differential expression of steroid receptor coactivators. This possibility is not mutually exclusive from differential AR expression. These molecules are recruited by the receptor to enhance the

rate of gene transcription, as opposed to corepressors, which serve to decrease transcription (Heinlein and Chang, 2002). Coregulatory proteins are able to dramatically change AR's transcriptional activity through molecular mechanisms such as acetylation, methylation, phosphorylation, or chromatin remodeling (Rosenfeld et al., 2006). Two coactivators that are particularly important for facilitating androgen action are cAMP response element binding protein (CREB)-binding protein (CBP) and steroid receptor coactivator-1 (SRC-1). They are the rate-limiting factor for increasing gene transcription (McKenna et al., 1999), and can act synergistically to coactivate nuclear receptors (Smith et al., 1996).

CBP is recruited by the DNA-bound receptor complex to the promoter region, where it facilitates recruitment of RNA polymerase II and other basic transcription factors. It has endogenous histone acetyltransferase (HAT) activity, which neutralizes the positively charged histone tails with the addition of negatively charged acetyl groups. When the histone tails lose their affinity for DNA (negatively charged), this enables the chromatin to decondense and DNA can be accessed more easily (Stanisic et al., 2010).

SRC-1 is a primary coactivator which aides in assembling transcription initiation complexes by bridging DNA-bound nuclear receptors and basal transcriptional machinery. It acts directly with the receptor complex and acts as scaffolding to recruit secondary coactivators as well as histone methyltransferases and acetyltransferases. In this way, CBP and SRC-1 can function together to enhance AR's transcriptional activity (Heinlein and Chang, 2002, Stanisic et al., 2010). Until the present work, CBP and SRC-1 had not been described in green anoles, although they have been investigated in birds as well as mammals (Auger et al., 2002, Charlier et al., 2002, Tetel, 2009). Either, or both, of these

coactivators are capable of affecting how AR is expressed, and potentially how it interacts with T.

Summary of Dissertation Experiments

Chapter 1: Investigate the potential contributions of AR, CBP, and SRC-1 in copulatory tissues and whole brain. Real-time qPCR was used to determine changes in mRNA expression of these three genes across sex, season, and hormone manipulation in the RPM muscle, hemipenis, and whole brain tissues. Western blots were performed in the RPM muscle and hemipenis for AR, CBP, and SRC-1 as well (Kerver and Wade, 2013).

Chapter 2: Evaluation of AR mRNA in the male and female anole brain across breeding season and testosterone exposure. Utilized *in situ* hybridization to examine the distribution in the steroid-sensitive brain regions regulating reproductive function in the anole. Western blots in preoptic-hypothalamic brain dissections were used to determine protein expression (Kerver and Wade, 2014).

Chapter 3: Investigation of the steroid receptor coactivator, SRC-1, to determine its distribution and hormonal regulation in the anole brain. *In situ* hybridization was employed to determine whether SRC-1 mRNA differs across sex or season in the brain, and whether these differences are regulated by circulating testosterone (Kerver and Wade, 2015).

Chapter 4: Examined CREB binding protein (CBP) in the male and female green anole forebrain. Used *in situ* hybridization to characterize the first description of this coactivator within the reptilian brain. Determined whether expression differed across sex, season, or hormonal manipulation (submitted manuscript).

CHAPTER 1

SEASONAL AND SEXUAL DIMORPHISMS IN EXPRESSION OF ANDROGEN RECEPTOR AND ITS COACTIVATORS IN BRAIN AND PERIPHERAL COPULATORY TISSUES OF THE GREEN ANOLE

Introduction

Steroid hormones are necessary for the display of courtship and copulation in most vertebrate species (Ball and Balthazart, 2002, Fabre-Nys and Martin, 1991, Fusani, 2008, Hull and Dominguez, 2007, Steel, 1981, Tennent et al., 1980). In particular, an annual rise in testosterone (T) facilitates seasonal breeding in many species of mammals, birds and reptiles (Baum, 2002). Despite the widespread nature of this type of behavioral activation, the molecular mechanisms associated with steroid hormone action remain largely unknown (McCarthy et al., 2012). More information is needed to understand the complex interplay between hormones and behavior, and the dynamics that govern changes across the brain and periphery.

Green anole lizards present a classic example of seasonal breeding, and are therefore an excellent model for investigation of responsiveness to T. These animals are primarily found in the southeastern United States, and exhibit a stereotyped suite of courtship and copulatory behaviors, which are easily observable in both the field and lab. Males mount receptive females and intromit one of two bilateral hemipenes. These organs are each controlled in part by a retractor penis magnus (RPM) muscle, which pulls the hemipenis back into the cloaca following copulation. The breeding season (BS) of green anoles in the field lasts from approximately April through July. At the end of the BS, these animals enter a non-breeding season (NBS), in which gonads of both sexes regress and reproductive behaviors are no longer displayed (Wade, 2005, 2011, 2012).

While some mammalian and avian species rely heavily on metabolites of T, including estradiol and dihydrotestosterone (DHT), for activation of male sexual behavior (Ball and Balthazart, 2002, Hull et al., 2002), T itself is the most potent activator in green anoles (reviewed in Wade (2011)). In this species, DHT is generally not sufficient to facilitate male sexual behavior (Rosen and Wade, 2000). Estradiol enhances motivation in adult male anoles, but systemic administration in castrated males does not activate reproductive behaviors. In parallel, inhibition of aromatase activity (the enzyme that catalyzes conversion of T to estradiol) does not limit these behaviors in this species (Winkler and Wade, 1998). Collectively, available data suggest that courtship and copulation in male green anoles is regulated by T binding to androgen receptors (AR).

T has limited effects in the NBS. For example, in the BS compared to the NBS, the same dose of this hormone in castrated males produces larger increases in male courtship and copulatory displays, whole brain aromatase activity, and soma size in a key region of the forebrain, as well as size of the hemipenis and fibers of the RPM (Cohen and Wade, 2010, Holmes and Wade, 2004, 2005, Lovern et al., 2004a, Neal and Wade, 2007, O'Bryant and Wade, 1999, 2002, Wade, 2005). The mechanism(s) by which this seasonal difference in responsiveness to androgens occurs at various levels of reproduction is unclear.

Perhaps the most parsimonious explanation is that AR levels differ across seasons. Previous work from our lab indicates that T increases the percentage of nuclei expressing AR protein in the RPM in both the BS and NBS; AR does not, however, differ across seasons in immunoreactivity in this tissue of gonadally intact males (Holmes and Wade, 2004, 2005). In the green anole brain, AR mRNA and protein have only been investigated in the BS. Males and females have generally comparable levels of AR mRNA within brain regions,

although the intensity of labeling is slightly greater in females when considered across multiple limbic areas (Rosen et al., 2002). AR protein was not quantified in this study, but qualitatively it parallels the pattern of mRNA detected using radiolabeled in situ hybridization. Additional information is necessary to determine whether AR plays a key role in the seasonal sensitivity to androgens in both brain and peripheral tissues, including a more detailed analysis of AR in these tissues across seasons.

Another potential mechanism for conferring seasonal changes in responsiveness to T is differential expression of steroid receptor coactivators. This possibility is not mutually exclusive from differential AR expression. These coactivators are recruited by the receptor to enhance gene expression (Heinlein and Chang, 2002). They are able to dramatically change transcriptional activity through molecular mechanisms such as acetylation, methylation, phosphorylation, or chromatin remodeling (Rosenfeld et al., 2006). Two coactivators that are particularly important for facilitating androgen action are CREB binding protein (CBP) and steroid receptor coactivator-1 (SRC-1). They are rate-limiting factors for transcription (McKenna et al., 1999), and can act synergistically to coactivate nuclear receptors, including AR (Smith et al., 1996). CBP and SRC-1 have not yet been described in reptiles.

The present set of studies was designed to begin to evaluate the idea that patterns in the expression of AR and/or these two coactivators, CBP and SRC-1, are consistent with a role in facilitating differential responsiveness to T across seasons. Real-time qPCR was performed on intact animals in the BS and NBS to examine AR, CBP, and SRC-1 mRNA expression in male copulatory tissues and the brains of both male and female green anoles. Because effects of season were detected in the copulatory tissues, follow-up studies were

conducted using hormone manipulations to more directly assess the role of circulating T in regulating seasonal changes in expression of AR, CBP, and SRC-1 mRNA. In addition, Western blot analyses were performed on RPM and hemipenis to examine seasonal and T-induced changes in relative protein levels.

Methods

Animals

Male and female green anole lizards were wild-caught and purchased from Charles Sullivan Co. (Nashville, TN) in the BS (April) and NBS (October). To control for the social environment, animals were housed individually in 10-gallon aquaria. Black dividers prevented visual contact between the cages, which contained a peat moss substrate and water dish, as well as sticks and rocks for climbing and basking. Cages were misted daily with water, and animals were fed crickets or mealworms 3 times per week in the BS or 2 times per week in the NBS. Along with fluorescent lights in the ceiling, which maintained a 14:10 light:dark cycle during the BS and 10:14 during the NBS, full spectrum bulbs and a heat lamp were present over each aquarium. Cage temperatures ranged from 28 to 38°C in the daytime (depending on proximity to the heat lamp) and were 18°C at night in the BS. During the NBS, temperatures were 24–30°C in the daytime and 15°C at night. Humidity was kept at 60–70% in both seasons. All procedures were approved by the Michigan State University Institutional Animal Care and Use Committee.

Treatment and Tissue Collection

Experiments examining naturally occurring differences across seasons in expression of AR, SRC-1, and CBP were performed on gonadally intact animals. To evaluate mRNA,

seven individuals per sex per season were rapidly decapitated at least two weeks after arrival in the lab. Brains were collected from all animals, and RPMs and hemipenes were removed from males. Tissues were individually snap-frozen in methyl butane and stored at 80°C. Breeding state was confirmed at that time by determining in males that testes were large and vascularized with visible seminiferous tubules (BS) or small, pale, and unvascularized (NBS). Females had at least one large yolking follicle or an egg in the oviduct (BS) and small, translucent follicles less than 1 mm in diameter (NBS).

RPM and hemipenis tissues were collected in an identical manner from an additional six males from each season to examine protein expression in intact males.

Experiments on treated male anoles were performed to determine whether circulating T levels regulate the seasonal changes we detected in the mRNA and protein of AR and its coactivators (see below). Seven males from each season were used for quantifying mRNA. However, only six hemipenis samples from blank and hormone-treated NBS groups could be used, as two did not produce enough RNA. Six males from each season were used for quantifying protein expression. These treatments involved gonadectomizing males from both the BS and NBS and providing either a hormone-filled or control capsule. One week after animals arrived in the lab, males were deeply anesthetized by hypothermia and bilaterally gonadectomized while on ice, as in Cohen and Wade (2012). Gonads were removed through a small incision made on each side of the animal. Each gonad was ligated with silk and then cauterized to ensure it was destroyed completely. During this procedure animals were subcutaneously implanted with a Silastic capsule (7 mm long 0.7 mm inner diameter 1.65 mm outer diameter) containing either 5 mm of packed testosterone propionate (TP) or left blank (Bl), as in previous studies (Cohen and Wade, 2010, Holmes

and Wade, 2005, Neal and Wade, 2007). Plasma from these animals was used in a different study, and the hormone implants produced mean detectable androgen concentrations of 48.69 ± 4.78 ng/ml, with no difference across seasons (Cohen and Wade, 2012). On average, these implants produced circulating levels of androgen higher than those of field collected animals, which are approximately 20 ng/ml in the BS and 11 ng/ml in the NBS (Lovern et al., 2001). However, the range of circulating T in these experimental animals overlapped with that of a control male in the original study (Cohen and Wade, 2012) that was incompletely castrated, suggesting that the levels were not extreme. The experimental dose was selected because it is effective in eliciting a variety of functions in the lab, including activating male sexual behavior, increasing RPM fiber size, and up-regulating activity of steroid metabolizing enzymes in the brain (Cohen and Wade, 2010, Holmes and Wade, 2004, Neal and Wade, 2007). Because the effects of this manipulation are often greater in the BS than NBS (see above), a modestly supraphysiological dose was provided to increase the chance of detecting potential effects in the NBS. Experimental males were euthanized by rapid decapitation one week after treatment. Absence of testes was confirmed at this time, as was the presence of appropriate capsule with some hormone remaining in those that had been filled with TP.

Real-time qPCR

RNA from RPM and hemipenis, as well as whole brain tissue, was extracted from individual samples using Trizol (Invitrogen, Carlsbad, CA) per manufacturer's instructions. Samples were cleaned and DNase treated with the RNeasy Mini Kit (Qiagen, Valencia, CA) per manufacturer's instructions. They were then ethanol precipitated, concentrations

determined by spectophotometry, and run on a 1% agarose gel to verify RNA integrity. cDNA was produced using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) per manufacturer's instructions. All samples within a tissue type were converted simultaneously. Primers for all genes were designed according to the sequences available on Ensembl (AR: ENSACAG00000009496; CBP: ENSACAG00000012380; SRC-1: ENSACAG00000000619) with Primer Express 2.0 (Applied Biosystems, Foster City, CA; Table 1), and reaction efficiencies were calculated for each primer pair using the equation E = 10(1/slope) on increasing concentrations of cDNA (efficiencies ranged from 1.83–2.00, similar to Pfaffl (2001)). To confirm these samples were free of genomic DNA, RNA from four individuals randomly selected across tissues and groups was used also used as a template. No amplification was observed.

A pilot study was performed to determine the most effective housekeeping gene to serve as a control for each tissue type, as their expression can differ across tissues and hormonal environment (Filby and Tyler, 2007). β-actin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and ribosomal protein s18 (RPs18) are three commonly used housekeeping genes that play roles in normal cell functioning (Fedrigo et al., 2010, Frost and Nilsen, 2003, Gorzelniak et al., 2001, Ingerslev et al., 2006, Morse et al., 2005, Radonic et al., 2005) that were tested on intact animals from BS and NBS. For each tissue, the gene with the smallest mean difference across groups was selected.

Each cDNA sample was run in triplicate, along with no-template controls, and contained 100 nM of each primer with 25 ng of cDNA. β-actin was run in parallel with each reaction and analyzed as a control for the brain and hemipenis samples; RPs18 was used as a control for the RPM samples. The reactions were run on an ABI Prism PE 7000 system

(Applied Biosystems, Foster City, CA) with Power SYBR green PCR Master Mix (Applied Biosystems). For each gene, an average was derived from the triplicate threshold cycles for each animal.

We were interested in whether each gene exhibited differences across groups. While AR, SRC-1 and CBP were of primary interest, specificity would be supported by lack of effects due to season, sex and treatment in the housekeeping genes. Therefore, each gene was first analyzed independently, using two-tailed t-tests to determine whether seasonal differences existed within the RPM and hemipenis of intact animals. Two-way ANOVAs were used to analyze the effects of sex and season in the brains of intact animals. In treated animals, peripheral tissues were analyzed for effects of season and hormone manipulation by 2-way ANOVA. Fold differences were calculated using $\Delta\Delta_{CT}$ (Livak and Schmittgen, 2001, Qi et al., 2012), normalized to a housekeeping gene as indicated above.

For cases in which housekeeping genes were unaffected by our manipulations (see Section 3), AR, SRC-1 and CBP values from individuals were corrected using their respective control gene (Burmeister et al., 2007, Pfaffl, 2001). These corrected values were analyzed as above using two-tailed t-tests and two-way ANOVAs. All analyses were completed using SPSS Statistics version 19 (IBM, Armonk, NY).

Table 1. Primers used for qPCR (5' to 3').

Gene	Forward	Reverse
AR	AGCGTGGACTACCCGGAAA	GGGCACCTGGACCGAGAT
CBP	AGCAAACCCCCTGATGAGTG	AGGTTCCCAGCATTCCCC
SRC-1	GCTCCTCCGCTACCTTCTTGA	GGGTTAGACGCCAGCTCCTT
β-actin	CTCCGCTTGGACCTGGCT	GATCTTCATGAGGTAGTCGGTCAA
RPs18	GGCAGTACAAGATCCCCGAC	CCTTGACGTCCTTCTGCCTG
GAPDH	AAACTGTGGCGAGATGGCAG	GGATGCTGGGATGATGTTCTG

Western Blot Analyses

Tissue from each animal was individually extracted in RIPA Lysis Buffer (Santa Cruz Biotechnology, Santa Cruz, CA) per manufacturer's instructions. Total protein concentration was determined using a Bio-Rad (Hercules, CA) Protein Assay with absorbance at 595 nm using a GeneQuant Pro Spectrophotometer (Amersham, Arlington Heights, IL).

Each tissue (RPM and hemipenis) was analyzed individually. Separate blots were prepared for each of the three proteins of interest (AR, CBP, and SRC-1). Each set of blots for each gene contained six protein samples from each group (intact, TP-treated, and blank) from both the BS and NBS. Although treated animals were analyzed separately from intact animals, they were run on the same blots to maintain consistency. Kaleidoscope Prestained Standards (Bio-Rad, Hercules, CA; 10µl) were used to visualize approximate molecular weights.

Protein was denatured by boiling at 100°C for 3 min and then chilling on wet ice. Total protein (30 µg) combined with 10 µl loading buffer was loaded into each well and run on a 4% stacking and 8% resolving polyacrylamide gel. The proteins were electrotransferred to a polyvinylidene fluoride (PVDF) membrane. Membranes were washed 2 5 min in 0.1% Tween-20 in PBS then blocked at room temperature for one hour in a solution of 5% nonfat dry milk, 0.1% Tween-20, and phosphate-buffered saline (PBS). They were then incubated in primary antibodies (Table 2) overnight at 4°C. They were then rinsed 3x5 min in PBS Tween-20 and incubated in secondary antibody (Table 2) at room temperature for 1 h. AR blots were processed for immunoreactivity using enhanced chemiluminescence (ECL) in the Western SuperStar Kit (Applied Biosystems, Foster City,

CA), and exposed to HyBlot CL autoradiography film (Denville Scientific Inc., Metuchen, NJ). CBP and SRC-1 blots were processed using ECL Plus Western Blotting Detection Reagents (Amersham, Arlington Heights, IL), which provided increased sensitivity with these primary antibodies. Following film exposure, the membranes were stripped for 15 min in Restore Plus Western Blot Stripping Buffer (Thermo Scientific, Waltham, MA), washed in PBS Tween-20, blocked again for 60 min, and reprobed for the loading control (Table 2). These proteins were visualized using the same procedures as above.

As negative controls, the primary antibodies for AR, CBP and SRC-1 were preadsorbed with excess peptide (Santa Cruz sc-815P, Santa Cruz sc-369P, and Thermo Scientific, PEP-044, respectively), before exposure to the membranes. All labeling for the bands of interest for these proteins were absent under these conditions (Fig. 1).

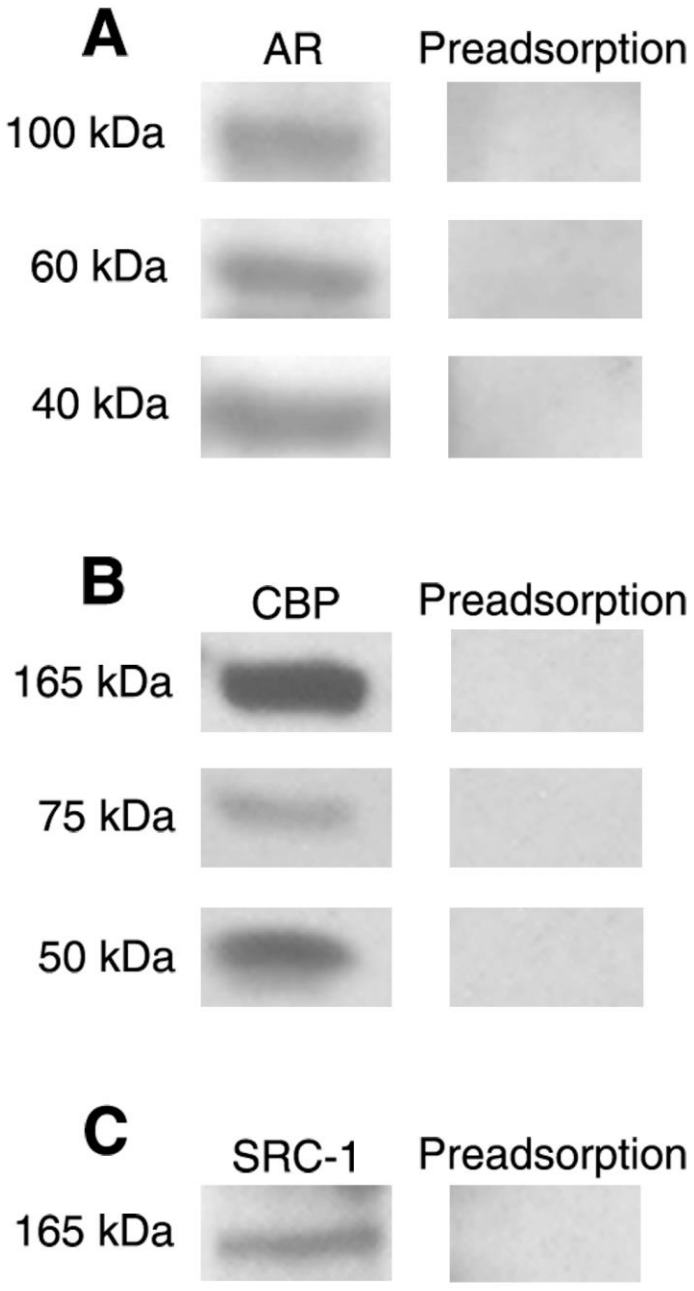
For all experimental animals, the mean optical density of each band of interest was quantified using Scion (NIH) Image. To control for loading, ratios of AR to β-actin and each of the coactivators to GAPDH were used in statistical analyses. In intact animals, optical density ratios of each of the three proteins were compared between BS and NBS animals by two-tailed t-test. In treated animals, the effect of TP and season were assessed by 2-way ANOVA. All analyses were completed using SPSS Statistics version 19 (IBM, Armonk, NY).

Table 2. Western blot antibodies and concentrations.

Protein	AR	CBP	SRC-1
1° Antibody	Rabbit polyclonal anti-AR[1] (sc-815)	Rabbit polyclonal anti-CBP[1] (sc-369)	Rabbit polyclonal anti-SRC-1[3] (PA1-840)
1° Concentration	1μg/5ml	1μg/200μl	2μg/ml
2° Antibody	Alkaline phosphatase (AP)-conjugated goat anti-rabbit[2] (T2191)	Horse radish peroxidase (HRP)-conjugated goat anti-rabbit[4] (7074)	Horse radish peroxidase (HRP)-conjugated goat anti-rabbit[4] (7074)
2° Concentration	0.033μl/ml	0.33μl/ml	0.33μl/ml
Control Protein	β-Actin	GAPDH	GAPDH
Control 1° Antibody	Goat polyclonal anti-β-Actin[1] (sc-1615)	Mouse monoclonal anti-GAPDH, clone 6C5[5] (MAB374)	Mouse monoclonal anti-GAPDH, clone 6C5[5] (MAB374)
Control 1° Concentration	1μg/mL	2μg/ml	2μg/ml
Control 2° Antibody	AP-conjugated donkey anti-goat[1] (sc-2022)	HRP-conjugated rabbit anti-mouse[6] (A9044)	HRP-conjugated rabbit anti-mouse[6] (A9044)
Control 2°Concentration	0.25μl/ml	0.0125μl/ml	0.0125μl/ml

[1]Santa Cruz Biotechnology, Santa Cruz, CA; [2]Applied Biosystems, Foster City, CA; [3]Thermo Scientific, Rockford, IL; [4]Cell Signaling Technologies, Beverly, MA; [5]Millipore, Bedford, MA; [6]Sigma-Aldrich, St. Louis, MO

Figure 1. Western blots depicting specific labeling for AR, CBP, and SRC-1. Multiple bands were detected for AR and CBP. In each case, these bands of interest were eliminated with a preadsorption control, indicating their specificity for the protein targets of the primary antibodies.



Results

Real-time qPCR in Copulatory Tissues

Intact RPM

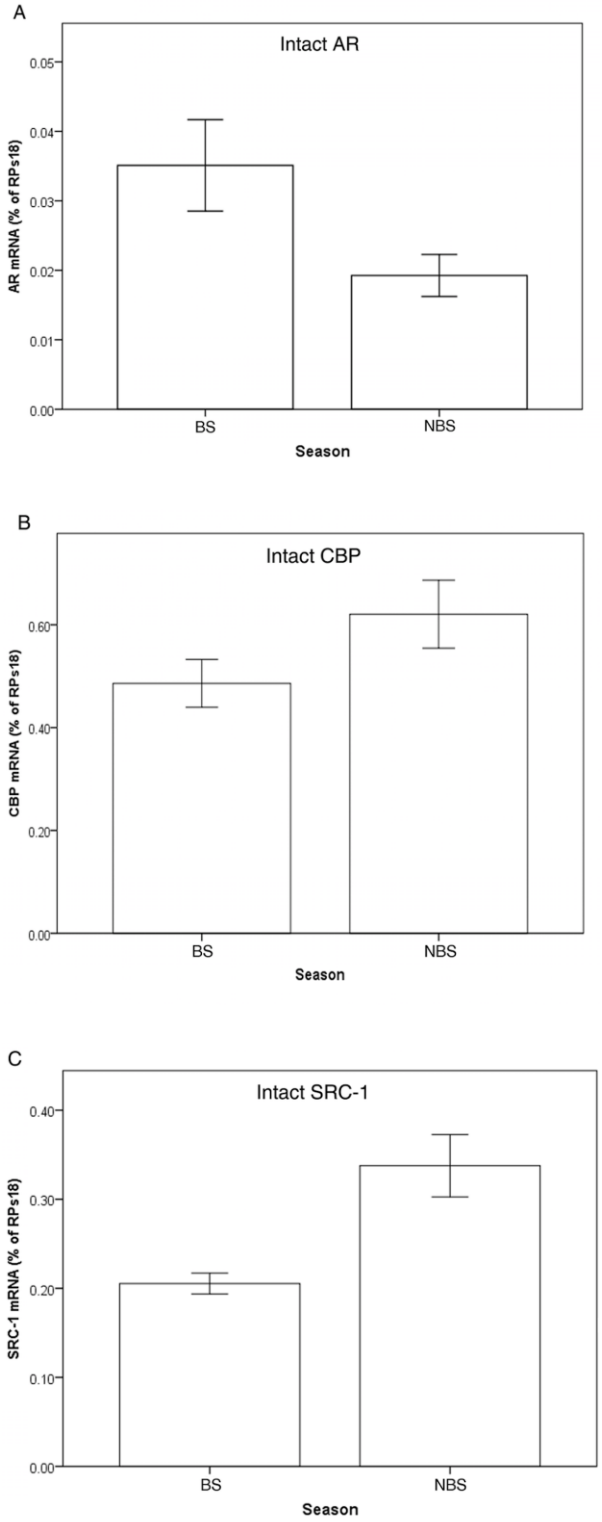
Individual analyses of each gene (Table 3) indicated that unmanipulated males had significantly greater expression of AR mRNA in the BS than NBS ($t(11) = 2.47$, $p = 0.031$). An effect of season was also detected for CBP ($t(11) = 2.23$, $p = 0.048$) and SRC-1 ($t(11) = 2.59$, $p = 0.025$), but in the opposite direction of AR. For both coactivators, more mRNA was detected in the NBS compared to the BS. The control gene, RPs18 showed no differences across seasons in intact animals (all $t(11) \leq 0.71$, $p \geq 0.493$). When individual values were corrected for RPs18 (Burmeister et al., 2007, Pfaffl, 2001) (Fig. 2), a trend remained in the same direction as the previous analysis, with more AR mRNA in the BS than NBS ($t(11) = 2.07$, $p = 0.063$). CBP no longer displayed a main effect of season when corrected for RPs18 ($t(11) = 1.70$, $p = 0.117$), but SRC-1 remained significantly increased in the NBS ($t(11) = 3.83$, $p = 0.003$).

Table 3. Effects of season on mRNA expression in intact RPM tissue.

Gene	BS:NBS*
AR	**1.76**
CBP	**0.76**
SRC-1	**0.67**

*fold-difference calculated by $\Delta\Delta_{CT}$, normalized to RPs18 (which did not differ across seasons or treatment). Significant main effects indicated with bold type.

Figure 2. Relative expression of AR (A), CBP (B), and SRC-1 (C) mRNA in the RPM of intact animals. Individual values were corrected for the housekeeping gene, RPs18. (A) shows a trend for a decrease in AR mRNA in the non-breeding season (NBS) compared to breeding season (BS). (B) CBP did not differ across the seasons. (C) documents a significant increase in SRC-1 in the NBS compared to BS. Means ± standard errors are depicted.



Treated RPM

A trend for an effect of treatment was detected for AR ($F(1,24) = 4.21$, $p = 0.051$; Table 4), with TP increasing its expression compared to controls. No main effect of season or interaction with treatment was found for AR (both $F(1,24) \leq 2.68$, $p \geq 0.115$), although the ratio of relative BS:NBS expression was slightly greater than in intact animals. Significant effects of season and treatment were not detected for CBP (all $F(1,24) \leq 0.55$, $p \geq 0.465$) or SRC-1 (all $F(1,24) \leq 1.37$, $p \geq 0.254$). No significant effects were detected for the RPs18 housekeeping gene (all $F(1,24) \leq 1.44$, $p \geq 0.241$).

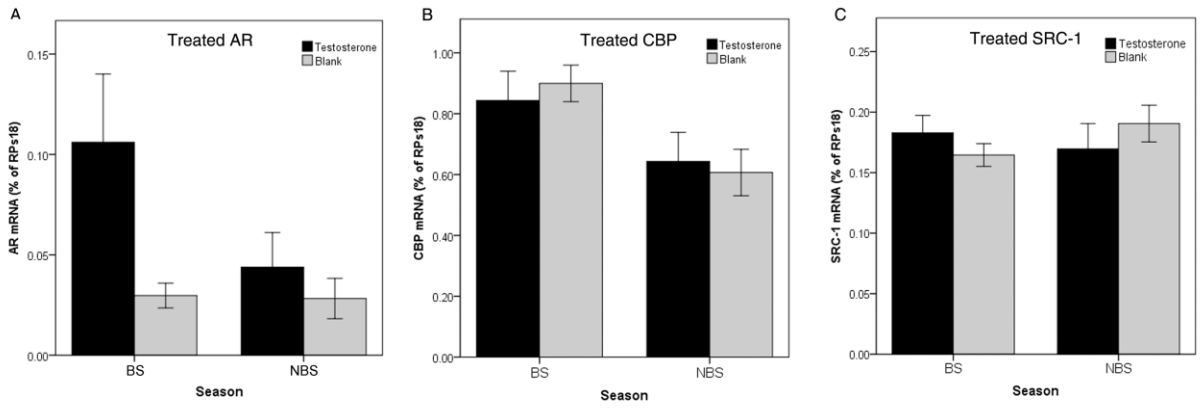
When values were corrected for RPs18 (Fig. 3), the effect of treatment on AR mRNA became statistically significant ($F(1,24) = 5.35$, $p = 0.030$); TP produced an increase compared to controls. As in the previous analysis, no main effect of season or interaction was found for AR (both $F(1,24) \leq 2.56$, $p \geq 0.122$). In contrast, CBP exhibited a main effect of season; this mRNA was increased in the BS compared to NBS ($F(1,24) = 8.80$, $p = 0.007$). No effect of treatment or interaction between treatment and season was found for CBP (both $F(1,24) \leq 0.31$, $p \geq 0.583$). As in the previous analysis, SRC-1 was not significantly different across season or treatment, and no interaction was detected, when individual values were corrected for RPs18 (all $F(1,24) \leq 1.60$, $p \geq 0.218$).

Table 4. Effects of season and treatment on mRNA expression in RPM tissue of hormone treated animals.

Gene	BS:NBS*	***TP:BL****
AR	2.01	***2.07***
CBP	1.50	0.98
SRC-1	1.02	0.97

*fold-difference calculated by $\Delta\Delta_{CT}$, normalized to RPs18 (which did not differ across seasons or treatment). Significant main effects indicated with bold type (italics denotes trend, p=0.051).

Figure 3. Relative mRNA expression of (A) AR, (B) CBP, and (C) SRC-1 in the RPM of hormone-manipulated animals. Values are corrected for RPs18. A main effect of treatment was detected, such that TP significantly increased AR mRNA, and a main effect of season was detected such that more CBP was expressed in the BS compared to NBS. SRC-1 did not differ across seasons or treatment conditions. Means ± standard errors are depicted in all graphs.



Intact Hemipenis

Intact animals displayed no differences across seasons in AR ($t(13) = 0.83$, $p = 0.424$) or CBP ($t(12) = 1.36$, $p = 0.199$) mRNA. In contrast, significantly more SRC-1 mRNA was detected in the BS than NBS ($t(12) = 2.36$, $p = 0.036$). This effect, however, was not specific, as β-actin that was run alongside SRC-1 also differed across season ($t(12) = 4.78$, $p < 0.001$). RPs18 and GAPDH were also run on a different plate to assess specificity of hemipenis effects, and like β-actin they exhibited increased expression in the BS compared to the NBS (both $t(12) \geq 3.33$, $p \leq 0.005$).

Treated Hemipenis

A variety of effects of season and treatment were detected in this tissue type. They are detailed here, but as was the case for the data from hemipenes of intact animals, it is clear that the results are not specific to the steroid-related genes of interest. β-actin differed significantly across both season and treatment in tissue from hormone-

manipulated animals. Its expression was greater in the BS than NBS, and in TP- compared to blank-treated males (both $F(1,22) \geq 1.71$, $p \leq 0.006$).

For AR, a main effect of season was detected ($F(1,22) = 5.45$, $p = 0.029$), with greater mRNA expression in the BS than NBS. No main effect of treatment was found ($F(1,22) = 1.41$, $p = 0.248$), but there was an interaction of season and treatment ($F(1,22) = 6.99$, $p = 0.015$). Pairwise comparisons revealed that within the BS, blank-treated males had more AR than TP-treated males ($t(12) = 3.19$, $p = 0.008$), and within blank-treated males, more AR was present in the BS than NBS ($t(11) = 2.84$, $p = 0.016$). No effect of treatment was revealed in the NBS ($t(10) = 0.88$, $p = 0.397$), and in TP-treated males there was no significant effect of season ($t(10) = 0.32$, $p = 0.754$).

For CBP, no significant main effects of season or treatment, and no interaction between these variables were found (all $F(1,22) \leq 2.04$, $\geq P\ 0.167$). For SRC-1, a significant main effect of treatment was found ($F(1,22)=5.61$, $p = 0.027$), with T increasing SRC-1 mRNA, but no effect of season or interaction existed (both $F(1,22) \leq 1.89$, $p \geq 0.183$).

Because of the lack of specificity, none of the results on mRNAs expressed in hemipenis are illustrated, however some general interpretations are provided below.

Real-time qPCR in Whole Brain from Intact Animals

Uncorrected analyses for individual genes showed a significant main effect of sex on AR ($F(1,24) = 15.80$, $p = 0.001$), with males having greater mRNA expression than females (Table 5). No effect of season was detected ($F(1,24) = 0.02$, $p = 0.889$), but a significant interaction of sex and season existed ($F(1, 24) = 7.93$, $p = 0.010$). Pairwise comparisons revealed that within the NBS, males had more AR mRNA than females ($t(12) = 4.87$, $p <$

0.001), and among males, more AR mRNA was present in the NBS ($t(12)=2.33$, $p = 0.038$). No effect of sex was detected in the BS ($t(12) = 0.81$, $p = 0.434$), and among females there was no significant effect of season ($t(12) = 1.73$, $p = 0.109$). Significant main effects of both sex and season were detected for CBP (both $F(1,24) \geq 5.03$, $p \leq 0.034$), but the variables did not interact ($F(1,24) = 1.97$, $p = 0.173$). Males had significantly more relative CBP mRNA than females, and there was also more CBP mRNA in the NBS than the BS. A significant sex difference was detected for SRC-1 ($F(1,23) = 9.35$, $p = 0.006$), such that males expressed SRC-1 mRNA at higher levels than females. There was no significant main effect of season ($F(1,23) = 0.42$, $p = 0.523$), and no interaction between sex and season ($F(1,23) = 3.37$, $p = 0.08$). In no case did β-actin differ across sexes or seasons in whole brain samples (all $F(1,24) \leq 2.51$, $p \geq 0.126$).

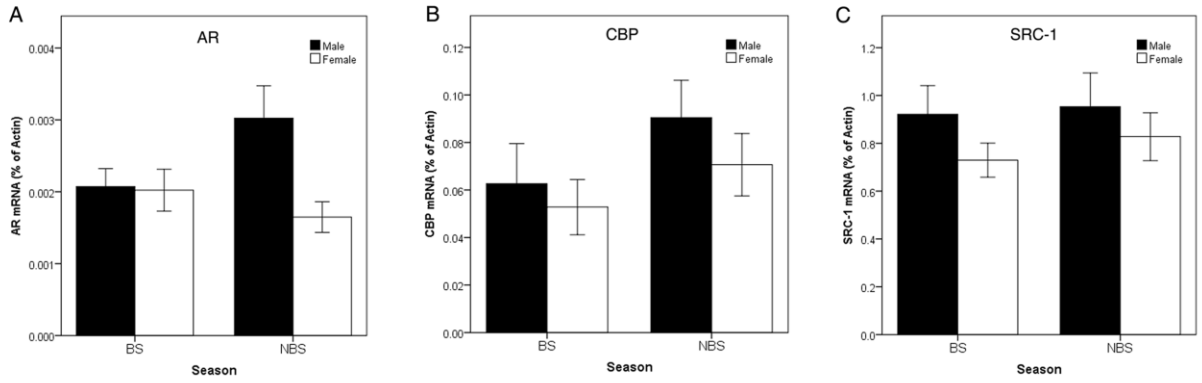
When individual values of AR mRNA were corrected for β-actin (Burmeister et al., 2007, Pfaffl, 2001), the sex difference remained ($F(1,24) = 4.29$, $p = 0.049$). No main effect of season was detected ($F(1,24) = 0.75$, $p = 0.395$), but there was a trend for the interaction between sex and season ($F(1,24) = 3.82$, $p = 0.062$). As in the analysis using uncorrected values, the male biased sex difference in AR mRNA is largely driven by the NBS (Fig. 4A). Despite the effects not quite reaching statistical significance for CBP or SRC-1 when using the corrected variables (all $F(1,24) \leq 2.50$, $p \geq 0.127$), the patterns were consistent across analyses (Fig. 4B, C and Table 5).

Table 5. Effects of season and sex on whole brain mRNA expression in intact animals.

Gene	BS:NBS*	Male:Female*	Pairwise Comparisons*
AR	0.92	**1.37**	NBS, M:F = **1.79** Males, BS:NBS = **0.70**
CBP	0.71	**1.21**	
SRC-1	0.94	**1.15**	

*fold-difference calculated by $\Delta\Delta_{CT}$, normalized to β-actin (which did not differ across seasons or sexes). Significant main effects indicated with bold type. Significant pairwise comparisons are also noted following an interaction between season and sex for AR only (see text).

Figure 4. Relative expression of (A) AR, (B) CBP, and (C) SRC-1 mRNA in whole brains of intact males and females. Values are corrected for β-actin (mean ± standard error). Panel A indicates a main effect of sex, such that males expressed more AR mRNA than females; an interaction of sex and season in this analysis indicated that the effect was driven by the NBS. CBP and SRC-1 (B and C) did not exhibit significant differences in this analysis.



Western Blot Analyses

Intact RPM

Two AR bands at 40 and 60 kDa were detectable in the RPM tissue. Relative optical density of the 40 kDa band was increased in the NBS compared to BS ($t(10) = 3.99$, $p = 0.003$) (Fig. 5A). In contrast, the 60 kDa band did not differ significantly across seasons in

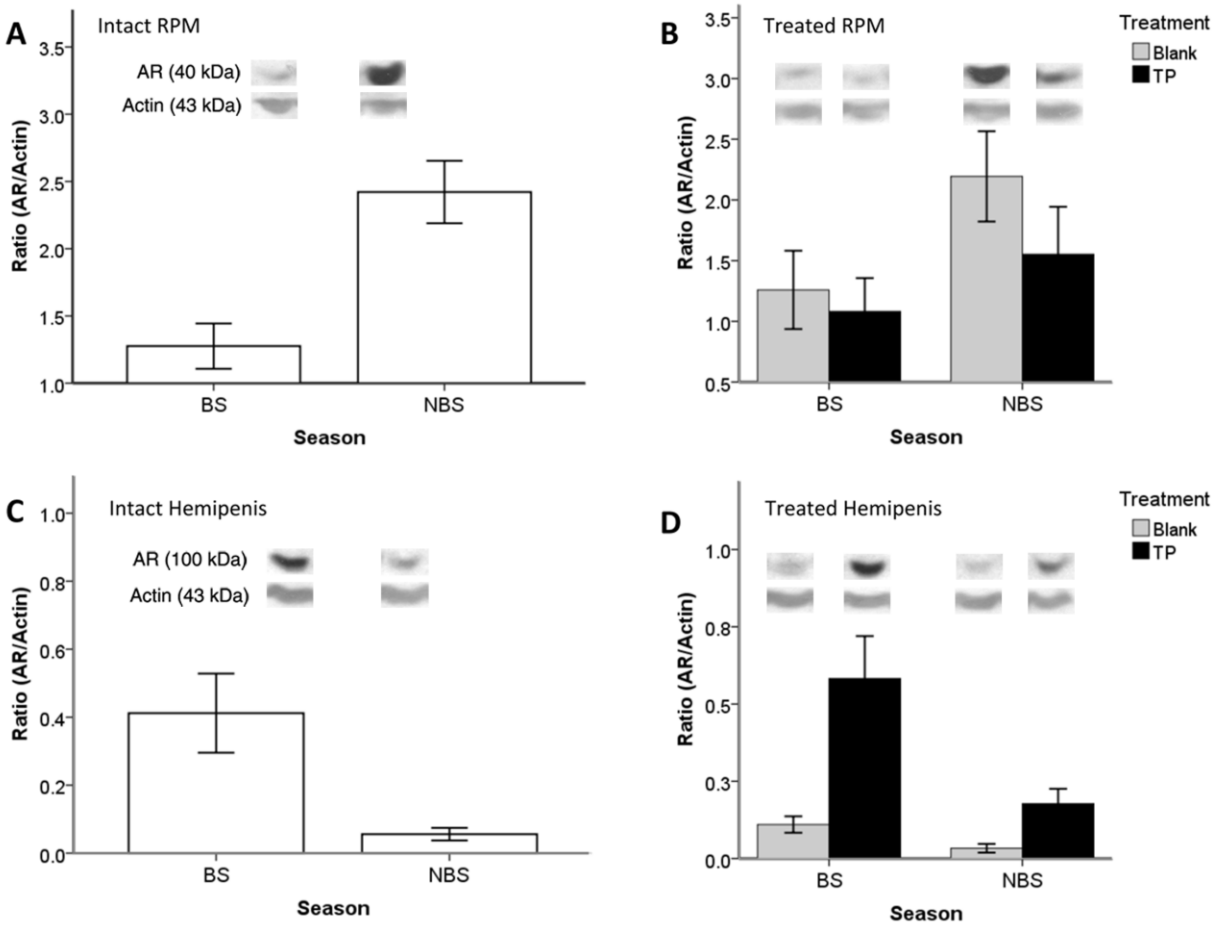
intact animals ($t(10) = 0.01$, $p = 0.994$; data not shown). Three bands were detected for CBP, with molecular weights of approximately 165, 75, and 50 kDa. No main effect of season was found in intact RPM for any of the bands (all $t(10) \leq 1.14$, $p \geq 0.280$; data not shown). One distinct band with a molecular weight of 165 kDa was detected for SRC-1. No main effect of season was found in intact animals ($t(10) = 1.13$, $p = 0.285$; data not shown). β-actin was used as the loading control for AR blots, and GAPDH was used for CBP and SRC-1. Neither of these was significantly different across season for any of the intact RPM Western blot analyses (all $t(10) \leq 0.76$, $p \geq 0.464$).

Treated RPM

For AR, the 40 kDa band exhibited a trend for greater expression in the NBS than BS in treated animals ($F(1,20) = 4.20$, $p = 0.054$). However, no effect of treatment or interaction between season and treatment was found (both $F(1,20) \leq 1.42$, $p \geq 0.247$; Fig. 5B). For the 60 kDa band, there were no main effects of season, treatment, or interaction between these variables (all $F(1,20) \leq 0.07$, $p \geq 0.796$; data not shown). The 165 kDa band for CBP differed significantly between seasons, with RPMs from the BS expressing a greater relative level of CBP than those from the NBS ($F(1,20) = 13.94$, $p = 0.001$; Fig. 6A). No main effect of treatment or interaction between treatment and season was found (both $F(1,20) \leq 2.81$, $p \geq 0.109$). Main effects of season and treatment were not detected in either the 50 kDa or the 75 kDa band (all $F(1,20) \leq 1.36$, $p \geq 0.257$; data not shown). For SRC-1, one outlier was identified using Dixon's Q test (Sokal and Rohlf, 1994) and removed from the TP-treated NBS group; his value was more than five standard deviations above the mean. There was a trend for an effect of season in treated males ($F(1,19) = 4.07$, $p = 0.058$; data

not shown), such that SRC-1 was increased in the BS over the NBS. There was no main effect of treatment or interaction in this tissue (both $F(1,19) \leq 2.60$, $p \geq 0.124$). Loading controls, β-actin and GAPDH, did not differ across season or treatment (all $F(1,20) \leq 2.77$, $p \geq 0.112$).

Figure 5. Relative expression of AR protein in copulatory tissues. Panels A and C show significant effects of season in gonadally intact males. AR was greater in the NBS than BS in the RPM and greater in the BS than NBS in the hemipenis. Panels B and D indicate relative protein expression of AR in the RPM and hemipenis, respectively, of treated animals. In the RPM, a trend for an increase in the NBS compared to BS was detected. In the hemipenis, main effects of season and treatment existed, such that AR was increased in the BS compared to the NBS and in TP-treated compared to control animals. An interaction between these variables was also detected, such that TP-treatment was more effective at increasing relative AR expression in the BS than NBS. Means ± standard errors are shown in all cases in the histograms, with representative bands from Western blots shown above for each group.



Intact Hemipenis

Three bands were detected in hemipenis tissue with the AR antibody at molecular weights of approximately 100, 60, and 40 kDa (Fig. 1A). In intact males, relative expression of the 100kD product was greater in the BS than NBS ($t(10) = 3.02$, $p = 0.013$; Fig. 5C). No significant differences were found in relative AR levels between the BS and NBS in the 40 kDa ($t(10) = 1.35$, $p = 0.207$) or 60 kDa bands ($t(10) = 0.92$, $p = 0.380$; data not shown). CBP expression across seasons did not differ between the seasons in intact males for any of the three bands (all $t(10) \leq 1.73$, $p \geq 0.114$; data not shown). SRC-1 expression did not differ between the seasons in intact animals ($t(10) = 0.08$, $p = 0.935$; data not shown). Actin was used as the loading control for AR blots, and GAPDH was used for CBP and SRC-1. Neither of the control proteins differed across season for any of the intact hemipenis Western blots (all $t(10) \leq 0.85$, $p \geq 0.416$).

Treated Hemipenis

Treated animals displayed a similar pattern of AR expression as intact animals. For the 100 kDa band, a main effect of season was detected ($F(1,20) = 10.53$, $p = 0.004$), such that relative AR protein was increased in the BS compared to NBS (Fig. 5D). A main effect of treatment was also detected ($F(1,20) = 17.29$, $p < 0.001$), with TP-exposed animals expressing more than animals than those that received a blank capsule. An interaction between season and treatment was also found ($F(1,20) = 4.88$, $p = 0.039$), reflecting a greater effect of T in the BS compared to NBS. No main effects of season or treatment were detected for the 40 or 60 kDa bands (all $F(1,20) \leq 2.53$, $p \geq 0.115$).

For CBP, one NBS TP-treated male was removed from the analysis, as his values for each band were greater than four standard deviations from the mean. This animal was a statistical outlier (Dixon's Q test; Sokal and Rohlf, 1994). Relative expression of the 75 kDa CBP band was increased in the BS compared to the NBS ($F(1,19) = 4.53$, $p = 0.047$), but no main effect of treatment or interaction was found (both $F(1,19) \leq 1.45$, $p \geq 0.244$) (Fig. 6B). Neither the 50 kDa band nor the 165 kDa band displayed a main effect for season or treatment, or interaction between them (all $F(1,19) \leq 3.73$, $p \geq 0.068$). For SRC-1, a main effect for treatment was detected ($F(1,20) = 8.59$, $p = 0.008$), such that animals that received TP-treatment had lower levels of SRC-1 than blank-treated animals (Fig. 7). No main effect for season or interaction was detected (both $F(1,20) \leq 2.01$, $p \geq 0.172$). Control proteins, β-actin and GAPDH, did not differ across season or treatment for any of the Western blots on tissues from treated animals (all $F(1,20) \leq 2.14$, $p \geq 0.159$).

Figure 6. **Relative expression of CBP protein levels in copulatory tissues of treated animals.** Panel A indicates a significant decrease in the NBS compared to the BS in the RPM. Panel B documents a parallel effect in hemipenis. Means ± standard errors are depicted in the graphs, with representative bands from the Western blots above.

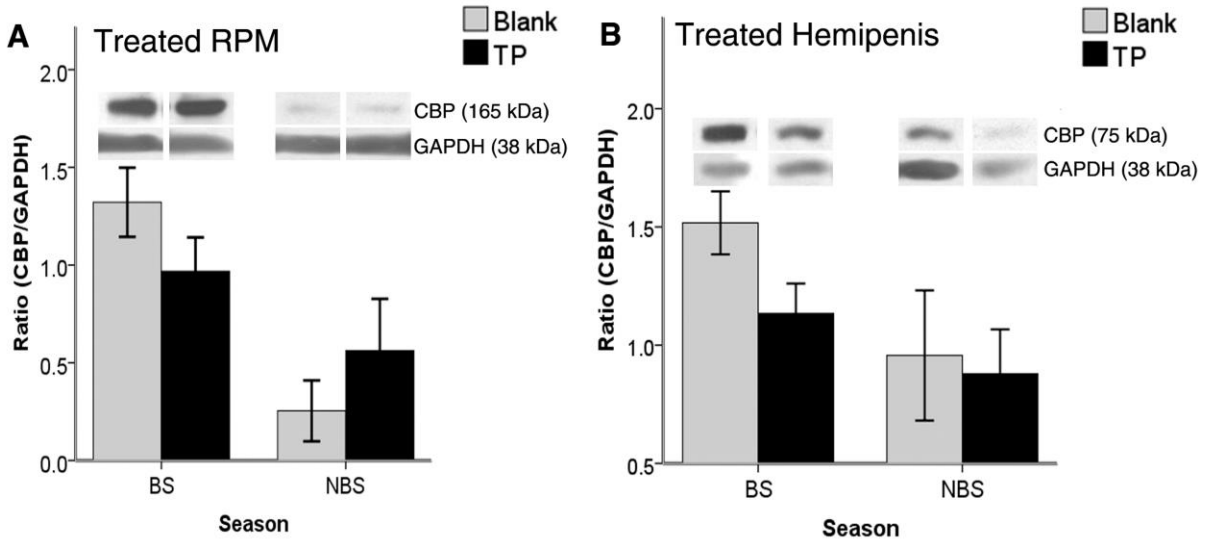
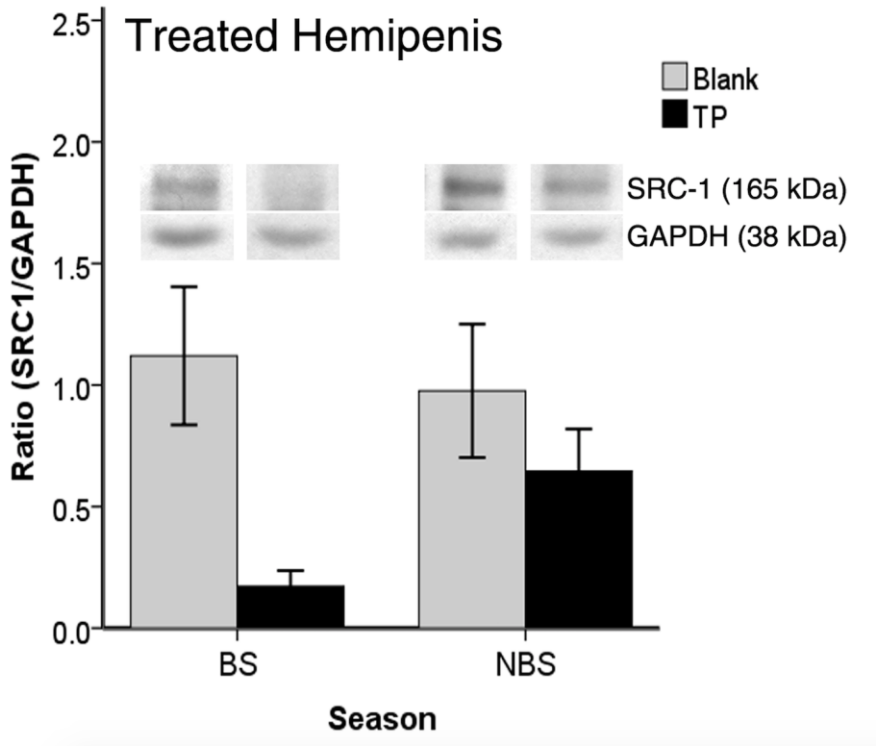


Figure 7. Relative protein expression of SRC-1 in the hemipenis of hormone manipulated animals. Means ± standard errors, plus representative images from Western blots are depicted. This value was significantly decreased in TP-treated compared to control animals.



Discussion

The present study evaluated the idea that differences in the expression patterns of AR and two of its coactivators, CBP and SRC-1, are consistent with differential responsiveness to T across seasons in tissues controlling male sexual motivation and behavior: the RPM, hemipenis and brain. These seasonal differences exist on behavioral, biochemical and morphological levels. Specifically, compared to the BS, the same dose of T in the NBS has a dramatically decreased effect on male courtship and copulatory displays, whole brain aromatase activity, soma size in the amygdala (a forebrain region important

for the display of male reproductive behavior; reviewed in Wade (2012)), as well as hemipenis and RPM fiber size (Cohen and Wade, 2010, Holmes and Wade, 2004, 2005, Lovern et al., 2004a, Neal and Wade, 2007, O'Bryant and Wade, 1999, 2002, Wade, 2005). The discussion below considers AR and its coactivators in each tissue of interest individually, and concludes with a synthesis across these anatomical levels required to coordinate the suite of sexual behaviors displayed by male green anoles.

Our interpretations consider two types of analyses for mRNA quantification. The first evaluated each target gene independently, including the housekeeping genes. The second utilized a ratio of expression to the control and accounted for efficiency of the qPCR (Pfaffl, 2001). Both analyses are informative; the first allows one to assess specificity of effects, and the second provides comparison of relative levels across conditions of interest.

The Western blot studies utilized standard analyses, correcting the target protein in each sample for its loading control after determining that the control proteins did not differ under any of the circumstances evaluated. Multiple bands were detected for AR. Three bands were present in hemipenis tissue at 40, 60, and 100 kDa, with the last being close in size to the 100–110 kDa band found in rats and humans (Brinkmann et al., 1989). Only the 60 and 40 kDa AR bands were detected in RPM tissue, suggesting tissue-specific differences in androgen signaling. Humans express multiple isoforms of AR with tissue-specific expression as well; six transcripts have been reported in Ensembl (ENSG00000169083), including an 87 kDa form expressed in lung, muscle, fat, kidney and reproductive structures (Wilson and McPhaul, 1994, 1996), and one at 45 kDa in heart and skeletal muscle (Ahrens-Fath et al., 2005). Further work is required to characterize the multiple sizes of AR detected in green anoles, and how they may relate to those identified in other

species. However, the detection of the 100 kDa AR isoform in hemipenis but not RPM suggests that it may play a specific role in hemipenis function.

CBP had three bands at 165, 75, and 50 kDa, all smaller than the predicted 265 kDa size in rodents, birds, and humans (Auger et al., 2002b, Chakravarti et al., 1996, Chrivia et al., 1993). However, two of our bands are similar in size to 78 and 58 kDa fragments reported in mice (Chrivia et al., 1993). These multiple bands may represent different forms of this protein; 12 human transcripts have been reported in Ensembl (ENSG00000005339), although half of these appear not to have protein products. All three CBP bands were detected in both hemipenis and RPM, although the isoforms that differed with season were not the same across tissues. This may suggest tissue-specific regulation attributed to a particular isoform of CBP. SRC-1 blots revealed only one band at 165 kDa, the same size as in humans and birds (Duncan and Carruth, 2011, Spencer et al., 1997), and close to the predicted 160 kDa in mice (Onate et al., 1995, Xu et al., 1998), suggesting evolutionary conservation of this protein.

RPM

AR mRNA expression was increased in the BS compared to NBS in unmanipulated (intact) males. This enhanced expression in the BS is likely regulated at least in part by circulating levels of T, as TP-treatment increased AR mRNA compared to castrated controls. However, other gonadal factors may be involved, as the effect of season detected in intact animals was eliminated in the animals in the hormone replacement study, all of which were gonadectomized. In contrast to the data on mRNA, relative levels of the 40 kDa AR protein

were higher in the NBS in the RPM in both intact and hormone treated males. Interestingly, TP did not mediate this seasonal difference.

We were somewhat surprised to see the opposite seasonal patterns in the RPM for AR mRNA and protein. However, they are not necessarily correlated (Krongrad et al., 1991); factors that can cause differences in patterns of mRNA and protein expression include regulation of transcription, translation, or post-translational modifications (Chen et al., 2002). These possibilities will need to be investigated in green anoles before we can have a complete understanding of relevant mechanisms. At present, our interpretations are largely based on the protein data. While mRNA quantification may reflect the rate of synthesis or turnover, protein is the level of expression most directly responsible for steroid hormone action.

This 40 kDa AR isoform in anoles is similar in size to a 45 kDa receptor isoform reported in humans, which can negatively regulate AR signaling, as shown in transfection studies (Ahrens-Fath et al., 2005). mRNA for this receptor has been detected in human heart, muscle, uterus, prostate, lung, and breast tissue, and the protein has been identified by Western blots in LNCaP lysates (Ahrens-Fath et al., 2005, Dehm and Tindall, 2011). We do not know if the 40 kDa AR protein in anoles is parallel to the 45 kDa form in humans. If they are functionally analogous, it may be involved in reducing copulatory function in these lizards at a time when they should not be breeding. This sort of inhibition at the receptor level might be particularly important, as adult male anoles have about 11 ng/mL circulating T in the NBS (Lovern et al., 2001). This is far lower than in the BS, but roughly twice that in a breeding adult male mammal (Feoktistova et al., 2010, Ferkin and Johnston, 1993).

Alternatively, this 40 kDa AR isoform could be positively regulating AR signaling. In this case, it seems reasonable to hypothesize that increased AR protein in the NBS could facilitate maintenance of the RPM during a time in which it is not used. While RPM fibers exhibit variation in size with large differences in circulating T due to gonadectomy and hormone replacement, the cross-sectional area is equivalent in unmanipulated animals in the BS and NBS, which exhibit a smaller difference in plasma T (Holmes and Wade, 2004, Neal and Wade, 2007). The purpose of such maintenance is not clear, but it might reflect a substantial cost that could occur with muscle regrowth prior to the following BS (Jimenez et al., 2011, Johnston et al., 2006).

Previous work in our lab has documented that seasonal changes in muscle fiber size and percentage of AR+ nuclei do not occur in intact animals, but exogenous T at the same level used in the current study increases percentage of AR+ nuclei in this muscle compared to castrated controls (Holmes and Wade, 2004). Considered in the context of the increased AR protein detected here in whole RPM without T regulation, the data collectively suggest that AR within each nucleus increases in the NBS in manipulated males. In contrast, the relatively large difference produced by a supraphysiological dose of T compared to the undetectable levels following gonadectomy results in more nuclei expressing AR with a simultaneous decrease in the amount within each.

Variations also exist in the morphology of other seasonally breeding mammalian neuromuscular systems critical for copulation, which grow larger in the breeding season due to both photoperiod and androgenic effects (Forger and Breedlove, 1987, Hegstrom and Breedlove, 1998, 1999, Hegstrom et al., 2002). AR is abundant in these muscles,

making them especially sensitive to androgens, which act locally to increase muscle fiber size, but not fiber number, in adult males (Sengelaub and Forger, 2008).

The coactivators analyzed in the current study exhibited seasonal changes in mRNA expression in the RPM. SRC-1 mRNA was consistently increased in this muscle in intact animals during the NBS compared to the BS. The fact that this effect of season was not detected in castrated males, regardless of hormone treatment, and that TP did not influence SRC-1 mRNA levels in the RPM of these animals, suggest that a gonadal factor other than T may be responsible for the seasonal difference in SRC-1 mRNA. In contrast to the mRNA, the single band detected for SRC-1 protein was not significantly modulated by season or by endocrine manipulation, although a trend for greater expression in the BS was detected. Collectively, the results suggest that SRC-1 mRNA levels in the RPM change seasonally, but the difference is not reflected in diminished protein. Thus, this coactivator in this copulatory muscle is not likely to have a substantial impact on structure or function related to copulation by adult male green anoles.

The data for CBP in the RPM are difficult to interpret because they were even less consistent. In intact animals, this mRNA was expressed to a greater degree in the NBS only when uncorrected values were analyzed. In treated animals, the pattern was the opposite (increased in the BS), but only using the corrected values. Relative levels of the 165 kDa protein were also increased in the BS compared to NBS in treated animals, but this effect was not seen in intact lizards.

Overall, further studies are necessary to determine why relative mRNA and protein levels differ in this tissue, as well as to uncover the functionality of the multiple isoforms of the proteins identified. At present, however, the data collectively suggest that increases in

AR and the two coactivators we investigated in the RPM do not appear to facilitate the greater responsiveness we have detected in behavior or muscle fiber size during the BS.

Hemipenis

Although there were no significant differences across season in the hemipenis from intact animals, more AR mRNA was present in this tissue in the BS than NBS in manipulated males. The differences between the two conditions might reflect increased sample sizes within seasons in the treated animals and suggest that some increase in AR during the BS could facilitate seasonal responsiveness to T in the hemipenis. However, this idea seems unlikely for two reasons. First, mean values were nearly identical across seasons in intact animals, which suggests that some characteristic(s) of gonadectomized animals may have revealed a difference not present in unmanipulated animals. Perhaps more importantly, mRNA of all three housekeeping genes was also increased in the BS compared to NBS in this tissue from treated animals. Thus, seasonal changes in mRNA do not appear specific.

In contrast, housekeeping controls for protein quantification did not change across season or treatment, so seasonal differences in at least one form of AR may be more functionally relevant. The 100 kDa AR protein was increased in the BS compared to NBS in both intact and treated animals. In parallel, TP increased this AR protein compared to the control manipulation in treated animals. Importantly, this effect was greater in the BS than NBS, suggesting a seasonal change in responsiveness to T in AR expression in this tissue, which could potentially contribute to the differences in T-facilitated copulatory behavior across season (see above). It would be useful to determine the extent to which this peripheral tissue might modulate behavior. It is also quite possible that the seasonal

increase in a form of AR is simply correlated with the time of enhanced behavioral expression to maximize the changes of productive copulation. While CBP mRNA did not differ across season or treatment in the hemipenis of intact and treated animals, relative levels of the 75 kDa band were increased in the BS compared to NBS in treated animals. These results are consistent with differences in the rate/efficiency of translation, but not transcription, across seasons.

SRC-1 mRNA, but not protein, was increased in the BS in the hemipenis of intact animals, although this effect was not specific as the housekeeping genes were also increased in the BS in this tissue. The increase in SRC-1 mRNA is likely due to circulating T levels; TP treatment increased SRC-1 mRNA expression over blank controls. However, SRC-1 protein was greater in control gonadectomized animals than those treated with TP. This suggests that SRC-1 is likely regulated by circulating T, but the effects of this hormone on the rate of synthesis of this gene and the actual protein expression are opposite. This coactivator is recruited to nuclear receptors in a hormone-dependent manner (Liu et al., 1999, Xu et al., 2009), so it is not surprising that circulating hormone regulates the activity of this gene. If T determines how SRC-1 is expressed, it could also, as a result, affect androgenic signaling through changes in AR coactivation. The increase in SRC-1 protein may serve as a compensatory mechanism to coactivate hemipenis AR when physiological T levels are low; however, further investigation is necessary. SRC-1 did not change in intact animals, so the protein may be upregulated in control castrated animals to compensate for the unnaturally low level of T. Some T action is necessary to maintain the structure and metabolic balance in this tissue (Traish and Kim, 2005). The patterns of SRC-1 are inconsistent with a role in T-facilitation of behavior.

The overall decrease in mRNA for two of the three genes of interest (AR and SRC-1), as well as housekeeping genes, may indicate a general decrease of hemipenis transcriptional function in the NBS. This type of inhibition makes sense at a time when the metabolic cost of copulation and the potential for increased predation during this behavior are not offset by the chance of successful reproduction (Buchanan et al., 2001, Hau, 2007, Lovern et al., 2004b, Marler and Moore, 1988). Like the RPM, the hemipenis does not appear to change in size in intact animals (Holmes and Wade, 2004), but its size is increased by exogenous T-treatment in castrated males, increases that appear greater in the BS than NBS (Holmes and Wade, 2004, Neal and Wade, 2007). Hemipenis structure appears to be more sensitive than the RPM to large increases in T, although our results indicate that this is not due to greater numbers of AR.

Brain

No seasonal differences in AR mRNA expression existed, which is somewhat surprising, as other seasonally breeding species show increases in AR in the forebrain. Specific regions, including the song control nuclei of passerine birds which are involved in the regulation of courtship behavior, as well as limbic areas involved in reproductive function, display increases in both AR mRNA (Fraley et al., 2010, Wacker et al., 2010) and protein (Pouso et al., 2010, Soma et al., 1999, Tetel et al., 2004) in breeding animals. However, AR mRNA was increased in the whole brain of male compared to female anoles, which is similar to other species, including mammalian and avian models (Scott et al., 2004, Voigt et al., 2009). This finding is consistent with the idea that T's action through AR is important for male-specific sexual behavior (Baum, 2002, Wade, 2011). It also suggests

that increased AR in males may facilitate the male-specific up-regulation of whole brain aromatase and 5a-reductase by T in anoles (Cohen and Wade, 2010). Local increases in AR in specific regions of the brain might also contribute to sex differences in the size and cell number of forebrain morphology (Beck et al., 2008, Beck and Wade, 2009), as well as motoneuron number and soma size (Lovern et al., 2004a, Ruiz and Wade, 2002).

Forebrain AR distribution has been investigated in anoles, but only in unmanipulated animals in the BS. In situ hybridization revealed that the distribution of AR mRNA is similar in males and females, but across limbic regions females have greater labeling intensity than males, particularly in the amygdala and a portion of the preoptic area (Rosen et al., 2002). The pattern of protein detected across areas was similar to that of mRNA, but protein was not quantified. Given that the present study found robust sex differences in AR mRNA, it will be important to quantify the number of cells expressing AR mRNA, as well as protein expression, within specific brain areas known to control male and female reproductive behavior, including the preoptic area, amygdala, and ventromedial hypothalamus (Wade, 2012).

Whole brain tissue revealed no seasonal differences in either CBP or SRC-1 mRNA, but both coactivators were increased in the male compared to female brain when values were uncorrected. These sex differences were, however, not statistically significant when values were corrected for β-actin. In mammalian and avian brains, both CBP protein and SRC-1 mRNA and protein are heavily expressed in steroid sensitive brain regions, including avian song control nuclei (Auger et al., 2002a,b, Charlier et al., 2002, Duncan et al., 2011, Meijer et al., 2000, Stromberg et al., 1999, Tetel et al., 2004). A male-biased sex difference of CBP occurs in early postnatal rats, but this sex difference disappears by the eleventh day

after birth (Auger et al., 2002a). SRC-1 mRNA is more dense in male canary song control nuclei than female (Duncan et al., 2011); however, females have a higher concentration than males in the POA of Japanese quail (Charlier et al., 2006). SRC-1 expression also increases with circulating T in both birds and mammals (Charlier, 2009, Charlier et al., 2002, 2003, 2006, Charlier and Balthazart, 2005, Tetel, 2009, Tetel et al., 2004). CBP and SRC-1 coactivate other transcription factors and steroid receptors (Smith et al., 1996, York and O'Malley, 2010), so their stable expression of mRNA across seasons may be necessary for other types of signaling. It will be important for us to follow up and examine these coactivators in a region-specific manner to determine whether they are locally regulated across season and hormonal environment.

Broader Context and Interpretations

AR and its coactivators are differentially regulated across brain, hemipenis and RPM tissue. Additionally, tissue-specific expression of the multiple sizes of AR proteins may possess different functional relevance for androgen signaling, as the 100 kDa receptor was present in hemipenis tissue only. For both CBP and AR, only one of the isoforms varied significantly in response to hormone and season, but the form exhibiting these effects differed across tissue. These protein isoforms will need to be studied further to fully understand their tissue-specific functions in regard to reproduction.

Overall, the patterns of AR, CBP, and SRC-1 expression in the RPM and brain are not consistent with a role in facilitating responsiveness to T in behavior, morphology, or biochemical function. The expression of AR protein in hemipenis tissue was most

consistent with a role in seasonal responsiveness to T, as AR was increased by T, and more so in the BS than NBS.

Interestingly, this study demonstrated that hemipenis tissue had a general inhibition in transcription in the NBS, regardless of circulating T, but this was not the case in the RPM. The hemipenis, unlike RPM, is not a muscle, but consists of fibrovascular tissue (Aughey and Frye, 2001). The cost of seasonal hemipenis regrowth may be minimal compared to RPM, which could explain the differential maintenance of these tissues year round. Extrapolating from Johnston and colleagues' optimal fiber size hypothesis, which suggests the number of small and large fibers in a given muscle is balanced by their metabolic costs, it may be the case that larger muscle fibers in the RPM may be metabolically cheaper to maintain than a cycle of regrowth (Johnston et al., 2006).

The present data may indicate that central control of male sexual behavior by T in this species is not as tightly regulated across seasons as at the peripheral level; changes in gene expression in copulatory tissues may be dictating the frequency by which sexual behaviors occur. Alternatively, it may simply be that shifts in the display of sexual behavior are paralleled by changes in the function of copulatory tissue to reduce costs when there are no benefits. In this case, patterns of expression within particular brain regions may vary across seasons in a manner that affects seasonal responsiveness to T. More information is needed to further characterize the roles of AR and its coactivators in seasonally breeding species. As rates of transcription and translation appear to differ dramatically in some cases, it will be critical to evaluate both mRNA and protein locally within regions such as the preoptic area and amygdala, which regulate male sexual behavior. Future work aims to evaluate the region-specific distribution of these genes

across season in the anole brain, as well as determine whether epigenetic mechanisms, DNA methylation in particular, are involved in the regulation of changing AR expression.

CHAPTER 2

RELATIONSHIPS AMONG SEX, SEASON, AND TESTOSTERONE IN THE EXPRESSION OF ANDROGEN RECEPTOR mRNA AND PROTEIN IN THE GREEN ANOLE FOREBRAIN

Introduction

Steroid hormones can modify the structure and function of both central and peripheral tissues across vertebrate species. When acting via genomic mechanisms, once a hormone is bound to its receptor, this complex interacts with response elements on DNA and causes changes in gene transcription. This process leads to differential protein expression, and can ultimately affect behavior and a variety of physiological processes. For example, androgen action through genomic mechanisms can cause dramatic changes in sexual differentiation and adult reproductive processes. Research in mammalian systems has documented that a diverse range of factors is involved in masculinization of the brain; however, it is clear that early exposure to gonadal steroids plays a critical role (McCarthy and Arnold, 2011). These actions occur following testosterone (T) or its metabolite, dihydrotestosterone, binding to androgen receptors (AR), or after aromatization of T to estradiol, which binds to estrogen receptors (Naftolin et al., 1975, Morris et al., 2004). Similarly, across species including the green anole, T maintains a number of sex differences in the brain in adulthood, and regulates seasonal changes in sexual behavior and brain morphology (Wingfield and Farner, 1978, Smith et al., 1997, Cooke et al., 1999, Soma et al., 1999, Beck et al., 2008, Beck and Wade, 2009a).

Green anole lizards provide a unique model to examine seasonal changes in relation to AR expression. They are seasonal breeders, with the breeding season (BS) lasting from approximately April through to July, followed by gonadal regression in both sexes during

the nonbreeding season (NBS) (Wade, 2005, 2011, 2012). It is primarily T, rather than its metabolites (estradiol or dihydrotestosterone), that is responsible for activating male sexual behavior in this species, presumably through AR (Winkler and Wade, 1998, Rosen and Wade, 2000, Wade, 2011).

AR distribution across vertebrate species is similar, and includes regions of the brain important for the display of sexual behavior, such as the preoptic area (POA), ventromedial amygdala (AMY), and ventromedial hypothalamus (VMH) (Sar et al., 1990, Balthazart et al., 1992, Smith et al., 1996b, Soma et al., 1999, Wood and Newman, 1999, Rosen et al., 2002, Tetel et al., 2004, Voigt et al., 2009). The POA and AMY (or its homolog) are critical for male typical sexual behavior across vertebrates (Christensen and Clemens, 1974, Wood and Newman, 1995, Thompson et al., 1998, Ball and Balthazart, 2004). The VMH is essential for female sexual behavior, controlling receptivity across species (Meisel et al., 1987, Flanagan-Cato, 2011).

In green anoles, lesions to the POA or AMY inhibit courtship and copulation, and hormone implants into these areas restore sexual behavior (Morgentaler and Crews, 1978, Wheeler and Crews, 1978, Crews and Morgentaler, 1979, Greenberg et al., 1984). The VMH is important for female sexual behavior in lizards, with lesions to this region preventing female sexual behavior in anoles and other species (Farragher and Crews, 1979, Kendrick et al., 1995). In green anoles, the POA is larger in volume in males than females and, in both sexes, the POA and VMH are larger in the BS than NBS (Beck et al., 2008).

While sex and seasonal differences in brain morphology and behavior may in some cases be due to differences in circulating T, other mechanisms must contribute to the sex differences and seasonal changes that occur in anoles. In gonadectomized males, the same

dose of T produces dramatically reduced responses in the NBS compared to the BS. These responses include masculine sexual behaviors (courtship and copulation), morphology (copulatory muscle fiber size, as well as soma size in the POA and AMY), and biochemical changes (brain aromatase activity) (O'Bryant and Wade, 1999, 2002, Holmes and Wade, 2004, Lovern et al., 2004a, Holmes and Wade, 2005, Wade, 2005, Neal and Wade, 2007, Cohen and Wade, 2010, 2011). This phenomenon of differential effects of T across breeding conditions is also seen in other reptiles and mammals (Crews et al., 1993, Holmes et al., 2007). One hypothesis is that the effectiveness of T is modulated by differences in AR expression across seasons in particular tissues. In the periphery, expression of AR protein in the hemipenises, bilateral tissues used for copulation in reptiles, is increased in the BS compared to NBS (Kerver and Wade, 2013). AR expression is also increased by T treatment in this tissue, and to a greater extent in the BS than NBS (Kerver and Wade, 2013). These results suggest that the function of T in this peripheral copulatory structure may be limited in the NBS due to the availability of its receptor.

It is unclear whether a similar mechanism may apply to seasonal differences in the brain. AR mRNA and protein have been examined in the green anole brain, but they have not been quantified in a region-specific manner across the seasons (Rosen et al., 2002, Kerver and Wade, 2013). In the BS, males and females generally have comparable levels of AR mRNA within the structures investigated, although, considered across limbic regions, the intensity of labeling (using analysis of silver grains following exposure to a radiolabeled probe) is greater in females. AR protein expression has not been quantified, but its distribution detected by immunohistochemistry generally mirrors that of mRNA (Rosen et

al., 2002). In whole brain homogenates, males have more AR mRNA than females, with no effect of season (Kerver and Wade, 2013).

The present study was completed to address open questions regarding relative levels of AR across sex and season within the areas of the green anole brain responsible for sexual behavior. In situ hybridization was performed on the brains of gonadally intact male and female anoles. AR-expressing cells were quantified in the POA, AMY and VMH to determine whether this variable might contribute to differential responsiveness to T at the central level. Western blots were also performed on microdissected portions of the brains of unmanipulated animals, which contained the POA and hypothalamus, to examine relative levels of AR protein. A second set of experiments (in situ hybridization and Western blots) examined the brains of male and female anoles that were gonadectomized and treated with T-propionate (TP) or blank capsules to determine how this hormone affects AR expression in the brain across the sexes and seasons.

Methods

Animals

Green anole lizards of both sexes were caught from the wild and sent to us by Charles Sullivan Co. (Nashville, Tenn., USA) near the start of each season (BS – April, NBS – October). Animals were housed in male/female pairs in 10-gallon aquaria that contained a peat moss substrate, natural sticks and rocks, and a water dish. Each day the cages were misted with water. During the BS, when animals are more active, they were fed with live crickets 3 times weekly; this occurred twice weekly in the NBS. Black dividers were placed in between cages to prevent visual contact. Animals were maintained on a 14:10-hour

light:dark cycle in the BS and a 10:14- hour cycle during the NBS. Fluorescent lighting, full-spectrum bulbs and a heat lamp were present over each cage. In BS conditions, the temperatures ranged from 28 to 38°C in the daytime, depending on the distance from the heat lamp, and 18°C was maintained at night. During NBS conditions, temperatures ranged from 24 to 30°C during the day and were maintained at 15°C at night. Humidity was kept at 60–70% in both seasons. All procedures were approved by the Michigan State University Institutional Animal Care and Use Committee.

Treatment and Tissue Collection

After arrival, animals were allowed 2 weeks to acclimate to the lab setting. To examine naturally occurring differences between seasons, gonadally intact males and females from each season were rapidly decapitated. Breeding state and reproductive maturity were confirmed by examining the gonadal state at the time of tissue collection. Sample sizes were initially 8 per group; however, a few animals were eliminated from the study because reliable measurements could not be obtained in specific brain regions. No more than 2 animals were removed from any one group, and these were due to histological artifact in the tissue. Final sample sizes are noted in the graphs (fig. 1–3), and degrees of freedom are indicated with the statistical results. Tissue was collected between the hours of 10 a.m. and 2 p.m. Brains were individually snap-frozen in methyl butane and stored at –80°C. The tissue was sectioned into four series at 20 μm, thaw-mounted onto SuperFrost Plus slides (Fisher Scientific, Hampton, N.H., USA) and stored at –80°C.

An additional six male and six female brains from gonadally intact animals were collected from both the BS and NBS and stored at –80°C for Western blot analyses. Not

enough protein was produced from attempts to take punches from individual brain regions, so, following rapid decapitation, the tissue containing the POA and hypothalamus was dissected from whole brain samples and snap-frozen in methyl butane before storage at –80°C. Microdissections were performed using landmarks including the optic chiasm, pituitary stalk and pyramidal tracts to reliably dissect the same region from each brain (Greenberg, 1982). The breeding state was confirmed by visual inspection of the reproductive organs at the time of tissue collection. Females had at least one large yolking follicle or an egg in the oviduct in the BS, and males had large, vascularized testes. Gonads were regressed in all NBS animals.

To determine whether T plays a role in AR expression in the anole brain, lizards were gonadectomized and implanted with either a TP-filled capsule or control (empty) capsule. These procedures were used on one set of males and females from both the BS and NBS for in situ hybridization (n = 8 per group) and another set for Western blots (n = 6 per group). After the animals were acclimated to the laboratory, males and females were injected with 10 µl of 0.2% lidocaine before being anesthetized with isoflurane, then bilaterally gonadectomized while on ice. During this procedure animals were subcutaneously implanted with a Silastic capsule (7 mm long × 0.7 mm inner diameter × 1.65 mm outer diameter) containing either 5 mm of packed TP or left blank, as in previous studies (Holmes and Wade, 2005, Neal and Wade, 2007, Cohen and Wade, 2010). This hormonal manipulation produces detectable plasma concentrations that average 48.69 ± 4.78 (SEM) ng/ml, with no difference across seasons (Cohen and Wade, 2012). These androgen levels average about twice that of endogenous androgen in field-collected animals (approx. 20 ng/ml in the BS and 11 ng/ml in the NBS); however, they overlap with

the range seen in breeding individuals (Lovern et al., 2001). This dose was chosen because it reliably activates male sexual behavior and facilitates biochemical and morphological changes in the brain (Neal and Wade, 2007, Cohen and Wade, 2010). These animals were euthanized by rapid decapitation and the brains were collected 1 week after treatment. Absence of testes or ovaries was confirmed at this time, and it was confirmed that some crystalline hormone remained in the TP capsules.

In Situ Hybridization

AR from the green anole was previously cloned into pBluescript (Rosen et al., 2002). T7 (antisense) and T3 (sense) polymerases were used to transcribe RNA probes using the Digoxigenin RNA Labeling Kit (Roche Diagnostics, Indianapolis, Ind., USA) according to the manufacturer's instructions. One series of slides was used for antisense labeling and a different series from a representative animal from each group was used for the sense reaction (thus, analyzed sections were 80 μm apart). All procedures were performed as in Cohen and Wade (2011, 2012), and all slides were processed simultaneously.

Briefly, slides were thawed and fixed in 4% paraformaldehyde, at a pH of 7.4. Following three PBS rinses, slides were treated with TEA-HCl with 0.25% acetic anhydride and rinsed in PBS. Slides were then placed in a hybridization buffer without the probe for 1 h at 55°C. Following prehybridization, slides were placed overnight at 55°C in hybridization buffer containing 250 ng/ml of either sense or antisense probe. The following day, slides were rinsed in 2× and 0.2× SSC at 60°C, followed by rinses in MAB with 0.1% Tween-20 (MABT) at room temperature. Two incubations with 0.9% hydrogen peroxide in MABT and 5% normal sheep serum in MABT were performed before a 2-hour

incubation in 0.5 µl/ml of anti-digoxigenin-AP Fab fragments (Roche) in MABT. Slides were treated with 4.5 µl/ml NBT and 3.5 µl/ml BCIP (Roche) in a detection buffer (pH 9.5) to produce a blue color reaction in antisensetreated slides, and no color in slides treated with sense probe. The color reaction was stopped with 1 M Tris and 0.5 M EDTA (pH 8.0). Tissue was then dehydrated and coverslipped with VectaMount (Vector Laboratories Inc., Burlingame, Calif., USA).

Stereological and Statistical Analysis

Slides were analyzed by an individual blinded to each animal's group under brightfield illumination using the optical fractionator function from Stereo Investigator (MicroBrightfield Inc., Williston, Vt., USA), as in Cohen and Wade (2011, 2012). The number of AR-positive cells, volume and density for each brain region were estimated for the POA, AMY and VMH. Labeled cells were defined by distinct dark blue cytoplasmic labeling, and brain regions were identified by comparison to a green anole forebrain atlas (Greenberg, 1982). The counting frame and grid size for each region, respectively, were as follows: POA: 50×50 and 100×100 μm^2, AMY: 30×30 and 60×60 μm^2, VMH: 30×30 and 80×80 μm^2. Sampling sites were placed randomly throughout each defined region by the Stereo Investigator software. Estimates of cell number and region volume were determined and a Gundersen coefficient of 0.1 or less was confirmed to ensure accurate estimates of cell counts.

The average values from the two sides of the brain were calculated for each region within each individual. In gonadally intact animals, the effect of sex and season were assessed by two-way ANOVAs within each brain region. Sex, season and hormone

manipulation were assessed by three-way ANOVA in treated animals, also within the POA, AMY and VMH. Interactions were broken down using two-tailed t tests, with a Bonferroni correction for multiple comparisons. All statistical analyses were completed using SPSS Statistics version 20 (IBM, Armonk, N.Y., USA).

Western Blot Analyses

Tissue from each animal was thawed and individually extracted in RIPA Lysis Buffer (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) according to the manufacturer's instructions. Total protein concentration was determined using a Bio-Rad Protein Assay (Bio-Rad, Hercules, Calif., USA) with absorbance at 595 nm using a GeneQuant Pro Spectrophotometer (Amersham, Arlington Heights, Ill., USA). Western blot procedures were performed as in Kerver and Wade (2013), using the same primary antibody that was validated in this previous report.

Protein was denatured at 100°C, and then 30 μg of total protein combined with 10 μl of loading buffer was loaded into each well and run on a 4–20% precast polyacrylamide gel (Bio-Rad). Samples from both gonadally intact and hormone-manipulated animals were run on the same blots to maintain consistency, although they were analyzed separately due to differences in exposure to surgery. Kaleidoscope prestained standards (Bio-Rad; 10 μl) were used to visualize approximate molecular weights. The proteins were electrotransferred to a polyvinylidene fluoride membrane. Membranes were washed, blocked and then incubated in primary antibody (AR C-19, sc-815, 1 μg/5 ml; Santa Cruz Biotechnology) overnight at 4°C. The relatively recent sequencing of the green anole genome (Alfoldi et al., 2011) allows comparisons of the amino acid sequence used to

generate this antibody, and it is 94% identical to the same region of the green anole AR. This is by far the best match to the anole AR of the commercial antibodies available. Membranes were then washed and incubated in secondary antibody (AP-conjugated goat-anti-rabbit, T2191 0.033 µl/ml; Applied Biosystems, Foster City, Calif., USA) at room temperature for 1 h. They were treated with Amersham ECL Prime Western Blotting Detection Reagent (RPN2232; GE Healthcare, Pittsburgh, Pa., USA) and exposed to HyBlot CL autoradiography film (Denville Scientific Inc., Metuchen, N.J., USA). Following film exposure, the membranes were stripped for 15 min in Restore Plus Western Blot Stripping Buffer (Thermo Scientific, Waltham, Mass., USA), washed, blocked and then reprobed for β-actin as a loading control, using the same specifications as in Kerver and Wade (2013).

Scion (NIH) Image was used to calculate the mean optical density of the bands produced on the film for each sample for both AR and β-actin. The ratio of AR to β-actin was used in statistical analyses. In intact animals, optical density ratios of each of the three proteins were compared between males and females from the BS and NBS animals by two-way ANOVA. In treated animals, the effect of sex, season and TP were assessed by three-way ANOVA. All analyses were completed using SPSS Statistics version 20 (IBM).

Results

The outcome of each study is described below. However, due to large number of results, all statistical analyses are presented in tables 6-8.

AR in Brains from Gonadally Intact Animals

In Situ Hybridization

In gonadally intact animals, no significant main effects of sex, season or interaction between these variables was detected in the POA, AMY or VMH (table 6). Variables quantified included the number of AR-expressing cells, the volume of the brain regions defined by AR mRNA labeling, and the density of AR+ cells.

Table 6. Statistical values for analyses based on labeling of cells expressing AR mRNA in intact animals. Significant main effects of sex and season were not detected, and the variables did not interact.

Region	Measure	Sex	Season	Interaction
POA	Cell number	$F(3,28)=0.11$, $p=0.743$	$F(3,28)=0.13$, $p=0.726$	$F(3,28)=0.89$, $p=0.353$
	Volume	$F(3,28)=2.21$, $p=0.148$	$F(3,28)=1.08$, $p=0.308$	$F(3,28)=0.13$, $p=0.722$
	Density	$F(3,28)=0.23$, $p=0.634$	$F(3,28)=0.17$, $p=0.686$	$F(3,28)=1.63$, $p=0.212$
AMY	Cell number	$F(3,27)=0.09$, $p=0.765$	$F(3,27)=0.05$, $p=0.823$	$F(3,27)=0.84$, $p=0.367$
	Volume	$F(3,27)=0.01$, $p=0.918$	$F(3,27)=0.00$, $p=0.951$	$F(3,27)=0.61$, $p=0.443$
	Density	$F(3,27)=0.41$, $p=0.527$	$F(3,27)=0.06$, $p=0.805$	$F(3,27)=0.00$, $p=0.978$
VMH	Cell number	$F(3,25)=2.04$, $p=0.166$	$F(3,25)=0.08$, $p=0.782$	$F(3,25)=0.07$, $p=0.799$
	Volume	$F(3,25)=3.43$, $p=0.076$	$F(3,25)=0.08$, $p=0.783$	$F(3,25)=0.11$, $p=0.745$
	Density	$F(3,25)=0.17$, $p=0.683$	$F(3,25)=0.06$, $p=0.812$	$F(3,25)=0.07$, $p=0.797$

Western Blots

One distinct band at 40 kDa was present in the POA-hypothalamic dissections. It matches one of the same molecular weight detected in copulatory tissues from the green anole (Kerver and Wade, 2013). No main effects of sex and season were detected, nor was there an interaction between the variables in relative levels of AR protein in the POA-hypothalamus dissections (table 7).

Table 7. Statistical values for Western blot analyses on protein extracted from POA-hypothalamus dissections of intact and hormone-manipulated animals.

	Sex	Season	Treatment	Interaction
Intact	$F(3,20)=0.94$, $p=0.343$	$F(3,20)=0.27$, $p=0.610$	N/A	$F(3,20)=0.12$, $p=0.732$
Treated	$F(7,46)=0.96$, $p=0.333$	$F(7,46)=2.23$, $p=0.142$	$F(7,46)=0.74$, $p=0.393$	**$F(7,46)=14.10$, $p<0.001$***

Table 8. Statistical values for analyses based on labeling of cells expressing AR mRNA in castrated animals with T or control implants. Significant effects of sex were detected in the AMY (indicated in bold), and interactions between variables were detected in the VMH (also bold).

Region	Measure	Sex	Season	Treatment	Interactions
Treated POA	Cell number	$F(7,51)=0.08$, $p=0.781$	$F(7,51)=0.00$, $p=0.963$	$F(7,51)=0.07$, $p=0.791$	All $F(7,51)\leq 1.98$, $p\geq 0.165$
	Volume	$F(7,51)=0.54$, $p=0.467$	$F(7,51)=0.00$, $p=0.988$	$F(7,51)=0.04$, $p=0.838$	All $F(7,51)\leq 1.59$, $p\geq 0.213$
	Density	$F(7,51)=0.89$, $p=0.351$	$F(7,51)=0.01$, $p=0.924$	$F(7,51)=0.02$, $p=0.904$	All $F(7,51)\leq 2.13$, $p\geq 0.151$
Treated AMY	Cell number	**$F(7,49)=7.12$, $p=0.010$**	$F(7,49)=0.09$, $p=0.762$	$F(7,49)=1.53$, $p=0.222$	All $F(7,49)\leq 0.43$, $p\geq 0.515$
	Volume	$F(7,49)=0.26$, $p=0.611$	$F(7,49)=0.02$, $p=0.901$	$F(7,49)=0.84$, $p=0.364$	All $F(7,49)\leq 1.43$, $p\geq 0.237$
	Density	**$F(7,49)=16.96$, $p<0.001$**	$F(7,49)=0.09$, $p=0.772$	$F(7,49)=0.36$, $p=0.551$	All $F(7,49)\leq 0.79$, $p\geq 0.377$
Treated VMH	Cell number	$F(7,54)=0.07$, $p=0.798$	$F(7,54)=0.03$, $p=0.865$	$F(7,54)=0.74$, $p=0.395$	**$F(7,54)=4.19$, $p=0.046$***
	Volume	$F(7,54)=1.25$, $p=0.269$	$F(7,54)=0.78$, $p=0.383$	$F(7,54)=0.87$, $p=0.356$	**$F(7,54)=4.91$, $p=0.031$***
	Density	$F(7,54)=2.75$, $p=0.103$	$F(7,54)=1.38$, $p=0.245$	$F(7,54)=0.09$, $p=0.767$	All $F(7,54)\leq 3.13$, $p\geq 0.082$

*Significant season x treatment interaction; none of the other possible interactions for either of these analyses were statistically significant (all $F(7,54)<3.75$, $p>0.058$). For VMH cell number, none of the pairwise comparisons were statistically significant (all $t(29) \leq 1.96$, $p \geq 0.060$; Bonferroni correction for multiple comparisons, $\alpha = 0.0125$). See Results section for pairwise comparisons on VMH volume.

AR in Brains from Hormone-Manipulated Animals

In Situ Hybridization

In the POA, no significant effects of sex, season, treatment or interactions among any of the variables were detected (Table 8). However, the AMY females had more total AR+ cells and a higher density of cells expressing AR mRNA than males (Table 8; Figure 8). No other significant main effects or interactions of sex, season or treatment in the number or density of AR+ cells or the volume defined by AR labeling were detected for the AMY (Table 8). In the VMH, a season × treatment interaction was detected in the total number of AR+ cells (Table 8; Figure 9). However, none of the pairwise comparisons were statistically significant. VMH volume defined by AR labeling also exhibited a significant season × treatment interaction with the same pattern (Table 8; Figure 9). Pairwise comparisons revealed a trend such that TP-treated animals in the BS had larger VMH volumes than TP-treated animals in the NBS ($t(30) = 2.35$, $p = 0.026$, Bonferroni $\alpha = 0.0125$). Also, within the

NBS, a trend existed for the volume to be greater in blank- compared to TP-treated animals ($t(29) = 2.11$, $p = 0.043$, Bonferroni $\alpha = 0.0125$). No other effects of sex, season, treatment or interactions among the variables were detected for total AR+ cell number in or volume of the VMH, and there were also no significant effects in the density of AR+ cells in this brain region.

Figure 8. AR mRNA detected with in situ hybridization in the AMY of hormone-manipulated animals. AR+ cells in this brain region were increased in females (A) compared to males (B). Both images are from blank-treated animals in the NBS, and a lack of labeling was apparent (C) when adjacent tissue sections were exposed to a sense, rather than antisense, probe. The main effects of sex (indicated with asterisks) were detected in both the total number (D) and density (E) of cells expressing AR. No other significant effects were detected in this brain region associated with sex, season or T treatment.

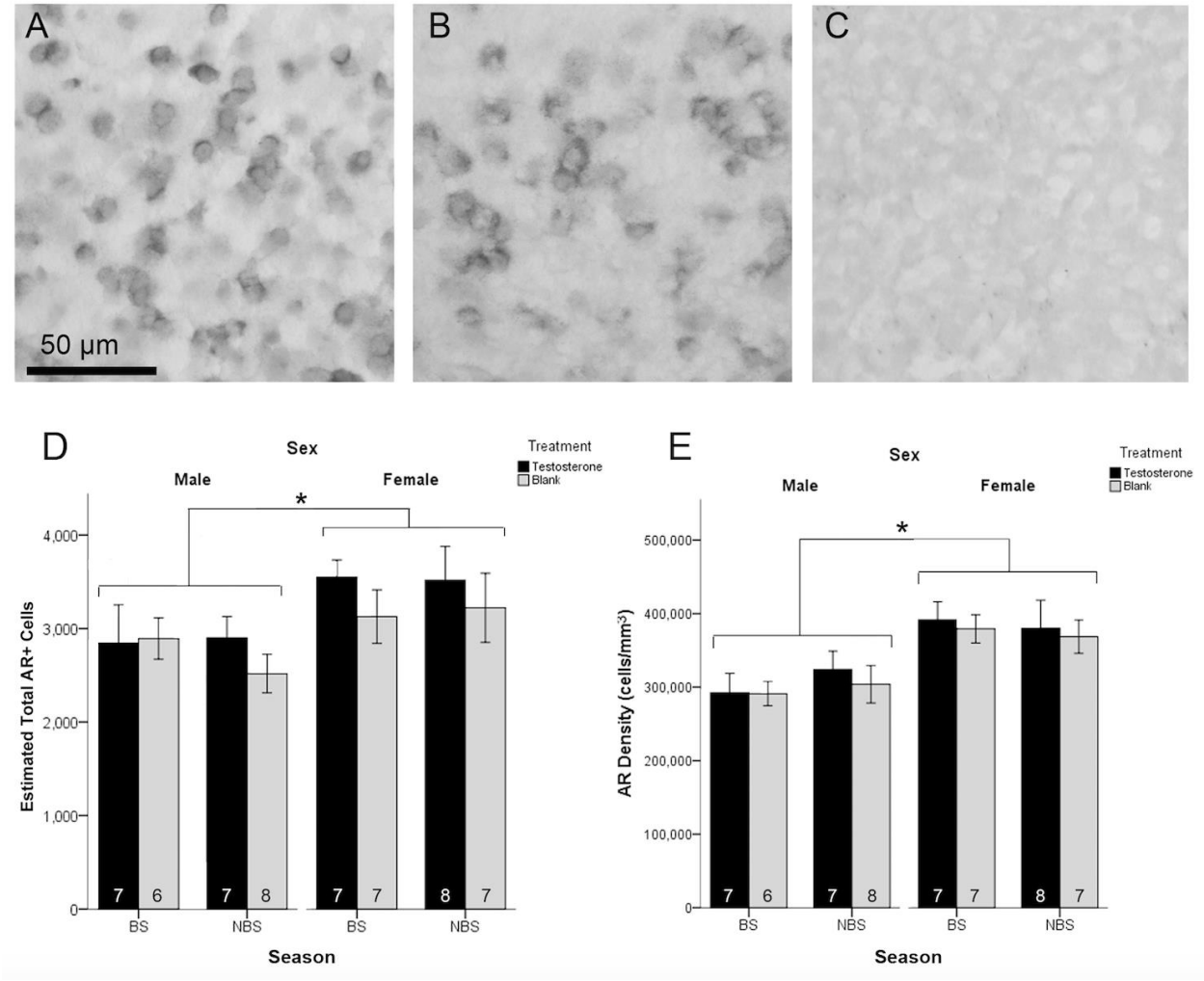
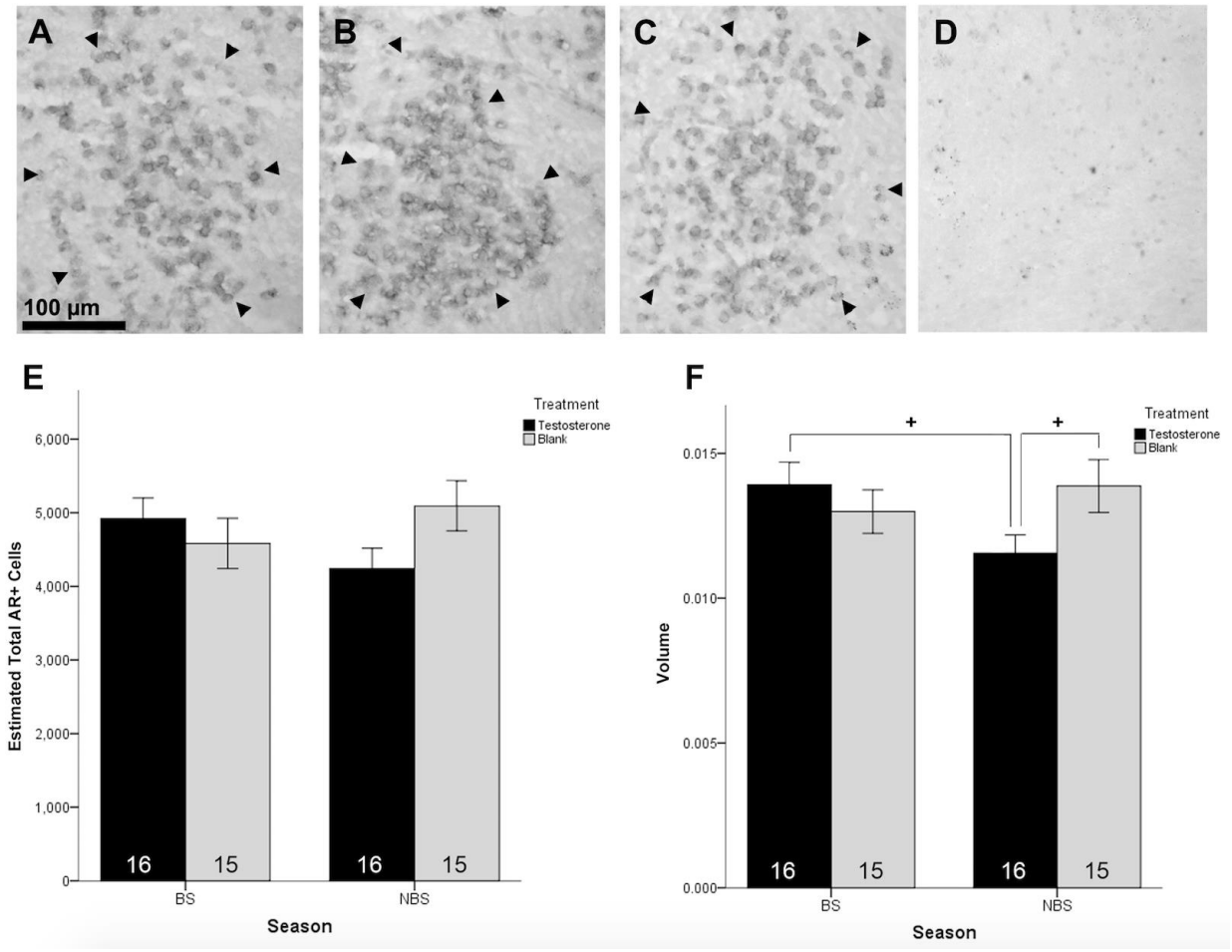


Figure 9. AR mRNA expression in the VMH. Photographs depict representative in situ hybridization labeling in T-treated animals from the BS (A) and NBS (B). The VMH in blank-treated NBS animal (C) and the lack of labeling in this region in tissue exposed to the sense, rather than antisense, probe (D). Arrowheads indicate the borders of the brain regions that were traced to obtain estimates of volume and cell number. While main effects of sex, season and hormone manipulation were not detected, significant season x treatment interactions existed in the number of AR+ cells (E) and the volume of the VMH defined by this labeling (F). Graphs are collapsed across sex because no significant effects involving sex were seen. Pairwise comparisons revealed no significant differences among the groups in the number of AR+ cells. Plus signs indicate trends for significant pairwise effects on VMH volume.

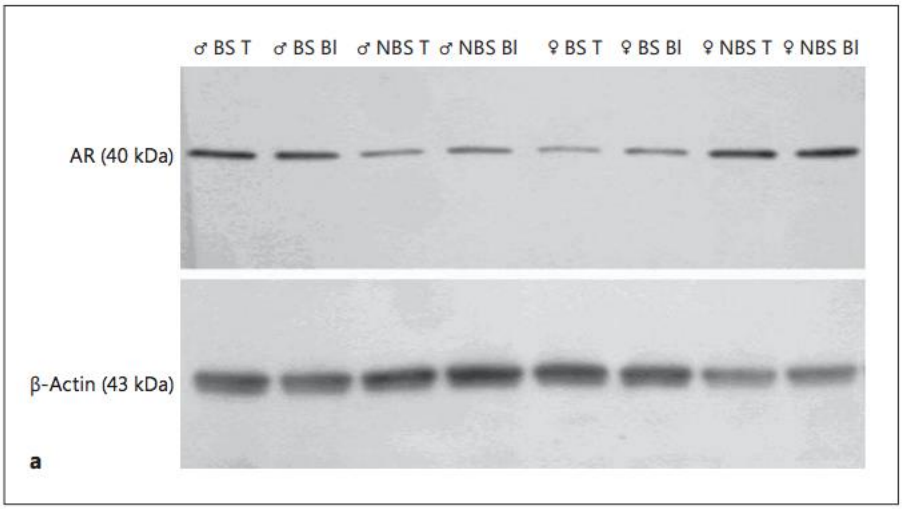


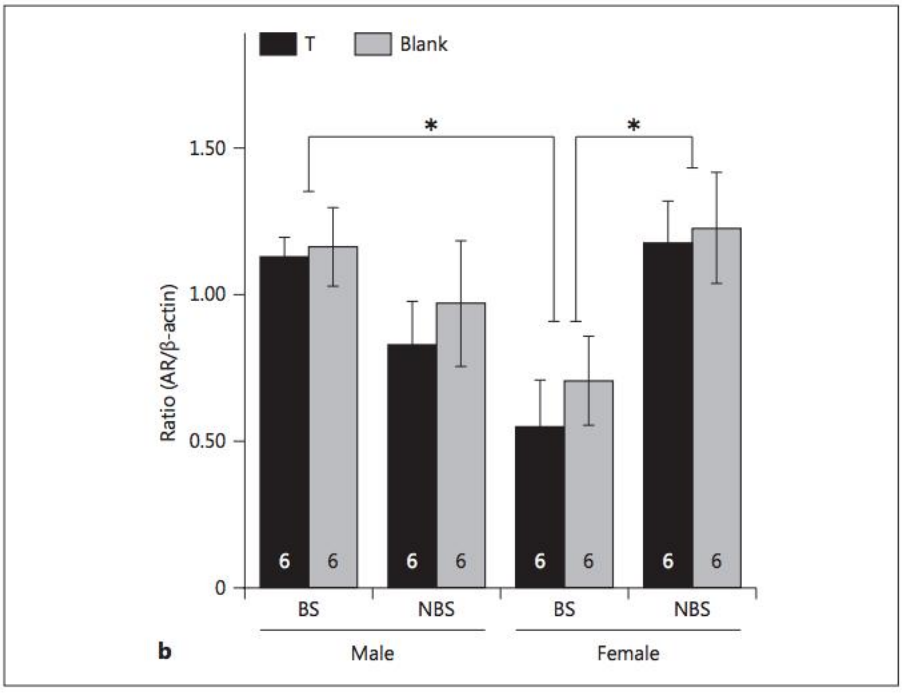
Western Blots

Main effects of sex, season and treatment were not detected for relative AR protein levels in the POA-hypothalamus (see Table 7). However, a significant interaction between sex and season existed (Figure 10). Pairwise comparisons, collapsed across treatment, revealed that within the BS males had higher relative levels of AR protein than females

(t(25) = 4.12, p < 0.001, Bonferroni α = 0.0125). There was no sex difference in the NBS (t(25) = 1.79, p = 0.86) and no difference in AR expression across seasons within males (t(25) = 1.70, p = 0.100). Among females, significantly more AR was expressed in the NBS than BS (t(25) = 3.74, p = 0.001, Bonferroni α = 0.0125).

Figure 10. Relative AR protein levels in preoptic-hypothalamic dissections of hormone manipulated animals. a) Western blot probed for AR and subsequently β-actin as a loading control. Bl = Blank. b) Significant sex × season interaction in relative AR protein levels, corrected for β-actin. Pairwise comparisons revealed that in the BS, males had significantly more AR protein than females. Also, among females, more AR protein was detected in the NBS than BS. * p ≤ 0.001.





Discussion

AR mRNA and Protein in the Brains of Gonadally Intact Animals

The distribution of AR mRNA in the green anole brain detected in the present study was very similar to what has been found previously, with high expression in areas including the POA, AMY and VMH (Rosen et al., 2002). This earlier study from our lab, which employed radioactive in situ hybridization on intact animals from the BS, found that the intensity of AR mRNA labeling (% area covered by silver grains) was greater in females than males across four limbic regions. This effect was largely due to the posterior POA and the AMY, although the areas were not analyzed individually (Rosen et al., 2002). The percentage of cells expressing AR mRNA did not differ between the sexes in that study. The present study found no effects of sex or season in the total number or density of cells expressing AR mRNA within the POA, AMY or VMH, or the volume of these brain regions defined by this marker. Similarly, relative AR protein in dissections of the POA and hypothalamus was equivalent in gonadally intact animals across the two sexes and seasons.

Taken together, the data suggest that females may have more AR mRNA per cell than males, but, overall, levels of AR are generally equivalent in the BS and NBS, as well as between males and females. Thus, the data from unmanipulated animals in the present study do not support the idea that seasonal differences in responsiveness to T in green anoles are due to increased AR mRNA or protein in brain areas associated with male or female sexual behavior. While it remains possible that protein levels did differ within specific portions of the POA and/or hypothalamus, the small size of these tissues would present a challenge for quantifying AR concentrations. Qualitative analysis of

immunohistochemistry from BS males and females (Rosen et al., 2002) does not provide support for sex differences.

AR mRNA Expression in Hormone-Manipulated Animals

Significant effects were detected only in hormone-manipulated lizards in the present study, which may be the result of larger sample sizes compared to the data collected from intact animals. It is also possible that removal of the gonads allowed particular effects to be revealed, perhaps by eliminating one or more endocrine factors released by these organs, or due to loss of neural connections to them. Previous studies from our lab have found a similar phenomenon of effects only becoming apparent following gonadectomy, in particular sex and seasonal differences in the volume and cell number across limbic forebrain regions (Beck et al., 2008, Beck and Wade, 2009a, b).

We detected the main effects of sex in the AMY, with females having a greater number and density of cells expressing AR mRNA than males; this pattern mirrors previous sex differences of AR in the anole brain (Rosen et al., 2002). In other reptilian and mammalian species, the sex difference is often in the opposite direction, with males having more AR mRNA and/or protein in the POA and hypothalamic areas (Lu et al., 1998, Wood and Newman, 1999, Fernandez-Guasti et al., 2000, Godwin et al., 2000, Moga et al., 2000). Although AR transcripts can be either up- or downregulated in response to androgen administration across vertebrate species (Krongrad et al., 1991, Gonzalez-Cadavid et al., 1993, Drengler et al., 1996, Godwin et al., 2000), increased AR in female anoles is not likely due to circulating androgens. Gonadally intact female anoles have only a fraction of the circulating T levels of males, regardless of the season (Lovern et al., 2001), and the sex

difference was not present in the intact animals in this study. In the animals that received hormone manipulations, the sex difference in AR expression in the AMY was apparent regardless of TP versus control treatment. It is possible that females respond differently than males to the same amount of TP. For example, increased estradiol in intact females compared to males might suppress AR expression in females. If so, removal of the gonads would eliminate the majority of endogenous estrogen and might release this increased inhibition. Consistent with this idea, estrogen reduces cytoplasmic AR in human breast cancer cells (Stover et al., 1987).

A season × treatment interaction existed for the estimated total number of AR-expressing cells in the VMH. However, a lack of significant effects detected among any of the pairs of groups suggests that this result is due to the generally opposing patterns across seasons (TP increasing AR+ cells in the BS, while decreasing them in the NBS) rather than substantial differences in the mean values of the four groups. The same set of relationships was detected in the overall volume of the VMH defined by AR labeling, with stronger trends for mean group differences. Caution should be exhibited in interpreting these data, both because the pairwise statistical results were not significant and because the difference in circulating T was greater between gonadectomized and hormone-implanted animals than is typical in unmanipulated animals across seasons. However, the trends suggest that T may have opposing effects on the VMH across seasons, appearing to increase VMH volume in the BS and decrease it in the NBS.

Previously, T was found to increase soma size in the POA and AMY, and in the AMY to a greater extent in the BS than NBS (O'Bryant and Wade, 2002, Neal and Wade, 2007). Therefore, it is possible that the changes seen in VMH volume across season may be due to

changes in soma size or AR expression in existing cells. These seasonal effects on VMH volume parallel findings in other mammals and reptiles (Crews et al., 1993, Holmes et al., 2007). In whiptail lizards, the male VMH reduces to a female-like size during the NBS, and T regulates this change in morphology (Wade and Crews, 1991, Wade et al., 1993).

No significant main effects or interactions existed in the POA of treated animals. Across limbic regions including the POA, the relative intensity of AR mRNA labeling was greater in females compared to males in gonadally intact animals from the BS (Rosen et al., 2002). Collectively, the data are consistent with the idea that more AR mRNA is expressed within each cell in the female POA. While the volume of this brain region has been found to differ across both sex and season in anoles using Nissl staining (O'Bryant and Wade, 2002, Neal and Wade, 2007, Beck et al., 2008), our study demonstrates that AR mRNA expression and volume of the POA defined by AR labeling remain stable across sex, season and hormonal exposure. Thus, a factor other than AR in this brain area contributes to these morphological changes. The lack of effects in AR mRNA expression in the POA in the present study is not consistent with a seasonal effect on responsiveness to T in this region

AR Protein Expression in Hormone-Manipulated Animals

The present study indicated that within the BS, males had a greater concentration of AR protein than females in POA-hypothalamic dissections. This is consistent with findings of male-biased sex differences in POA and hypothalamic AR from other species (Lu et al., 1998, Wood and Newman, 1999, Moga et al., 2000). Photoperiod and androgen treatment together have also been shown to increase AR in the hamster POA (Tetel et al., 2004). AR protein (cell density and number) is also more abundant in the song nuclei of male white-

crowned sparrows during breeding compared to nonbreeding conditions (Soma et al., 1999). Our findings are consistent with the idea that T signaling through AR protein may facilitate male sexual behavior in the green anole during the BS.

In contrast, the increase in AR protein in the POA-hypothalamus of females during the NBS specifically is not consistent with a purpose related to facilitating sexual behavior. AR levels did not differ between TP and blank-treated groups, so differences in protein are not androgen mediated. However, because this effect in females was detected only in hormone-manipulated and not intact animals, it is possible that gonadectomy revealed the seasonal change, potentially by reducing exposure to non-androgenic gonadal hormones. This type of increase in a steroid-related effect at a time when animals are not reproductively active is reminiscent of increased sensitivity to the negative feedback effects of T in castrated hamsters exposed to short days (Turek, 1977).

There may also be a unique feature of NBS environmental conditions that increases AR in females, such as their tendency to spend more time in social groups (Lovern et al., 2004b). An enhancement of the effect of T by upregulating AR in NBS females could increase social investigation, as T does in female mice (Tejada and Rissman, 2012). It is also conceivable that the photoperiod could affect AR levels, even independent of hormone condition, as it does in Siberian hamsters (Bittman et al., 2003, Tetel et al., 2004).

The presence of a single band of AR at 40 kDa in the Western blots from our brain samples is interesting given our previous finding of multiple AR bands in analyses of copulatory tissues at 40, 60 and 100 kDa (Kerver and Wade, 2013). The single form detected in brain may indicate that peripheral AR is regulated differently compared to central AR. There are six transcripts of human AR reported in Ensembl

(ENSG00000169083), and the 40-kDa fragment we detected in the anole brain is similar in size to a 45-kDa AR human variant found mainly in heart and skeletal muscle (Ahrens-Fath et al., 2005). Its expression remained stable across seasons in the male brain in the present study, parallel to data from hemipenis tissue (Kerver and Wade, 2013). However, the 40-kDa AR in a muscle that controls the hemipenis had a similar pattern of expression as in the female brain, increasing in the NBS compared to BS (Kerver and Wade, 2013). AR may play a different role in muscle than in brain or reproductive structures. The 100-kDa AR band found previously in hemipenis tissue is increased in the BS compared to NBS, and could be involved in facilitating a seasonal responsiveness to T in the periphery. It is possible that some forms of AR are more responsive to circulating T than others. The variable expression of multiple and tissue-specific forms of AR in this species is intriguing and should be followed up.

General Conclusions and Future Directions

We identified a set of sex and seasonal differences in AR expression among lizards that received hormone manipulations. These results provide potential mechanisms for some differences in androgen-mediated functions. However, the data are generally inconsistent with the hypothesis that alterations in AR are responsible for seasonal differences detected in responsiveness to T related to masculine brain morphology and function. The lack of seasonal differences found in AR expression in regions involved with male-typical behaviors (POA and AMY) in both gonadally intact and hormone-manipulated anoles suggests that a mechanism other than an upregulation of AR in the BS is facilitating differences in T activity in these specific regions. The sex differences in the number and

density of cells expressing AR mRNA in the AMY were female biased, which is consistent with previous findings in anoles (Rosen et al., 2002), and unrelated to T levels. The other effects detected were in the VMH, a region primarily associated with female receptivity. Statistical interactions there indicate that T may differentially affect the number of cells expressing AR mRNA and the brain region volume defined by them, depending on the season.

The sex × season interaction in AR protein from the POA-hypothalamus supported the role of AR in facilitating male sexual behavior in the BS. However, among females, protein in POA-hypothalamic dissections was increased in the NBS compared to BS. While it appears that peripheral changes in AR expression in male copulatory tissues may facilitate T's actions across seasons (see Kerver and Wade, 2013), this is not the case for central regulation of reproductive behavior in the areas investigated.

It should be noted that the differences in mRNA and protein expression might be due to the method of quantification. Individual mRNA analyses occurred in the POA, AMY and VMH, while protein expression was more broadly examined in microdissected portions of the brain containing the POA, VMH and surrounding hypothalamic areas, but not containing the AMY. Alternatively, differences in the expression of mRNA and protein are common for AR (Krongrad et al., 1991, Kerver and Wade, 2013), and may suggest a posttranslational mechanism, such as stabilization or degradation of the protein, that may differ in this instance.

Successful reproduction is carried out through a suite of behaviors controlled by both central and peripheral targets (Bai and Deng, 2006, Hull and Dominguez, 2007), and mechanisms other than differential AR distribution may be involved in the brain. For

example, steroid receptor coactivators, such as SRC-1 and CREB-binding protein, may also facilitate a seasonal responsiveness to T at the molecular level. A productive AR transcriptional complex requires coactivators, which are the rate-limiting factors for transcription and can act synergistically to coactivate nuclear receptors. Once recruited by a ligand-activated AR complex, SRC-1 and CBP are able to dramatically increase the transcriptional activity of AR once it binds to an androgen response element on the DNA (Smith et al., 1996a, McKenna et al., 1999, Heinlein and Chang, 2002, Rosenfeld et al., 2006, Heemers and Tindall, 2007). This possibility is currently being investigated in the anole brain.

CHAPTER 3

HORMONAL REGULATION OF STEROID RECEPTOR COACTIVATOR-1 mRNA IN THE MALE AND FEMALE GREEN ANOLE BRAIN

Introduction

Steroid hormones and their receptors are necessary for courtship and copulation across vertebrates (Ball and Balthazart, 2002, Hull and Dominguez, 2007, Steel, 1981). Testosterone (T) is particularly important for facilitating seasonal breeding in males of various mammalian, avian, and reptilian species (Baum, 2002). Species that breed seasonally provide the opportunity to investigate how hormones govern annual shifts in sexual behaviour, and how responses to the hormones are regulated at the molecular level. One such species is the green anole lizard, which in addition to changes in circulating T, displays a seasonal difference in responsiveness to the hormone that may serve to limit morphological, biochemical, and behavioural effects during times of reproductive inactivity (see below). The genome of the green anole was recently sequenced (Afoldi et al., 2011), which provides access to a new set of tools for examination of this phenomenon at the molecular level.

The breeding season (BS) of the green anole lasts from approximately April through July. The gonads of both sexes regress as they enter the non-breeding season (NBS), and reproductive behaviours are no longer displayed (Wade, 2011). It is T rather than its metabolites, dihydrotestosterone and oestradiol, that is the primary activator of male sexual behaviour in the green anole (Wade, 2011, Rosen and Wade, 2000, Winkler and Wade, 1998).

Although T activates an array of reproductive functions in the BS, the same dosage of T has reduced effects in the NBS on courtship, copulation, copulatory muscle fiber size, soma size in brain regions responsible for male sexual behaviour, and also brain aromatase activity (Cohen and Wade, 2010, Holmes and Wade, 2004, 2005, Lovern et al., 2004, Neal and Wade, 2007, O'Bryant and Wade, 1999, 2002). We have investigated whether expression of androgen receptor (AR) across seasons is related to this change in responsiveness to T. While it appears that AR levels in the copulatory organs (hemipenises) may facilitate an increase in T activity during the BS (see Kerver and Wade, 2013), the pattern of AR mRNA expression in the brain was not consistent with this role, with no overall differences between breeding and non-breeding animals of either sex (Kerver and Wade, 2014). Males did have more AR protein than females in the dissections of the preoptic area and hypothalamus in the BS specifically, but other mechanisms are likely responsible for facilitating T's seasonal actions in the brain (Kerver and Wade, 2014).

Coregulatory proteins can interact with steroid receptors to modulate their transcriptional activity. Ligand-bound AR dimerizes and is translocated to the nucleus, where the receptor complex can recruit coactivators. These molecules enhance the rate of gene transcription, as opposed to corepressors, which serve to decrease transcription (Henlein and Chang, 2002). Coregulatory proteins can dramatically change AR's transcriptional activity through molecular mechanisms such as acetylation, methylation, phosphorylation, or chromatin remodeling (Rosenfeld et al, 2006), and they are the rate-limiting factor for increasing gene transcription of steroid hormone receptors (McKenna et al, 1999). The first known coactivator, steroid receptor coactivator-1 (SRC-1), was

discovered less than twenty years ago, and since then over 350 coregulator proteins have been identified (York and O'Malley, 2010).

SRC-1 aides in assembling transcription initiation complexes by bridging DNA-bound AR's (as well as other nuclear receptors) and basal transcriptional machinery. It acts directly with the receptor complex and serves as a scaffolding to recruit secondary coactivators as well as histone methyltransferases and acetyltransferases. Thus, SRC-1 can act synergistically with other coactivators to increase the rate of transcription of AR and other nuclear receptors (Heinlein and Chang, 2002, Smith et al., 1996). SRC-1 is necessary for the androgen-mediated development of masculine behaviours in both rodent and bird species (Charlier et al., 2005, Auger et al., 2000). For example, knockdown of SRC-1 with antisense oligonucleotides reduces androgen (and oestrogen) - dependent male sexual behaviours in the Japanese quail (Charlier et al., 2005). Similarly, reducing SRC-1 protein in the neonatal rat brain prevents defeminising actions of T (Auger et al., 2004).

In both rodents and birds, neural SRC-1 is differentially expressed across the two sexes, hormonal conditions, and photoperiods (Charlier et al., 2006, Tetel et al., 2004, Bian et al., 2011). Even time of day affects SRC-1 protein expression within the hippocampus, hindbrain and optic lobes, although the pattern of SRC-1 fluctuation varies across the areas (Charlier et al., 2006). Thus, SRC-1 is sensitive to both endogenous and exogenous cues, which can exhibit tissue and even regional specificity within the brain. It is therefore plausible that SRC-1 may be expressed in the anole brain in a manner that facilitates a seasonal responsiveness to T. We hypothesized that males express more SRC-1 in the brain than females, and that increased SRC-1 expression in breeding males may facilitate an increased neural responsiveness to T during the BS.

Three brain regions involved in the control of sexual behaviour were investigated in the green anole: the preoptic area (POA), amygdala (AMY), and ventromedial hypothalamus (VMH). The POA and AMY are critical for male typical sexual behaviour across vertebrates (Ball and Balthazart, 2004, Thompson et al., 1998, Wood and Newman, 1995), and the VMH is essential for female sexual behaviour, receptivity in particular (Flanagan-Cato, 2011, Meisel et al., 1987). Lesions to these areas cause a deficit in sexual behaviour in anoles and other species (Wade 2011, Kendrick et al., 1995, Wheeler and Crews, 1978). The distribution of SRC-1 has not yet been investigated in the reptilian brain, but is widely expressed in the mammalian and avian brain, particularly in the brain areas mentioned above (Charlier et al., 2002, Meijer et al., 2000, Misiti et al., 1998).

The present study utilised in situ hybridisation to investigate relative levels of SRC-1 mRNA in gonadally intact males and females from the BS and NBS with a focus on areas of the brain important for reproduction. Additionally, to assess potential effects of T on SRC-1 mRNA, in situ hybridisation was performed on males and females from both seasons that were gonadectomised and treated with either a capsule containing T-proprionate (TP) or a blank capsule (control).

Methods

Animals

Male and female green anole lizards were caught in the wild during both the BS and NBS and shipped to us by Charles Sullivan Co. (Nashville, TN). BS animals were collected in April, NBS animals in October. Animals were housed one male and one female to each 10-gallon aquarium, containing peat moss substrate, a rock and stick for basking, and a water

dish. Black dividers were placed in between cages to prevent visual contact. During breeding conditions animals were maintained on a 14:10 light:dark cycle, and a 10:14 cycle during the NBS. Each individual cage was exposed to fluorescent lighting, full spectrum bulbs, and a heat lamp. Temperatures ranged from 28 to 38°C in the daytime during the BS, depending on distance from the heat lamp, and 18°C at night when the lights were off. During NBS conditions, temperatures ranged from 24-30°C during the day and 15°C at night. Humidity was kept at 60-70% in both seasons. All procedures were approved by the Michigan State University Institutional Animal Care and Use Committee.

Treatment and Tissue Collection

Animals were given two weeks to acclimate upon arrival in the lab. Gonadally intact males and females from the BS and NBS (n=9 per group) were rapidly decapitated, brains were collected, and breeding state was confirmed through visual inspection of the gonads. Breeding females had at least one large yolking follicle or an egg in the oviduct. Breeding males had large, vascularised testes. The gonads in the NBS were regressed in all animals.

Another set of animals with males and females from the BS and NBS (n=8 per group) were gonadectomised and given either a TP-filled or empty (control) capsule. Prior to bilateral gonadectomy, animals were injected with 10µl of 0.2% lidocaine before being anaesthetized with isofluorane. Animals remained on ice during this procedure, and were subcutaneously implanted with a Silastic capsule (7 mm long × 0.7 mm inner diameter × 1.65 mm outer diameter) containing either 5 mm of packed testosterone propionate (TP) or left blank (Bl). This dose has been used in previous studies and reliably activates male sexual behaviour and associated biochemical and morphological changes in the brain

(Cohen and Wade, 2010, Neal and Wade, 2007). These capsules produce detectable plasma concentrations that average 48.7 ± 4.8 (SEM) ng/ml of androgen, with no difference across seasons (Cohen and Wade, 2012). Intact males collected in the wild average approximately 20ng/ml in the BS and 11ng/ml in the NBS, but the concentrations produced by our TP capsules overlap with those of breeding individuals (Lovern et al., 2001). One week following gonadectomy, animals were rapidly decapitated and brains were collected. Confirmation that TP remained in the hormone capsule, along with the complete absence of gonadal tissue, was completed at this time.

Cloning of SRC-1

RNA was extracted from the whole brain of one male from each of the BS and NBS. The tissue was homogenised in Trizol (Invitrogen Corporation; Carlsbad, CA), chloroform was added, and total RNA was extracted from the phase separation. RNA was isolated using RNeasy minicolumns (Qiagen; Valencia, CA) and ethanol precipitated. The RNA was reconstituted in RNase-free water and stored at −80°C. A SuperScript III Reverse Transcription Kit (Invitrogen) was used to convert the RNA to cDNA per manufacturer's instructions, and the cDNA was stored at −20 °C until it was used.

Primers were designed for the green anole SRC-1 gene (GenBank: XM_003225921.2) using the Oligo Analysis Tool program (Eurofins MWG Operon; Huntsville, AL). The primers spanned 815 base pairs within the SRC-1 sequence. The forward primer sequence 5' to 3' was TCGTGTTTGTGTCAGAGAATGTGA and the reverse primer 5' to 3' was TGTAGACAGAGGCATACCTT. The sequence was PCR-amplified and cloned into pGEM-T Easy Vector per manufacturer's instructions (Promega Corp; Madison, WI). The ligated

vector was transformed into One Shot TOP10 Chemically competent E. coli cells (Invitrogen). Transformed cells were grown overnight on LB agar plates containing 100 μg/ml ampicillin and X-Gal. The following day colonies were selected and grown overnight in LB broth with 100 μg/ml ampicillin. DNA was isolated using Wizard Plus Miniprep kits (Promega), and the sequence was confirmed in both directions. DNA was then isolated using a Qiagen Plasmid Maxi Kit and stored at -20°C. The restriction enzyme NotI was used to linearise the plasmid, and the template was stored at -20°C.

In Situ Hybridisation

Antisense (T7) and sense (SP6) probes were transcribed with the Digoxigenin RNA Labeling Kit (Roche Diagnostics; Indianapolis, IN), per manufacturer's instructions. The probes were run through a column made with G50 Sephadex beads and stored at -80°C until use. In situ hybridisation was performed as in (Kerver and Wade, 2014). Slides were thawed, fixed in 4% paraformaldehyde (pH 7.4), and washed three times in PBS. Following a treatment with TEA-HCl with 0.25% acetic anhydride, slides were rinsed in PBS and placed in hybridisation buffer that did not contain the probe at 55°C for one hour. Slides were then placed in hybridisation buffer, containing 250ng/ml of either antisense or sense probe, overnight at 55°C.

The following day, the tissue went through a series of rinses in 2x and 0.2x SSC at 60°C, then MAB with 0.1% Tween-20 (MABT) at room temperature. Slides were incubated in 0.9% hydrogen peroxide in MABT, 5% normal sheep serum in MABT, and finally in 0.5μl/mL anti-digoxigenin-AP Fab fragments (Roche) in MABT for two hours. A color reaction was performed with 4.5 μl/ml NBT and 3.5 μl/ml BCIP (Roche) in detection buffer

(pH 9.5). This produced a blue labeling in antisense treated slides and no labeling in sense treated slides (Figure 11). Tissue was dehydrated and coverslipped with VectaMount (Vector Laboratories Inc., Burlingame, CA).

Stereological Analysis

Sense and antisense slides were examined under brightfield illumination using the optical fractionator function from Stereo Investigator (MicroBrightfield, Inc.; Williston, VT), as in (Kerver and Wade, 2014) by an individual blind to the experimental groups. The software placed a grid area and sampling sites randomly within each defined region (POA: 50 x 50 μm^2 and 100 x 100 μm^2, AMY: 30 x 30 μm^2 and 60 x 60 μm^2, VMH: 30 x 30 μm^2 and 80 x 80 μm^2). A Gundersen coefficient of 0.1 or less was confirmed to ensure accurate estimates of cell counts. The number of SRC-1+ cells and the volume of each brain region defined by this labeling was calculated for the POA, AMY and VMH. The density of SRC-1 expressing cells in each brain region was calculated by dividing the total number of SRC-1+ cells by the volume. Cells that contained SRC-1 mRNA were defined by distinct dark blue cytoplasmic labeling. A green anole forebrain atlas was used to define and trace the brain regions (Greenberg, 1982).

Values for each region within individuals were averaged between the right and left side of the brain. The effect of sex and season were assessed by two-way ANOVA within the POA, AMY and VMH in gonadally intact animals. In hormone-manipulated animals, the effects of sex, season and TP-treatment were assessed using a three-way ANOVA within each brain region. Two-tailed t-tests, with a Bonferroni correction for multiple

comparisons, were used to break down significant interactions. All statistical analyses were completed using SPSS Statistics version 21 (IBM, Armonk, NY).

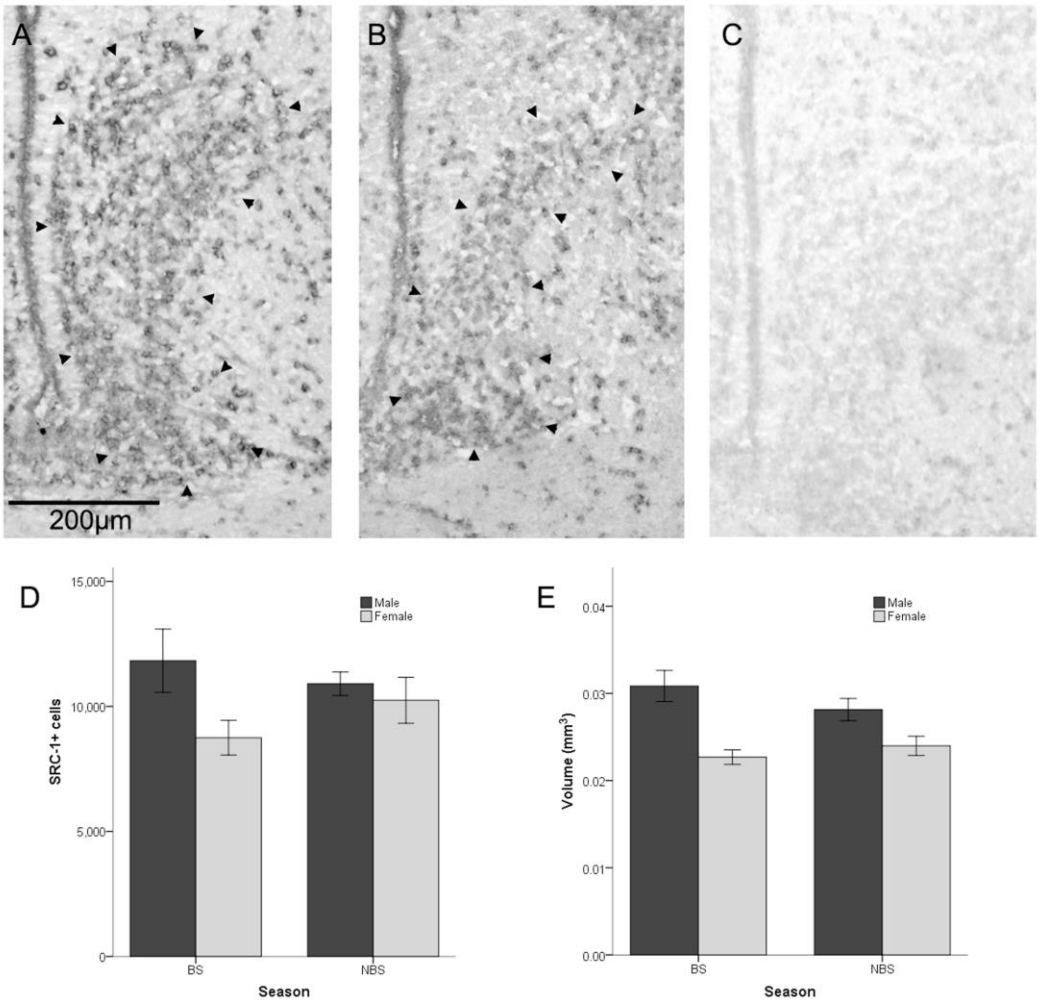
Results

The outcome of each study is described below. However, due to the large number of results, values for the initial analyses (ANOVAs) are reported in Table 9 for gonadally intact animals, and in Table 10 for those that were hormonally manipulated.

Table 9. Statistical values for analyses of SRC-1 mRNA in intact animals. Significant effects are indicated in bold type.

Region	Measure	Sex	Season	Interaction
POA	Cell number	***F*(3,30)=4.60, *p*=0.040**	*F*(3,30)=0.11, *p*=0.744	*F*(3,30)=1.92, *p*=0.176
	Volume	***F*(3,30)=22.75, *p*<0.001**	*F*(3,30)=0.28, *p*=0.590	*F*(3,30)=2.41, *p*=0.131
	Density	*F*(3,30)=0.48, *p*=0.494	*F*(3,30)=1.00, *p*=0.325	*F*(3,30)=0.34, *p*=0.565
AMY	Cell number	*F*(3,27)=1.07, *p*=0.310	*F*(3,27)=0.02, *p*=0.903	*F*(3,27)=2.93, *p*=0.098
	Volume	*F*(3,27)=1.10, *p*=0.304	*F*(3,27)=0.42, *p*=0.522	*F*(3,27)=3.30, *p*=0.080
	Density	*F*(3,27)=0.22, *p*=0.643	*F*(3,27)=0.54, *p*=0.469	*F*(3,27)=0.03, *p*=0.855
VMH	Cell number	***F*(3,27)=5.86, *p*=0.022**	***F*(3,27)=5.08, *p*=0.033**	***F*(3,27)=17.00, *p*<.001**
	Volume	***F*(3,27)=4.42, *p*=0.045**	***F*(3,27)=9.29, *p*=0.005**	***F*(3,27)=6.12, *p*=0.020**
	Density	*F*(3,27)=0.09, *p*=0.767	*F*(3,27)=1.14, *p*=0.295	***F*(3,27)=7.74, *p*=0.010**

Figure 11. SRC-1 mRNA detected with in situ hybridisation in the POA of gonadally intact males and females. Both the number of SRC-1+ cells and volumes were increased in males (A) compared to females (B); both images from breeding season (BS) animals. (C) Showing the lack of labelling in tissue exposed to the sense, control, probe in an adjacent tissue section from the same BS male as in (A). Main effects of sex are depicted in the graphs for both number of SRC-1+ cells (D) and the volume of the POA defined by this labeling (E).



SRC-1 mRNA in Gonadally Intact Animals

In the POA, males had more SRC-1+ cells, and the volume of the brain region defined by these was also larger than in females (Figure 11). No main effect of season existed for

either of these measures, and sex and season did not significantly interact. No significant main effects or interactions were detected on the density of SRC-1 expressing cells in the POA.

No main effects of sex or season were detected for any of the measures in the AMY of intact animals, and the variables did not interact.

In the VMH of gonadally intact animals, significant main effects of both sex and season (males>females and BS>NBS) were detected, as well as an interaction between the two variables for both the number of cells expressing SRC-1 mRNA and VMH volume (Figure 12). Pairwise comparisons revealed that among males, the volume was greater and more SRC-1+ cells were present in the BS than NBS (both $t(13) \geq 4.15$, $p \leq 0.001$; $\alpha = 0.0125$; Figure 12). Within the BS, males had more cells containing SRC-1 mRNA than females in the VMH ($t(13)=4.24$, $p < 0.001$; Figure 12), and a trend existed for a larger VMH volume defined by this labeling in males compared to females ($t(13)=2.85$, $p=0.014$; $\alpha=0.0125$; Figure 12). Within females, these measures did not differ according to the season, and sex differences were not detected in the NBS for either measure (all $t(13) \leq 1.44$, $p \geq 0.171$).

The density of cells expressing SRC-1 mRNA in the VMH was not different across sex or season. There was, however, an interaction between sex and season, although none of the pairwise comparisons reached significance following a Bonferroni correction (all $t(13) \leq 2.60$, $p \geq 0.017$; data not depicted).

Figure 12. SRC-1 mRNA in the VMH from gonadally intact males and females. In situ hybridisation labeling from gonadally intact males from the breeding season (BS) (A) and nonbreeding season (NBS) (B), as well as gonadally intact females from the BS (C) and NBS (D). The graphs indicate significant main effects of sex and season, as well as a significant interaction between the two variables for both the number of SRC-1+ cells (E) and volume of the ventromedial hypothalamus (VMH) (F). Asterisks (*) denote significant pairwise comparisons after Bonferroni correction ($P < 0.0125$). A hash symbol (#) denotes a trend for an increase in males compared to females within the BS ($P = 0.014$).

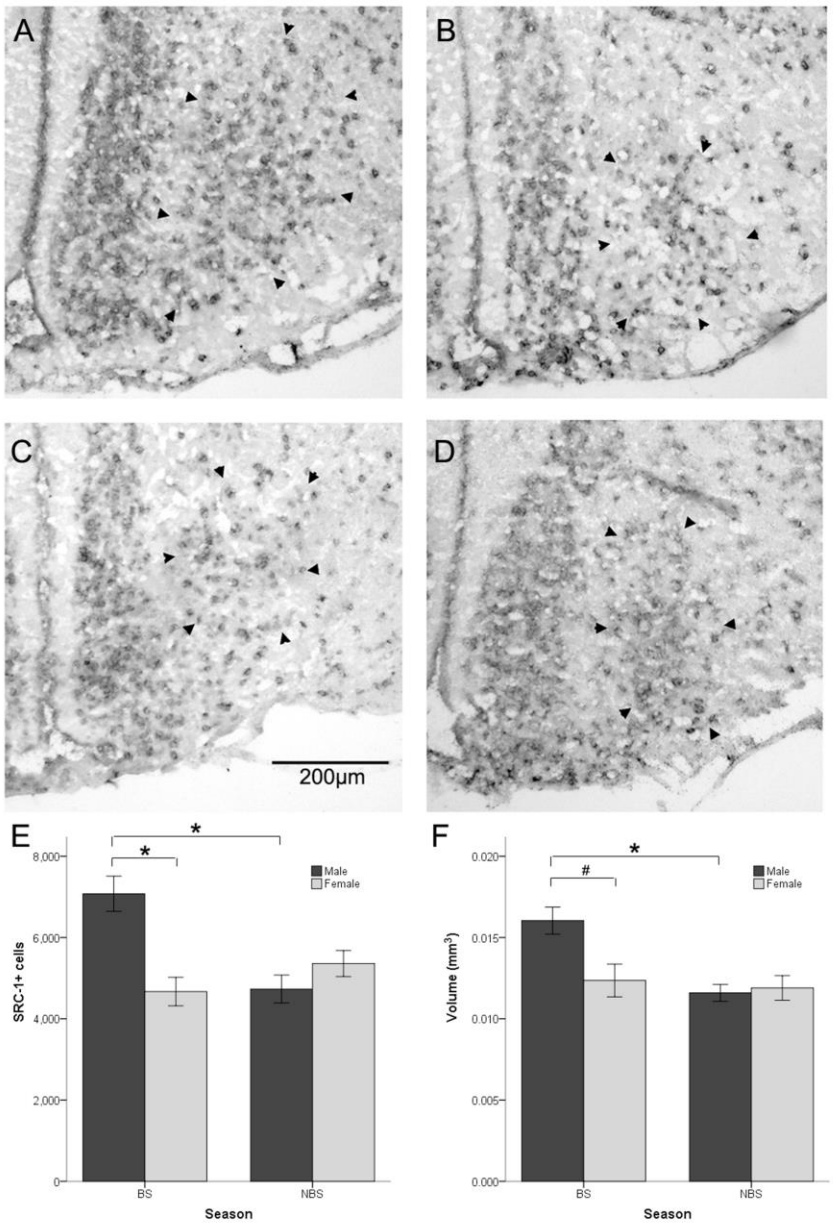


Table 10. **Statistical values for analyses of SRC-1 mRNA in castrated animals with TP or control implants.** Significant effects are indicated in bold.

Region	Measure	Sex	Season	Treatment	Interactions
POA	Cell number	$F(7,54)=0.62$, $p=0.436$	$F(7,54)=0.00$, $p=0.949$	**$F(7,54)=11.69$, $p=0.001$**	**$F(7,54)=4.92$, $p=0.031$***
	Volume	$F(7,54)=2.29$, $p=0.136$	$F(7,54)=1.20$, $p=0.278$	**$F(7,54)=8.47$, $p=0.005$**	All $F(7,54)\leq 3.82$, $p\geq 0.056$
	Density	$F(7,54)=0.61$, $p=0.440$	$F(7,54)=1.21$, $p=0.277$	$F(7,54)=2.66$, $p=0.109$	All $F(7,54)\leq 1.18$, $p\geq 0.282$
AMY	Cell number	$F(7,52)=1.56$, $p=0.218$	$F(7,52)=0.00$, $p=0.975$	**$F(7,52)=5.09$, $p=0.028$**	All $F(7,52)\leq 0.69$, $p\geq 0.409$
	Volume	$F(7,52)=0.76$, $p=0.388$	$F(7,52)=1.03$, $p=0.314$	**$F(7,52)=5.22$, $p=0.026$**	All $F(7,52)\leq 1.29$, $p\geq 0.262$
	Density	**$F(7,52)=5.83$, $p<0.019$**	$F(7,52)=1.54$, $p=0.221$	$F(7,52)=0.17$, $p=0.680$	All $F(7,52)\leq 0.62$, $p\geq 0.436$
VMH	Cell number	$F(7,53)=0.11$, $p=0.742$	$F(7,53)=3.70$, $p=0.060$	$F(7,53)=0.13$, $p=0.724$	All $F(7,53)\leq 1.06$, $p\geq 0.309$
	Volume	$F(7,53)=1.40$, $p=0.241$	**$F(7,53)=7.49$, $p=0.008$**	$F(7,53)=0.32$, $p=0.572$	All $F(7,53)\leq 3.25$, $p\geq 0.077$
	Density	$F(7,53)=0.09$, $p=0.763$	$F(7,53)=0.40$, $p=0.532$	$F(7,53)=0.16$, $p=0.691$	All $F(7,53)\leq 2.41$, $p\geq 0.126$

*Significant sex x season interaction; none of the other possible interactions were statistically significant (all $F(7,54) \leq 1.16$, $p\geq 0.286$). None of the pairwise comparisons were statistically significant for this interaction (see results).

SRC-1 mRNA in Hormone-Manipulated Males and Females

In the POA, both the number of cells containing SRC-1 mRNA and the volume defined by this labeling were increased in TP-treated compared to control animals (Figure 13). A sex x season interaction was also detected for the number of cells expressing SRC-1 mRNA, and a trend for a sex x season interaction existed for POA volume. However, none of the pairwise comparisons were statistically significant (all $t(27)\leq 2.20$, $p\geq 0.035$; $\alpha=0.0125$). There were no other significant main effects or interactions among the variables in the POA for these measures, or for the density of cells expressing SRC-1 mRNA.

In the AMY of hormone-manipulated animals, TP-treatment increased both the number of cells containing SRC-1 mRNA and the volume of the AMY defined by them, compared to blank-treated animals (Figure 14). There were no other significant main effects or interactions among the variables for these two measures. The density of cells containing SRC-1 mRNA in the AMY was higher in females than in males (Figure 14). No other main effects or interactions of the variables existed for SRC-1 cell density in the AMY.

A trend was detected for an increased number of cells expressing SRC-1 in the BS compared to the NBS in the VMH (Figure 15), but no other main effects or interactions between the variables were significant for the number of cells in this brain region. VMH volume, as defined by labeling of SRC-1+ cells, was significantly increased in BS animals as opposed to NBS (Figure 15). No other significant main effects or interactions for were detected for VMH volume, or for the density of cells expressing SRC-1 mRNA.

Figure 13. SRC-1 mRNA in the POA of hormone-manipulated animals. Photographs show a representative testosterone proprionate (TP)-treated (A) and control (blank; BL) (B) animal; both males from the breeding season (BS). A main effect of this hormone manipulation was detected for both the estimated total number of SRC-1+ cells (C) and the volume of the POA defined by this marker (D).

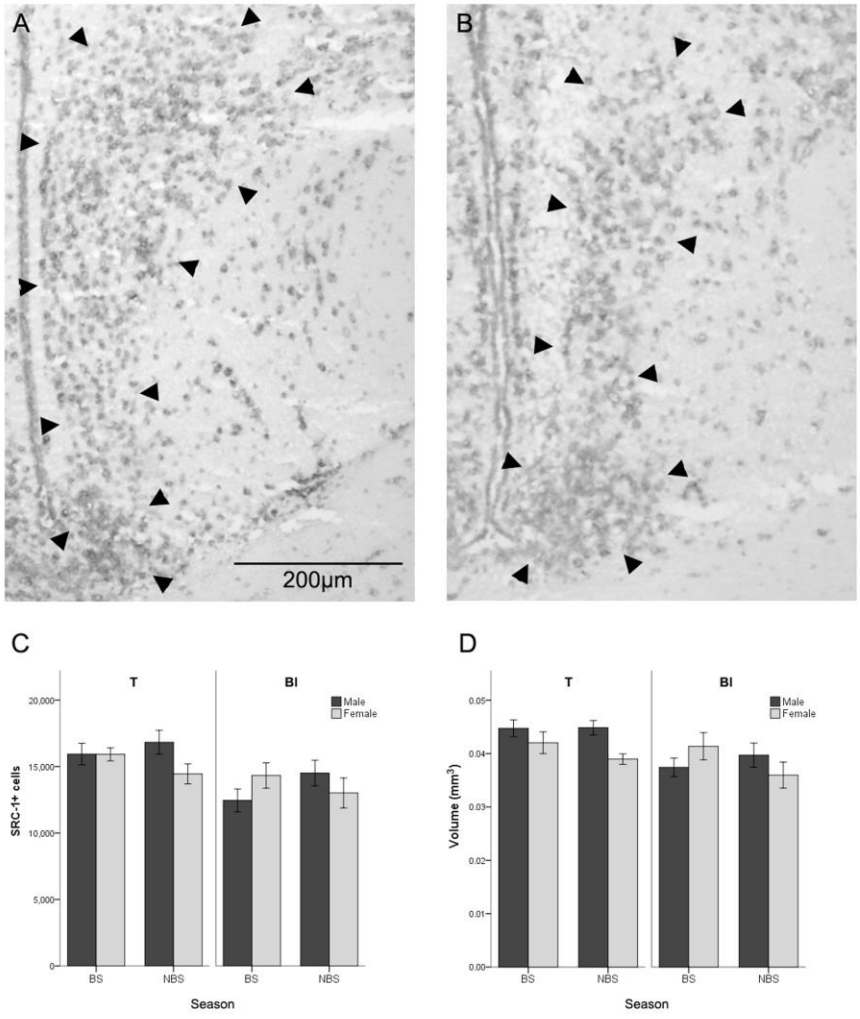


Figure 14. SRC-1 mRNA expression in the AMY of hormone-manipulated animals. Representative photomicrographs are shown from breeding season (BS) animals; (A) blank-treated male (BL), (B) testosterone proprionate (TP)-treated male, (C) TP-treated female. Androgen significantly increased the number of SRC-1+ cells (D) and the volume of the AMY as defined by these cells (E). The density of SRC-1+ cells in the AMY was higher in females than in males (F).

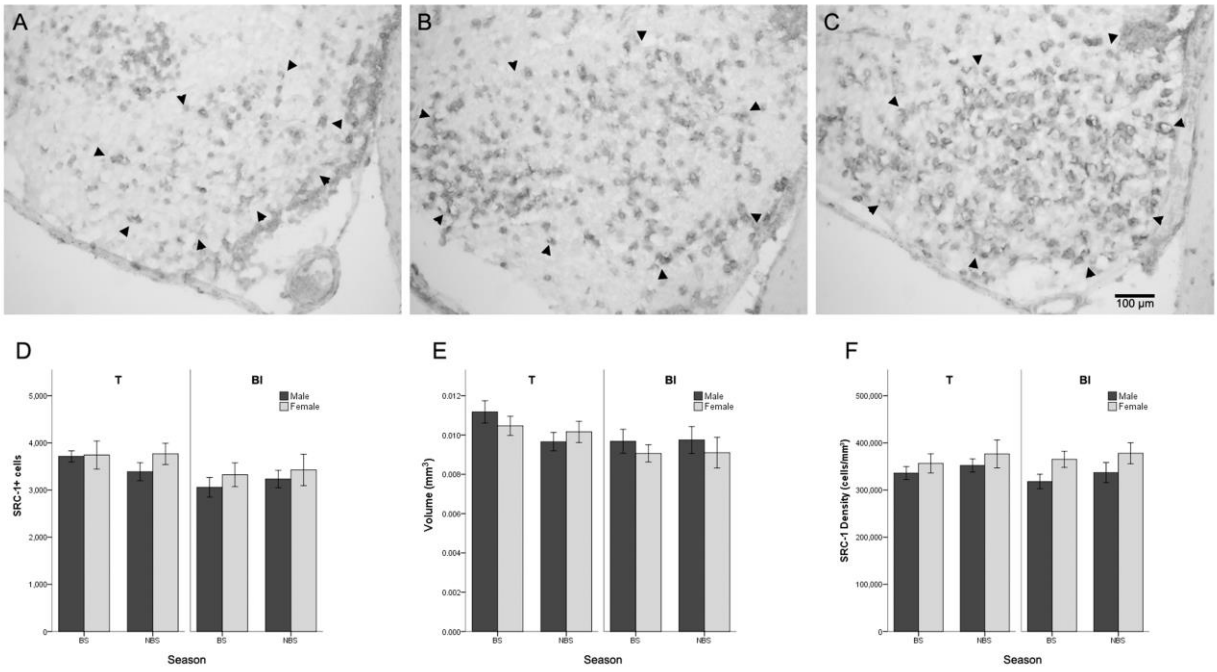
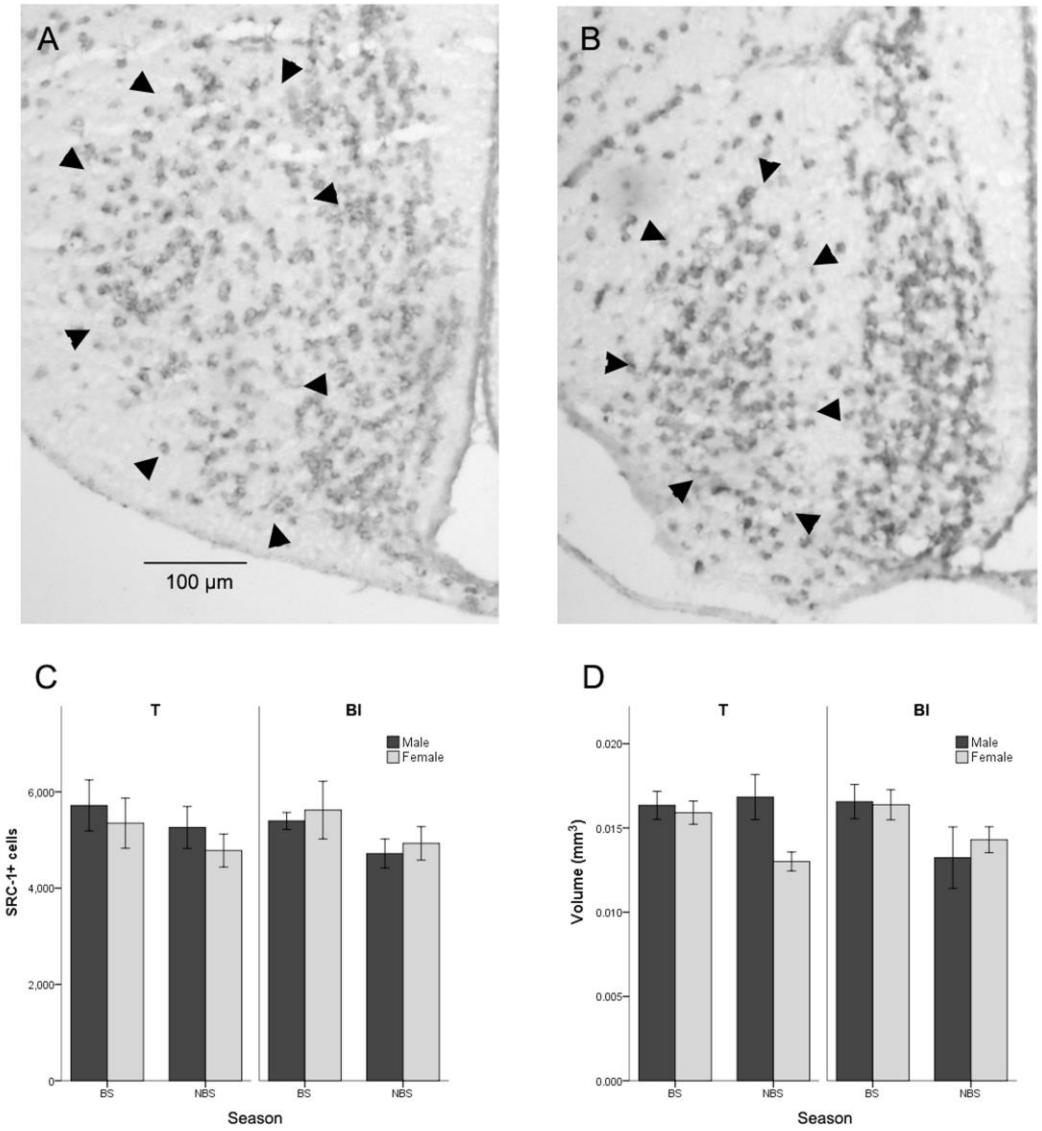


Figure 15. SRC-1 mRNA in the VMH of hormone-manipulated animals. Images represent blank-treated (BL) males from the breeding season (BS) (A) and nonbreeding season (NBS) (B). The graph on the left (C) depicts a trend for an effect of season (increased SRC-1+ cells in the BS, P = 0.060). (D) shows a significant main effect of season (BS > NBS) on VMH volume in hormone-manipulated animals. TP, testosterone proprionate.



Discussion

The distribution of SRC-1 mRNA in the green anole brain was consistent with other findings of mRNA and protein expression in birds and rodents (Bian et al., 2011, Charlier et al., 2002, Meijer et al., 2000, Duncan et al., 2011), with high levels in steroid-sensitive regions. The pattern of expression across sexes and seasons in brain regions important for the regulation of reproductive behaviors suggests that SRC-1 may contribute to these sexually dimorphic functions, and is regulated by circulating T in a regionally specific manner.

SRC-1 mRNA in the POA

Compared to females, male anoles had both more cells expressing SRC-1 mRNA in the POA and a larger volume of the region defined by that labeling. These sex differences parallel findings in the song system of canaries and zebra finches, in which males express more SRC-1 mRNA (Charlier et al., 2003) and protein (Duncan et al., 2011) than females. An initial finding of a male-biased sex difference was also reported in the preoptic area/hypothalamus region in Japanese quail (Charlier et al., 2002). However, a later study in the Japanese quail documented females having more SRC-1 mRNA than males in this region (Charlier et al., 2006). The authors suggest that this discrepancy may be due to an endogenous seasonal rhythm in expression of this coactivator that occurs independent of the light cycle. Since the two studies with conflicting results were performed in different breeding states, the Japanese quail may demonstrate a seasonal change in SRC-1 expression. In contrast, no effect of season was seen in SRC-1 mRNA in the POA of gonadally intact green anoles in the present study. Thus, while increased SRC-1 in the POA

may facilitate an increase in the male sexual behavior facilitated by this region, the present results suggest that SRC-1 within the POA of gonadally intact animals does not contribute to seasonal changes in responsiveness to T.

In the POA of males and females that received hormonal manipulations, TP-treatment significantly increased the number of cells expressing SRC-1 mRNA, as well as the volume of this region as defined by these cells. This is consistent with findings from other species, which suggest that this coactivator is sensitive to hormonal stimuli. SRC-1 mRNA expression in the Japanese quail is upregulated by T in the male preoptic area/hypothalamus (Charlier et al., 2006). In mouse, removal of the testes significantly reduces the amount of SRC-1 protein in several brain regions, including the POA and VMH (Bian et al., 2014).

Collectively, our results suggest that an increase in T in males compared to females may upregulate SRC-1 expression in the anole POA, but that seasonal differences in circulating T may not be sufficient to create this effect. Data on hormone levels are consistent with this idea. Unmanipulated males from the BS and NBS average around 20ng/ml and 11ng/ml, respectively, both of which are much higher than female anoles in either the BS or NBS, which average less than 1ng/ml (Lovern et al., 2001). Similarly, the difference in circulating T due to our implants and the undetectable levels in their castrated controls with empty capsules was on average somewhat greater than the difference between normal males and females (Cohen and Wade, 2012).

SRC-1 mRNA in the AMY

No significant effects were detected in the AMY of gonadally intact animals, but in hormone-manipulated animals TP-treatment significantly increased the number of cells expressing SRC-1 mRNA, as well as the volumes of the region defined by these cells, compared to blank controls. Similarly, differences in SRC-1 across photoperiod have been demonstrated in Syrian hamsters in the posterodorsal medial amygdala and posteromedial bed nucleus of the stria terminalis. Daylength and androgen treatment interacted to affect SRC-1 protein in the anterior medial amygdala of those hamsters as well (Tetel et al., 2004). As in the case of the anole POA, this pattern of results may suggest that relatively large differences in circulating T are required to produce a detectable difference in SRC-1+ cell number in the AMY of green anoles.

The density of SRC-1+ cells in the AMY of hormone treated animals was higher in females than males. It is difficult to know how to interpret these data, as this sex difference was not detected in gonadally intact lizards, and because significant effects of sex were not detected on the estimated number of cells expressing SRC-1 mRNA or the volume defined by this labeling (the two measures that went into this calculation). However, if this increase in females represents a greater concentration of SRC-1, it may facilitate female-specific functions. For example, SRC-1 coactivates oestrogen as well as androgen receptors. It is possible female behaviors are mediated through oestrogen receptor action in the AMY. This general region of the brain is important for receptivity in rodents, as demonstrated with lesion and electrochemical stimulation (Masco and Carrer, 1980). It also mediates other social behaviors in male and female rodents (Newman, 1999). Its roles beyond those

associated with sexual and aggressive behaviors in the male anole (Greenberg et al., 1984) are largely unknown.

The current finding of a significant sex difference in hormone-treated, but not gonadally intact animals, may be the result of removing the gonads. This phenomenon of effects only becoming apparent following gonadectomy, regardless of hormone level following implant, has been detected in the anole brain for other steroid-related molecules. For example, a male-biased sex difference in the volumes of the AMY and VMH as defined by ERα-expressing cells was detected in gonadectomized animals, regardless of oestradiol treatment, but these effects were not seen in gonadally intact animals. In the same study a seasonal difference in the number of cells expressing ERα mRNA in the VMH was seen in gonadectomised, but not intact, animals (Beck and Wade, 2009). A similar phenomenon was detected in AR mRNA expression in the anole AMY and VMH (Kerver and Wade, 2014).

SRC-1 mRNA in the VMH

Analyses of the VMH in gonadally intact animals suggest increased SRC-1 specifically in males during the BS. As noted above, among unmanipulated animals, BS males have the highest levels of T, with NBS males averaging approximately half of these, and BS and NBS females even less (Lovern et al., 2001). Thus, one might expect that the increased T in BS males was responsible for these sex and seasonal differences. The sex difference, however, was not present in gonadectomized animals (with or without T). Because males and females within treatment groups had equated levels of androgen, these results are consistent with the idea that levels of one or more gonadal hormones other than T is/are responsible for the sex difference in VMH SRC-1 in unmanipulated lizards. Oestradiol from

the ovaries may play a role in decreasing SRC-1 in females. However, given the role of the VMH in female receptivity (see Introduction), oestradiol would more likely be expected to increase SRC-1 instead of decrease it. Previous studies have shown that SRC-1 modulates oestrogen receptor and progesterone receptor action in the ventromedial nucleus of the hypothalamus in female rats, and influences female sexual behaviour (Molenda-Figueira et al., 2006).

Across both intact and hormone-manipulated animals, BS animals had more SRC-1+ cells (although only a trend in treated animals) and larger VMH volumes than NBS animals. There was no effect of hormone treatment on any measure, so these seasonal effects on this coactivator are not the result of variations in levels of circulating T. The possibility that other hormones, such as oestradiol, are contributing to seasonal differences remains plausible. Alternatively, SRC-1 may have an endogenous seasonal variation in expression in the VMH, which has been suggested for SRC-1 protein in the posterodorsal medial AMY and bed nucleus of the stria terminalis in Siberian hamsters (Tetel et al., 2004). A correlation in SRC-1 mRNA expression and time of day was also revealed in several brain areas in the Japanese quail, including the optic lobes, hippocampus, hindbrain and telencephalon (Charlier et al., 2006). Specific mechanisms in the green anole require further investigation.

The present results from analyses of in situ hybridisation within the VMH are consistent with the idea that SRC-1 is sexually dimorphic in the anole brain, and could increase steroid receptor activation within the male brain. The increase in SRC-1 within the VMH seen specifically in BS males may also facilitate some aspects of the seasonal responsiveness to T that governs male sexual behaviour across season. Lesions of this region in rats prevent the full suite of sociosexual behaviors in males, including T-mediated

ultrasonic vocalisations and scent marking (Harding and McGinnis, 2005). Lesions of the basal hypothalamus in male anoles decreased courtship behaviors (Farragher and Crews, 1979), suggesting the VMH may contribute to male reproductive behavior. The current data are consistent with the idea that SRC-1 in this region may play a role in either male or female sexual behaviour of the green anole during BS conditions.

General Conclusions

Taken together, the present results suggest that SRC-1 is selectively regulated by T in specific brain regions, as demonstrated by male-biased sex differences within the POA and VMH, and effects of TP in the POA and AMY. While the patterns of SRC-1 mRNA expression are consistent with the idea that this coactivator promotes some aspects of male sexual behaviour within the BS, our data suggest it is not likely a candidate for facilitating the seasonal responsiveness to T within the green anole. However, analysis of relative levels of SRC-1 protein within the three regions of interest should be conducted. SRC-1 is able to act synergistically with other coactivators such as CREB binding protein, so perhaps there are other proteins that are more involved in coactivation, or even corepression, of AR across seasons. CREB binding protein is currently being investigated in the green anole brain; the AR-specific coactivator, ARA70, is also of interest. Research on steroid receptor coregulators in reptiles will provide a comparative perspective to the regulation of brain and behavior that should eventually allow broader conclusions about mechanism to be drawn.

CHAPTER 4

SEXUALLY DIMORPHIC EXPRESSION OF CREB BINDING PROTEIN IN THE GREEN ANOLE BRAIN

Introduction

Sexual behavior is regulated by steroid hormones and their cognate receptors across vertebrate species. The receptors and hormones that facilitate sexual behavior have largely been identified, but many of the molecular mechanisms involved in steroid action in the brain have yet to be elucidated (McCarthy et al., 2012). In particular, a number of factors leading to sex and seasonal differences in reproductive behaviors remain to be determined. The green anole lizard is an excellent model in which to address this topic. The genome of this species was recently sequenced (Afoldi et al., 2011), which facilitates investigations at the genetic and molecular levels.

Green anoles have a breeding season (BS) that lasts from April through July, followed by a non-breeding season (NBS) in which the gonads of both sexes regress and reproductive behaviors cease (Wade, 2011). Male sexual behavior in this species is primarily regulated by testosterone (T), rather than its metabolites, dihydrotestosterone and estradiol (Wade, 2011; Rosen and Wade, 2000; Winkler and Wade, 1998). T has specific effects on stereotyped reproductive behaviors, as well as morphological and biochemical changes within the brain. However, responsiveness to this hormone varies across seasons. Specifically, the same dose of T given to castrated males in the NBS has reduced effects compared to the BS on courtship, copulation, copulatory muscle fiber size and the size of cells in brain regions responsible for male sexual behavior (O'Bryant and Wade, 1999, 2002; Holmes and Wade, 2004; Lovern et al., 2004; Neal and Wade, 2007). In

addition, T increases brain aromatase activity only in males and only during the BS (Cohen and Wade, 2010).

To investigate how these changes in hormone responsiveness may occur at the molecular level, we initially examined whether androgen receptor expression changes across season in three regions of the anole brain: the preoptic area (POA), ventromedial amygdala (AMY) and ventromedial hypothalamus (VMH). Across vertebrates, including green anoles, the POA and AMY (or its homolog) are critical for male typical sexual behaviors (Christensen and Clemens, 1974; Wood and Newman, 1995; Thompson et al., 1998; Ball and Balthazart, 2004; Greenberg et al., 1984; Wheeler and Crews, 1978) and the VMH regulates female receptivity (Meisel et al., 1987; Flanagan-Cato, 2011). While androgen receptor mRNA was detected in all three regions, the pattern of its expression was not consistent with a role in facilitating a seasonal responsiveness to T. No differences in expression were found between the seasons for either sex (Kerver and Wade, 2014).

We then began to consider steroid receptor coactivators. These coregulatory proteins are rate-limiting factors for transactivation of the nuclear receptor complex McKenna et al., 1999). When a ligand-activated androgen receptor complex binds to an androgen response element on DNA, it recruits coactivators to the complex, which increase transcriptional activity. Coactivators serve to bridge the receptor complex with basal transcriptional machinery. They can also have endogenous histone acetyltransferase activity, which alters how tightly DNA is wound around histones and thus allows transcription factors to bind more easily. Additionally, coactivators recruit RNA polymerase II and general transcription factors to the promoter of a gene to enhance transcription (Heinlein and Chang, 2002).

Two nuclear receptor coactivators of particular interest are steroid receptor coactivator-1 (SRC-1) and cAMP response element binding protein (CREB)-binding protein, abbreviated as CBP. They act synergistically to coactivate androgen and other nuclear receptors (Smith et al., 1996). We recently investigated whether SRC-1 might play a role in the seasonal responsiveness to T. While the patterns of mRNA detected were consistent with facilitation of male sexual behavior within the BS, differences in SRC-1 expression probably do not regulate changes in responsiveness to T across seasons in the anole (Kerver and Wade, 2015). The present set of studies investigates CBP mRNA within the anole brain.

CBP was discovered in 1993 as a protein that binds specifically to phosphorylated CREB, and thus was named CREB-binding protein (Chrivia et al., 1993). It has since been shown to interact with hundreds of different transcription factors (Vo and Goodman, 2001). CBP is expressed in steroid-sensitive brain areas that are involved with reproduction in rodents (Auger et al., 2002a; Tetel, 2009). Immunocytochemistry studies demonstrate that CBP is widely expressed in the brain, with high levels in the POA, amygdala, hypothalamus, thalamus, hippocampus, cortex and cerebellum (Molenda et al., 2002; Stromberg et al., 1999).

CBP is necessary for AR-dependent transactivation (Aarnisalo et al., 1998). The concentration of available coactivators can cause genome-wide changes due to competition for their recruitment (Rosenfeld et al., 2006). We therefore hypothesized that the availability of CBP could influence the responsiveness to T across seasons. We examined the expression of CBP mRNA in males and females from both the BS and NBS in three brain regions critical for sexual behavior in the anole – the POA, AMY and VMH. Additionally, we

used gonadectomized animals treated with either T or a blank control implant to determine the effect that T has on CBP expression in the brain.

Methods

Animals

Male and female green anoles were wild caught and shipped to us during the BS and NBS by Charles Sullivan Co. (Nashville, TN). BS animals were received in April and NBS animals in October. Male/female pairs were housed in individual 10-gallon aquaria, which each contained peat moss substrate, a rock and stick for basking, as well as a water dish. Cages were misted daily with water. Black dividers between the cages prevented visual contact. Animals were exposed to fluorescent lighting, full spectrum bulbs and a heat lamp directly over each cage. BS conditions were maintained on a 14:10 light:dark cycle with temperatures ranging from 28 to 38°C in the daytime, depending on the proximity to the heat lamp. Temperatures averaged 18°C when the lights were off overnight. During NBS conditions, daytime temperatures ranged from 24 to 30°C and averaged 15°C at night. Humidity was maintained at 60-70% in both seasons. All procedures were approved by the Michigan State University Institutional Animal Care and Use Committee.

Treatment and tissue collection

Following a two-week period of acclimation to the lab, eight gonadally intact animals of each sex were rapidly decapitated. Final sample sizes were 7 or 8 per group; degrees of freedom for each analysis are identified within the results. Following decapitation, breeding state was confirmed through inspection of gonadal state, and brains

were snap frozen in methyl butane and stored at -80°C. Breeding males had large, vascularized testes and breeding females had at least one yolking follicle or an egg in the oviduct. Non-breeding gonadal state was confirmed by the observation of regressed gonads in all animals.

Additional males and females from the BS and NBS were used for a separate study to assess the effect of T on CBP mRNA expression. Final sample sizes were 6-8 per group; degrees of freedom for each analysis are identified within the results. These animals were anaesthetized with isofluorane following a 10µl injection of 2% lidocaine, then gonadectomized and given either a subcutaneous TP-filled or empty (blank control) capsule. The animals remained on ice throughout this procedure. The Silastic capsule (7mm long x 0.7mm inner diameter x 1.65 mm outer diameter) contained either 5mm of packed testosterone propionate (TP) or remained empty (Bl). This dose reliably activates male sexual behavior and associated morphological and biochemical changes in the brain (Neal and Wade, 2007; Cohen and Wade, 2010), and produces plasma concentrations that average 48.7 ± 4.8 (SEM) ng/ml of androgen, regardless of season (Cohen and Wade, 2012). Gonadally intact males collected in the wild average approximately 20ng/ml in the BS and 11ng/ml in the NBS, but concentrations produced by our TP capsules overlap with BS individuals (Lovern et al., 2001). Brains were collected after rapid decapitation, one week following gonadectomy. At the time of tissue collection it was confirmed that some TP remained in the hormone-filled capsules and that there was a complete absence of gonadal tissue.

Cloning of CBP

Procedures were performed as for SRC-1 in (Kerver and Wade, 2015). Briefly, total RNA was extracted from the male anole whole brain and isolated using RNeasy minicolumns (Qiagen; Valencia, CA). RNA was reconstituted and stored at -80°C. A SuperScript III Reverse Transcription Kit (Invitrogen; Carlsbad, CA) was used to convert RNA to cDNA per manufacturer's instructions, and the cDNA was stored at -20°C.

Primers were designed for the green anole CBP gene (GenBank: XM_008120890.1) using the Oligo Analysis Tool program (Eurofins MWG Operon; Huntsville, AL). The primers spanned 900bp within the CBP sequence. The forward primer sequence was 5'-GCGGAGGCAGTGGCAACAGCTC-3' and the reverse primer sequence was 5'-GCTACGACGCGGACAGAAACCTC-3'. The sequence was PCR-amplified and cloned into pGEM-T Easy Vector per manufacturer's instructions (Promega Corp; Madison, WI). One Shot TOP10 Chemically Competent *E. coli* cells (Invitrogen) were used for the transformation. These cells were grown overnight on LB agar plates, and the following day colonies were selected and grown overnight in LB broth. DNA was isolated using a Wizard Plus Miniprep kit (Promega), and the sequence was confirmed in both directions. DNA was isolated with a Qiagen Plasmid Maxi Kit and stored at -20°C. The plasmid was linearized with NotI, and the template stored at -20°C until use.

In situ hybridization

Brains were coronally sectioned at 20 μm and thaw-mounted in four series onto SuperFrost Plus slides (Fisher Scientific; Hampton, NH). Tissue was stored at -80°C until processing. Antisense (SP6) and sense (T7) probes were transcribed using MAXIscript In

Vitro Transcription Kit with SP6/T7 RNA polymerases (Ambion; Austin, TX), and were labelled with ^{33}P-UTP (Perkin Elmer; Waltham, MA). *In situ* hybridization procedures were performed as in (Qi et al., 2012).

Briefly, tissue was brought to room temperature, rinsed in phosphate-buffered saline (PBS), and fixed in 4% paraformaldehyde. Following dehydration in a series of ethanols, slides were pre-hybridized for 2 hours and then exposed to ^{33}P-UTP-labeled RNA probes overnight. Tissue was washed in saline-sodium citrate (SSC) buffers to remove excess probe and dehydrated in a series of ethanols. Slides from one animal from each group were exposed to autoradiography film (HyBlot CL, Denville Scientific Inc.; Metuchen, NJ) to confirm adequate levels of signal. Slides were then dipped into NTB emulsion (Eastman Kodak; Rochester, NY) and stored at 4ºC in the dark for 4 weeks. They were developed using Kodak Professional D-19 Developer and Fixer (Eastman Kodak) and lightly counter-stained with thionin to facilitate identification of specific brain regions.

An experimenter blind to the sex or season of the animal completed all analyses from the emulsion-coated slides. Brain regions of interest (POA, AMY and VMH) were located with a green anole forebrain atlas (Greenberg, 1982) using brightfield microscopy and then captured in darkfield using Image J (National Institute of Health). Two 0.06 mm^2 boxes were analyzed for each brain region: one on the left side of the brain and one on the right. These boxes were placed within the middle of each brain region in the center of the rostro-caudal distribution of the specific region. The percent area covered by silver grains was measured using the threshold function of the software, and values for right and left sides were averaged. Background labeling, which was defined by that detected on neighboring sense-treated sections for each individual, was subtracted from the antisense.

Resulting percentages were averaged across the right and left side for each individual brain area. A 2-way ANOVA (IBM SPSS statistics version 22; Armonk, NY) was used to analyze the effects of sex and season on the percent area covered by silver grains within each of the three brain regions (POA, AMY and VMH).

Results

Gonadally intact animals

Females expressed significantly more CBP mRNA than males in the AMY (Figures 16 and 17). However, this value did not differ across season in the AMY, and the variables did not interact. No main effects of sex, season, or interactions between the two variables were detected in the POA or VMH. Statistical values for gonadally intact animals can be found in Table 11.

Table 11. Statistical values for analyses of CBP mRNA in intact animals. Significant effects are indicated in bold type.

Brain Region	Sex	Season	Interaction
POA	$F(3,26)=3.18$, $p=0.086$	$F(3,26)=0.56$, $p=0.462$	$F(3,26)=0.58$, $p=0.452$
AMY	**$F(3,26)=18.73$, $p<0.001$**	$F(3,26)=2.36$, $p=0.137$	$F(3,26)=1.61$, $p=0.216$
VMH	$F(3,26)=1.63$, $p=0.213$	$F(3,26)=0.44$, $p=0.515$	$F(3,26)=0.92$, $p=0.345$

Table 12. Statistical values for analysis of CBP mRNA in hormone manipulated animals. No significant values were found in this study.

Brain Region	Sex	Season	Treatment	Interaction
POA	$F(7,51)=0.41$, $p=0.527$	$F(7,51)=1.42$, $p=0.238$	$F(7,51)=0.31$, $p=0.579$	All $F(7,51)\leq0.99$, $p\geq0.325$
AMY	$F(7,51)=0.94$, $p=0.336$	$F(7,51)=1.36$, $p=0.249$	$F(7,51)<00.01$, $p=0.973$	All $F(7,51)\leq0.98$, $p\geq0.326$
VMH	$F(7,51)=0.29$, $p=0.591$	$F(7,51)=0.58$, $p=0.451$	$F(7,51)=0.04$, $p=0.848$	All $F(7,51)\leq3.89$, $p\geq0.054$

Hormonally manipulated animals

There were no main effects of sex, season, or treatment in any of the three brain regions investigated, nor were there any significant interactions among the variables (Table 12). The only value that approached statistical significance was a three-way interaction for the VMH. However, two-way ANOVAs designed to further evaluate this potential interaction produced no statistically significant effects.

Figure 16. CBP mRNA levels in AMY in males and females from the BS and NBS. A significant female-biased sex difference was detected. Values represent means ± 1 standard error.

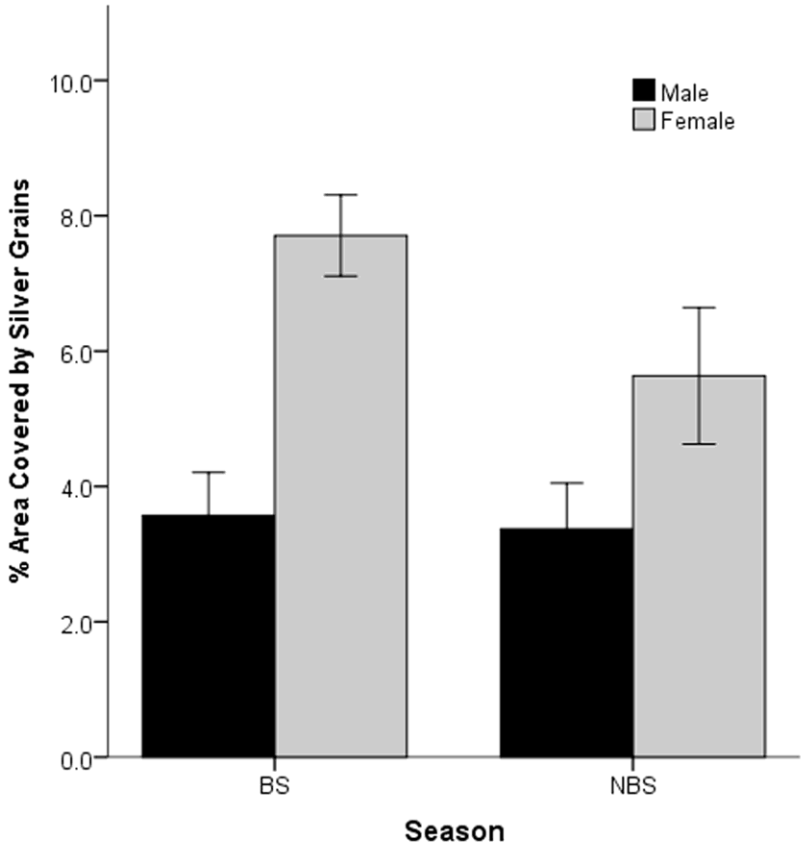
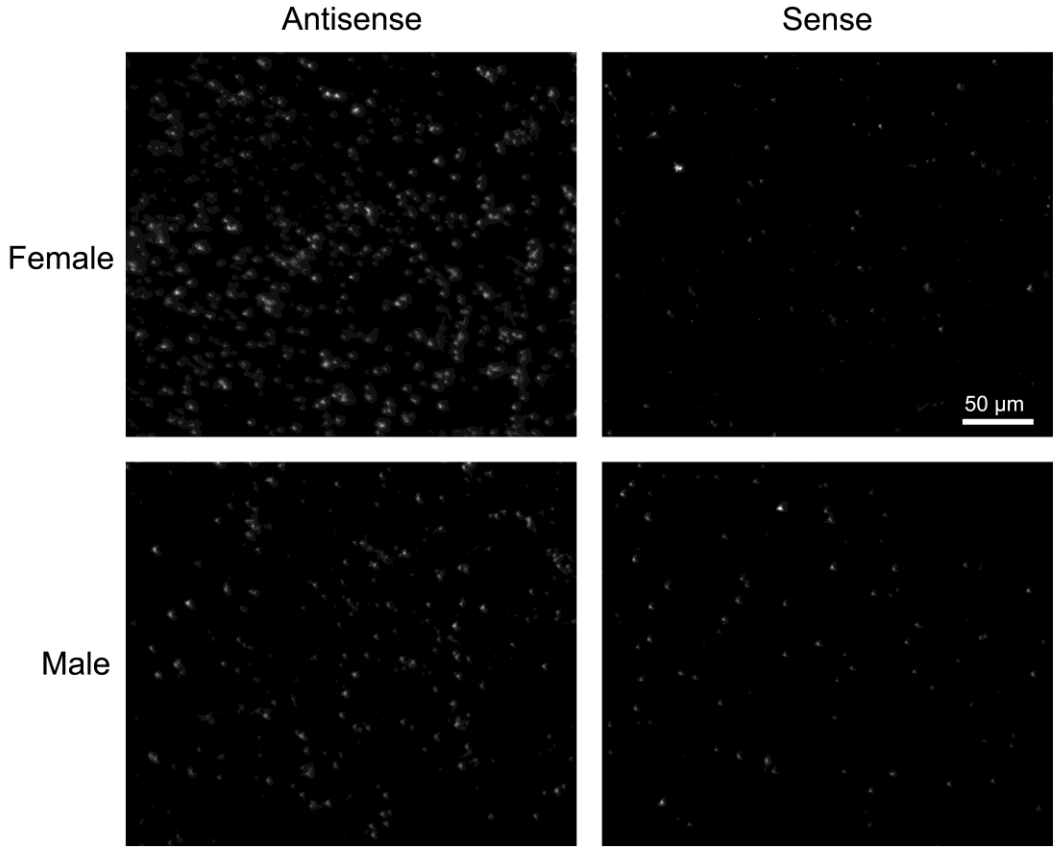


Figure 17. Darkfield images depicting representative CBP mRNA labeling in the AMY. Images are from the center of the region from a male and female from the breeding season. Females exhibited greater expression than males in this brain region, regardless of season.



DISCUSSION

Sexually Dimorphic Expression in the AMY

CBP distribution in the anole brain was very similar to what has been described in other species, with relatively widespread expression across the brain and particularly high expression in steroid-sensitive regions (Stromberg et al., 1999; Auger et al., 2002a; Auger et al., 2002b; Duncan and Carruth, 2011). However, the results were generally not consistent with our prediction that CBP expression would be increased in the BS,

particularly in POA or AMY of males. The only significant effect detected was greater expression in the AMY of gonadally intact *females*, which did not vary across the seasons.

These females had nearly twice the level CBP expression of males in this brain region (a 92% increase). This contrasts with other findings of sex differences in CBP. In neonatal rats, males express more CBP protein than females in the VMH, medial POA and arcuate nucleus, although this male-biased sex difference disappears by postnatal day 11 (Auger et al., 2002a). To our knowledge, sex differences in CBP expression have not been quantified in adult vertebrates outside of the present study. We found a parallel female-biased sex difference in the number of cells expressing androgen receptor mRNA in the AMY of anoles, although this occurred only in hormone-manipulated animals (Kerver and Wade, 2014), and the density of cells expressing SRC-1 mRNA in the AMY of manipulated animals is higher in females than in males (Kerver and Wade, 2015). While the specific roles these molecules play remain to be identified, collectively the data suggest that the AMY may be an important region for steroid hormone receptor and coactivator action, especially in females.

Interestingly, the hormone-manipulated animals in the present study exhibited no significant differences in CBP expression across any of the groups in the brain regions investigated. In contrast to the large effect in gonadally intact animals, treated females on average expressed only 16% more CBP than males in the AMY. This phenomenon of results differing across intact and hormonally manipulated groups has been detected in the anole brain for other steroid-related molecules. For example, a male-biased sex difference in the volumes of the AMY and VMH as defined by estrogen receptor alpha-expressing cells was detected in gonadectomized animals, regardless of estradiol treatment, but these effects

were not seen in gonadally intact animals. In the same study, a seasonal difference in the number of cells expressing estrogen receptor alpha mRNA in the VMH was detected in gonadectomized, but not intact, animals (Beck and Wade, 2009a). Additionally, females had a greater density of both androgen receptor and SRC-1-expressing cells in the AMY of hormone-manipulated, but not gonadally intact animals, and also a female-biased sex difference in the number of androgen receptor-expressing cells (Kerver and Wade, 2014, 2015). The presence or absence of functioning gonads appears to influence whether particular effects are revealed, perhaps due to the endogenous endocrine factors released by these organs.

In the present study, it is clear that the sex difference in CBP expression in the AMY is not due to T, as no significant effects resulted from TP treatment. However, increased estradiol or other ovarian hormones could activate the increase in expression. This idea should be tested. In male anoles, the AMY is associated with sexual and aggressive behaviors, but its role in the female anole brain is largely unknown (Greenberg et al., 1984). This brain region is important for female receptivity in rodents, as demonstrated by lesion and electrochemical stimulation studies (Masco and Carrer, 1980). The amygdala is also important for mediating other social behaviors in rodents of both sexes (Newman, 1999).

The role of CBP within the rodent amygdala is also important for functions not directly related to reproduction. For example, CBP histone acetyltransferase activity is critical for consolidation and reconsolidation of amygdala-dependent fear memories (Maddox et al., 2013). CBP histone acetyltransferase activity is also necessary for normal memory consolidation. Transgenic mice that express CBP in which its histone

acetyltransferase activity is eliminated can still acquire new information and have intact short-term memory, but they have impaired ability to consolidate information into long-term memory (Korzus et al., 2004). The role of increased CBP in the AMY of female anoles is not clear, but future studies should consider functions related to memory, as well as receptivity and other reproductive behaviors.

Detection of Relatively Few Differences Between the Sexes and Seasons

While we were initially surprised to find largely consistent CBP expression across the sexes and seasons, the multiple roles of CBP may be one reason why there were so few differences in CBP expression across groups. This coactivator is involved in numerous functions within the brain and periphery, not all of which are associated with steroid receptor function (Goodman and Smolik, 2000). Importantly, in addition to androgen receptors, which are of primary interest in relation to seasonal changes in responsiveness to T in green anoles, CBP coactivates other steroid receptors, including those for estrogen, progesterone and glucocorticoids (Smith et al., 1996; Kino et al., 1999; McKenna et al., 1999). Thus, while CBP may facilitate androgen receptor function, its expression may not differ across the sexes or seasons in some brain regions due other functions.

Specifically, in addition to potential roles associated with ovarian estrogens (see above), seasonal differences in glucocorticoid function may be influenced by CBP in the anole brain. Baseline corticosterone levels are higher in NBS males than BS males in both green and brown anoles (Tokarz et al., 1998; Husak et al., 2007). Androgen receptor mRNA expression does not differ across season in the anole brain (Kerver and Wade, 2014), but it is possible that available CBP preferentially coactivates androgen receptors in the BS, and

glucocorticoid receptors in the NBS. Expression patterns of glucocorticoid receptors in the anole brain require further investigation.

Two other factors warrant consideration. First, mRNA rather than protein was quantified in the present study. It is possible that relative protein levels do not fully match the pattern of mRNA expression. Second, in addition to CBP availability, the expression of other steroid receptors should be considered, as competition among them for the coactivator may limit the availability of CBP to androgen receptor during the NBS. Thus, even with similar levels in the BS and NBS, CBP could still be important in the seasonal change in responsiveness to T because its interaction with this receptor could be diminished in the NBS compared to BS. This type of competition for intracellular coactivators like CBP is common, and can result in inhibited transcription (Aarnisalo et al., 1998; Van Orden et al., 1999; Rosenfeld et al., 2006).

Conclusions and Future Directions

While the detection of higher levels of CBP mRNA in the AMY of gonadally intact females compared to males is interesting, the present data do not provide support for the idea that CBP contributes to the seasonal responsiveness to T in the green anole lizard. We have also investigated the patterns of androgen receptor and SRC-1 expression, and the results for these genes within the brains of gonadally intact animals do not support the predictions that they play a significant role in seasonal responsiveness to T (Kerver and Wade, 2014, 2015). Overall, investigations in gonadally intact animals suggest that androgen receptor mRNA expression does not change across sex or season in intact animals (Kerver and Wade, 2014), but CBP is important in the female AMY, perhaps for

estrogen receptor signaling. SRC-1 mRNA is increased in the male VMH and POA of gonadally intact animals (Kerver and Wade, 2015). SRC-1 may facilitate aspects of male sexual behavior in the male VMH and POA, and this is likely regulated by circulating T in the POA, as T manipulations increase SRC-1 mRNA in this region (Kerver and Wade, 2015). However, further investigation is still necessary into alternative changes at the molecular level that contribute to the decreased effects of T seen on behavior, morphology and biochemical functions in the NBS, as the expression of these three genes in the anole brain does not appear to contribute. In addition to the ideas presented above, alternative coactivators, such as the androgen receptor-specific coactivator, ARA70, and corepressor proteins, should be investigated.

CONCLUSION

While the receptors and hormones that regulate sexual behavior have largely been identified, there is still a relative paucity of information on the molecular mechanisms that are involved in steroid action in the brain. In particular, factors leading to long lasting sex differences, as well as transient changes such as seasonal differences, that contribute to reproductive behaviors remain to be determined. The green anole lizard displays a seasonal change in responsiveness to T, and is therefore an excellent model to address changes at the molecular level that govern reproductive behavior across seasons.

The experiments in this dissertation set out describe expression patterns of AR and two of its coactivators, SRC-1 and CBP, and how differences at these levels may facilitate a seasonal responsiveness to T in limbic forebrain regions and copulatory structures related to sexual behavior in this species. Additionally, these experiments tested how T regulates expression of these genes across tissues. The distribution of AR was examined across season for the first time in the anole brain, and this is also the first description of the coactivators SRC-1 and CBP in a reptilian brain.

My results document that AR mRNA is increased in males compared to females in whole brain homogenates, but does not differ across season in the brains of gonadally intact males and females. Specific effects became apparent following gonadectomy in hormone-manipulated animals, such as an increase in the number of AR+ cells in the AMY and the volume of this region in females compared to males, as well as a T-induced increase in the volume of the VMH in the BS but not the NBS. Additionally, differences in AR protein expression became apparent following gonadectomy. In POA-hypothalamic dissections, AR protein is increased in males compared to females, specifically within the BS, and among

females there is more AR protein in the NBS compared to the BS. Collectively, these results indicate that differences in central AR expression are not likely facilitating a seasonal responsiveness to T, but may be regulating some differences between the sexes in the display of reproductive behaviors or other functions associated with these limbic brain regions.

AR expression in the copulatory tissues, however, appears to be more tightly regulated across seasons. Expression in the RPM muscle is not consistent with a role in responsiveness to T across season, but changes in the expression of AR in the hemipenis may be dictating the frequency by which sexual behaviors occur. Transcriptional activity appears generally diminished in the NBS in hemipenis tissue. Additionally, the expression of AR protein in hemipenis tissue was consistent with a role in seasonal responsiveness to T. Gonadally intact males have more AR protein in the BS compared to the NBS. AR was also increased by T in this tissue and more so in the BS than NBS. Future work may aim to determine whether epigenetic mechanisms, DNA methylation in particular, are involved in regulating this pattern of changing AR expression.

SRC-1 appears to be the most responsive to circulating T in these studies, both at peripheral and central levels. T treatment in gonadectomized animals decreases SRC-1 protein in hemipenis tissue. SRC-1 protein does not differ in POA-hypothalamic dissections in the brain in either gonadally intact or hormone treated animals. SRC-1 mRNA, however, appears to be selectively upregulated by T in specific brain regions, as demonstrated by male-biased sex differences within the POA and VMH, and increases in the number of cells and volumes due to T in the POA and AMY. Although the patterns of SRC-1 mRNA expression are consistent with the idea that this coactivator facilitates some aspects of

male sexual behavior within the BS, these data suggest that it is not likely to be a candidate for regulating the seasonal responsiveness to testosterone within the green anole.

CBP protein is upregulated in the BS compared to NBS in hemipenis tissue, which may facilitate coactivation of increased levels of AR protein in the BS. CBP does not differ across season or hormone manipulation within the brain regions responsible for sexual behavior, but is increased by 92% in females compared to males in the AMY. This female-biased sex difference in the AMY also exists in AR, for both the number of AR+ cells and volume, as well as a female-biased increase in the density of SRC-1+ cells in this region. While the specific roles these molecules play remain to be identified, the data suggest that the AMY may be an important region for steroid hormone receptor and coactivator action, especially in females.

The implications for this body of work suggest that these molecules have tissue specific expression and regulation across sexes, seasons, and hormone-manipulations. While the expression patterns in the brain are not consistent with a role for these molecules in the seasonal responsiveness to T in the green anole lizard, the data do suggest potential roles for AR, SRC-1, and CBP in the regulation of male and female specific sexual behavior. The changes in AR in the hemipenis are the most consistent with facilitating seasonal responsiveness to T in the anole, but this possibility should be further investigated. Future work should examine the role of corepressor proteins in T-mediated AR signaling, as well as the kinetics of coactivator binding across seasons. Although coactivator levels may not change seasonally, they may be preferentially binding to AR during the BS.

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