

Changes in androgen receptor mRNA expression in the forebrain and oviduct during the reproductive cycle of female leopard geckos, *Eublepharis macularius*

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Abstract

Successful reproduction requires the coordination of reproductive physiology with behavior. The neural correlates of reproductive behavior have been elucidated in a variety of amphibians, mammals, and birds but relatively few studies have examined reptiles. Here we investigate differences in androgen receptor (AR) mRNA expression in the forebrain and oviduct between previtellogenic and late vitellogenic female leopard geckos, *Eublepharis macularius*. Plasma concentrations of testosterone (T) are low when females are previtellogenic and sexually unreceptive but increase dramatically during late vitellogenesis when females are receptive. In addition, receptivity can be induced by treatment with exogenous T. The relative abundance of AR-mRNA across various nuclei was greater in late vitellogenic than in previtellogenic females. This general pattern was observed in the medial preoptic area, anterior hypothalamus, external nucleus of the amygdala, dorsolateral aspect of the ventromedial hypothalamus, lateral septum, and periventricular hypothalamus. There were also clear differences in AR-mRNA expression among these nuclei. The pattern of gene expression observed in the brain was reversed within stromal cells of the oviduct where expression of AR-mRNA decreased from the previtellogenic stage to the late vitellogenic stage. Overall, these data demonstrate that T concentration in the plasma, abundance of AR-mRNA in the brain and oviduct, and sexual behavior change coordinately during the reproductive cycle of female leopard geckos. Although the function of AR in the female leopard gecko is not yet clear, our results are in accord with growing evidence that androgens regulate numerous aspects of female physiology and behavior in vertebrates.

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1. Introduction

Ovarian hormones play a key role in the regulation of female sexual behavior and uterine function in mammals. During the ovarian cycle in the rat, 17 β -estradiol (E2) concentrations slowly rise as ovulation approaches, and four hours before ovulation, progesterone (P4) concentrations surge (reviewed in Pfaff et al., 1994).

These changes in hormone concentrations lead to biochemical changes in neurons of the ventromedial hypothalamus (VMH) that facilitate the display of lordosis. The increase in E2 produces an increase in progesterone receptor (PR) expression in the VMH, which thereby sensitizes the brain to the subsequent surge in P4 (Lauber et al., 1991; Pfaff et al., 1994). The same pattern of PR induction by E2 occurs in stromal cells of the uterus, priming these cells for decidualization in response to P4 and the implanting blastocyst (Rider, 2002). Although reproductive and behavioral endocrinology have been well studied in certain mammals, much less is known about the endocrine regulation of the reproductive cycle in females of other species and vertebrate classes. Such comparative information is

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essential for a full understanding of the unique and evolutionarily conserved aspects of endocrine function. For example, there is increasing evidence that androgens, including non-aromatizable ones, are involved in normal female development and reproduction in many vertebrates (Lutz et al., 2001; Mahendroo and Russell, 1999; Staub and De Beer, 1997).

Concentrations of androgens change during the reproductive cycle and are often higher than estrogen concentrations in a number of reptiles. In the Kemp's ridley sea turtle, *Lepidochelys kempi*, testosterone (T) and E2 concentrations increase above basal concentrations during vitellogenesis, and concentrations of T are approximately 10-fold higher than E2 throughout the year but are 20-fold higher at the time of mating (Rostal et al., 1998). In the Galápagos tortoise, *Geochelone nigra*, concentrations of T and E2 increase during the mating season, and T concentrations are roughly 2- to 10-fold higher than E2 concentrations, with the largest difference between T and E2 concentrations during the mating season (Schramm et al., 1999). An increase in T also occurs in female American alligators at the time of mating (Guillette et al., 1997). Testosterone and E2 concentrations, however, are equivalent during late vitellogenesis in the alligator. Although species vary in the relative concentrations of T and E2 and the exact relationship of these hormones to follicular development and mating behavior, concentrations of both hormones generally increase in a correlated manner during the reproductive cycle. These findings suggest that T may have a conserved physiological role in the reproductive biology of female reptiles (also see discussions in Guillette et al., 1997; Rostal et al., 1998).

Recent studies in our laboratory have documented the behavioral and hormonal changes that occur during the reproductive cycle of female leopard geckos, *Eublepharis macularius* (Rhen et al., 2000). Female leopard geckos are not receptive when previtellogenic, a few are receptive during early vitellogenesis, and most are receptive during late vitellogenesis. Females are again unreceptive after ovulation. Concentrations of dihydrotestosterone (DHT) and T are low when females are previtellogenic, increase slightly during early vitellogenesis, increase dramatically during late vitellogenesis (i.e., prior to ovulation), and then decrease to previtellogenic levels after ovulation. Concentrations of E2 increase gradually from the previtellogenic stage to a peak during late vitellogenesis and then drop to previtellogenic levels after ovulation. In contrast, concentrations of P4 in the female leopard gecko only increase slightly from the previtellogenic stage to the early vitellogenic stage, remain at the same level during late vitellogenesis, and then decrease after ovulation. Though all hormones examined changed with the reproductive cycle, it is likely that T and E2 play the most important roles in regulating receptivity. Among late

vitellogenic individuals, receptive females have significantly higher concentrations of DHT, T, and E2 than unreceptive females. Ovariectomy eliminates receptive behavior and treatment with exogenous T or E2 induces receptive behavior (Rhen and Crews, 2000; Rhen et al., 1999). Treatment with DHT alone does not elicit receptivity thus indicating that T may induce receptivity after aromatization to E2. Injections of P4 after E2 treatment do not appear to enhance or inhibit receptive behavior in ovariectomized females (unpublished observations, Rhen and Crews). Together, these studies suggest that cyclical changes in T and E2 levels may control receptivity in female leopard geckos.

The neuroendocrine mechanisms underlying hormonal and behavioral changes during the female reproductive cycle remain to be elucidated in this species. To begin to address this problem and to explore the potential role of androgen receptors (AR) in reproductive behavior and physiology, we examined the expression of AR-mRNA in various forebrain nuclei and in oviduct in previtellogenic and late vitellogenic female leopard geckos. Using in situ hybridization, we found that the relative abundance of AR-mRNA across a number of nuclei was greater in late vitellogenic than in previtellogenic females. This pattern was reversed in the reproductive tract where expression of AR-mRNA in stromal cells of the oviduct was lower in vitellogenic females than in previtellogenic females.

2. Materials and methods

2.1. Animals

Animals were treated according to a research protocol approved by the Institutional Animal Care and Use Committee at the University of Texas. The methods used to produce the animals for this experiment have been described in detail previously (Rhen and Crews, 1999; Rhen and Crews, 2000). Briefly, leopard gecko eggs from the captive-breeding colony at the University of Texas were collected. Fertile eggs were placed in individual cups filled with moist vermiculite (1 part water:1 part vermiculite) and split among four constant incubation temperatures (i.e., 26, 30, 32.5, and 34 ± 0.1 °C). Incubation of leopard gecko eggs at 26 °C produces only females, 30 °C produces a female-biased sex ratio (~30% males), 32.5 °C produces a male-biased sex ratio (~70% males), and 34 °C again produces virtually all females. Geckos hatched from these eggs were raised in isolation to reproductive maturity and then placed in cages with one adult male and two or three other females. The 14 adult females used in this study had been incubated at 30 °C, were of similar age, and were reproductively active (i.e., they had produced at least one viable clutch of eggs).

The reproductive status of females was monitored every two to three days. In the leopard gecko, reproductive status can be determined easily because follicles and eggs are visible through the abdominal wall. Females were randomly assigned to two groups. One group of females was behavior tested when they were previtellogenic (i.e., had no visible follicles or eggs; $n=7$), a time when sex steroid concentrations are low (Rhen et al., 2000). In accord with our previous study, all females in this group were unreceptive to courting males. The second group of females was behavior tested when their follicles were >12 mm in diameter (i.e., during late vitellogenesis; $n=7$), when sex steroid concentrations are elevated (Rhen et al., 2000). These females were sexually receptive as observed in our previous study. After behavior tests were completed, females were euthenized by rapid decapitation, their brains immediately frozen in isopentane, and stored at -80°C until sectioning. The same was done for oviducts from two females in each group.

2.2. Tissue preparation, *in situ* hybridization, and silver grain quantification

In brief, frozen sections of tissue $10\ \mu\text{m}$ thick were melted onto RNase-free, poly-L-lysine-coated microscope slides (Sigma). Sections from experimental animals were placed on a series of slides for hybridization to different probes, and, all together, 10 series of slides were collected. Sections were dried at room temperature and then stored in slide boxes at -80°C . Oviducts from four females were also sectioned at $10\ \mu\text{m}$ to examine AR-mRNA expression within the reproductive tract. The procedures used to clone a 374 bp fragment of the leopard gecko AR-mRNA have been described previously, as have the nucleotide and predicted amino acid sequences (Rhen and Crews, 2001). The cloned fragment of the leopard gecko AR spans from the A/B domain into the ligand-binding domain of the receptor.

The method used for *in situ* hybridization has also been described in detail (Rhen and Crews, 2001). In brief, radiolabeled (^{35}S -CTP, NEN) antisense and sense cRNA probes were synthesized by *in vitro* transcription using T7 RNA polymerase. Unlabeled antisense cRNA probes were made in the same manner, except that cold CTP replaced ^{35}S -CTP in the transcription reaction. Probes were separated from unincorporated nucleotides by ethanol precipitation in ammonium acetate. cRNA probes were resuspended, denatured at 85°C , and added to the hybridization solution at a final concentration of $0.3\ \mu\text{g probe} \times \text{length in kb per ml}$. For the first control, an additional 100-fold excess of competing unlabeled antisense probe was added to the hybridization solution. The hybridization solution consisted of 50% deionized formamide, 10% dextran sulfate, 0.3 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, $1\times$ Denhardt's solution, 10 mM dithiothreitol, and 0.5 mg/ml tRNA.

Prior to prehybridization, tissues were fixed in 4% paraformaldehyde (pH 7.2) for 20 min, rinsed in $3\times$ phosphate-buffered saline (PBS) for 5 min, rinsed twice for 5 min (10 min total) in $1\times$ PBS, dipped in RNase-free water, and washed in fresh 0.1 M triethanolamine (pH 8.0) and 0.25% acetic anhydride for 10 min. Tissues were then rinsed in $2\times$ SSC (0.3 M sodium chloride, 0.03 M sodium citrate, pH 7), dehydrated in 70%, 95%, and 100% ethanol for 3 min each, delipidated in chloroform for 5 min, rinsed in 100% and 95% ethanol, and air dried.

Tissues were prehybridized for 16 h at 45°C in hybridization solution in which the cRNA probe was replaced with tRNA at a final concentration of 2.5 mg/ml. Prehybridization took place in plastic containers containing moist paper towels. Tissues were rinsed clean of the prehybridization solution using $2\times$ SSC for 3 min. Tissues were then dehydrated in 70% and 95% ethanol for 3 min each and air dried.

Hybridization solution was then applied to the tissues and incubated overnight at 45°C . After hybridization, tissues were washed twice for 15 min at 50°C in $1\times$ SSC, 50% formamide, and 0.1% β -mercaptoethanol. Tissues were then RNase A (20 $\mu\text{g/ml}$) digested in a buffer consisting of 10 mM Tris-HCl, 100 mM sodium chloride, and 2 mM EDTA for 30 min at 37°C . RNase A was then washed off using the same buffer for 30 min at 37°C . Two consecutive washes for 15 min each at 50°C were done in $1\times$ SSC, 50% formamide, and 0.1% β -mercaptoethanol. Two washes for 30 min each at 50°C were then done in $0.1\times$ SSC and 1% β -mercaptoethanol. Final washes were 50% and then 85% ethanol and ammonium acetate for 3 min each and 100% ethanol for 3 min. Tissues were dried and dipped in Kodak NTB-2 autoradiographic emulsion, dried, exposed at 4°C for 15 days for AR-mRNA. The slides were developed in Kodak D-19 developer, fixed, rinsed in water, stained in cresyl violet, and coverslipped using Permount. Tissues were examined using darkfield and brightfield microscopy and images captured digitally. Brightness and contrast were adjusted using Adobe Photoshop 5.0 to improve image quality. The specificity of *in situ* hybridization for AR-mRNA was demonstrated by using (1) slides hybridized to radioactively labeled sense probe, (2) slides hybridized to labeled antisense probe in the presence of 100-fold excess unlabeled antisense probe, or (3) slides hybridized to labeled antisense probe after slides were pretreated with RNase A (20 $\mu\text{g/ml}$ for 30 min). All slides were counter-stained with cresyl violet to facilitate identification of nuclei.

The procedure for darkfield quantification of silver grains in labeled cells has been described previously (Fleming and Crews, 2001; Godwin and Crews, 1999; Wennstrom and Crews, 1998; Young et al., 1995). Slides were coded and randomized so that the reproductive status of individual females was unknown to the researcher.

Brain nuclei were located at 10 \times , and silver grain density was quantified at 40 \times . All readings were done unilaterally, and the side was chosen randomly. The Grains image analysis program (v. 1.2—University of Washington) selected five of the most densely labeled clusters, then counted the number of silver grains in all the clusters. For each reading of a nucleus, we took background measure-

ments of silver grain density at a location adjacent to the nucleus, and we subtracted this background reading from each reading of the nucleus. This became our measurement of signal intensity above background, and we used this number to calculate the average grains/cluster.

We analyzed AR-mRNA expression in rostral and caudal divisions of the medial preoptic area (MPOAr

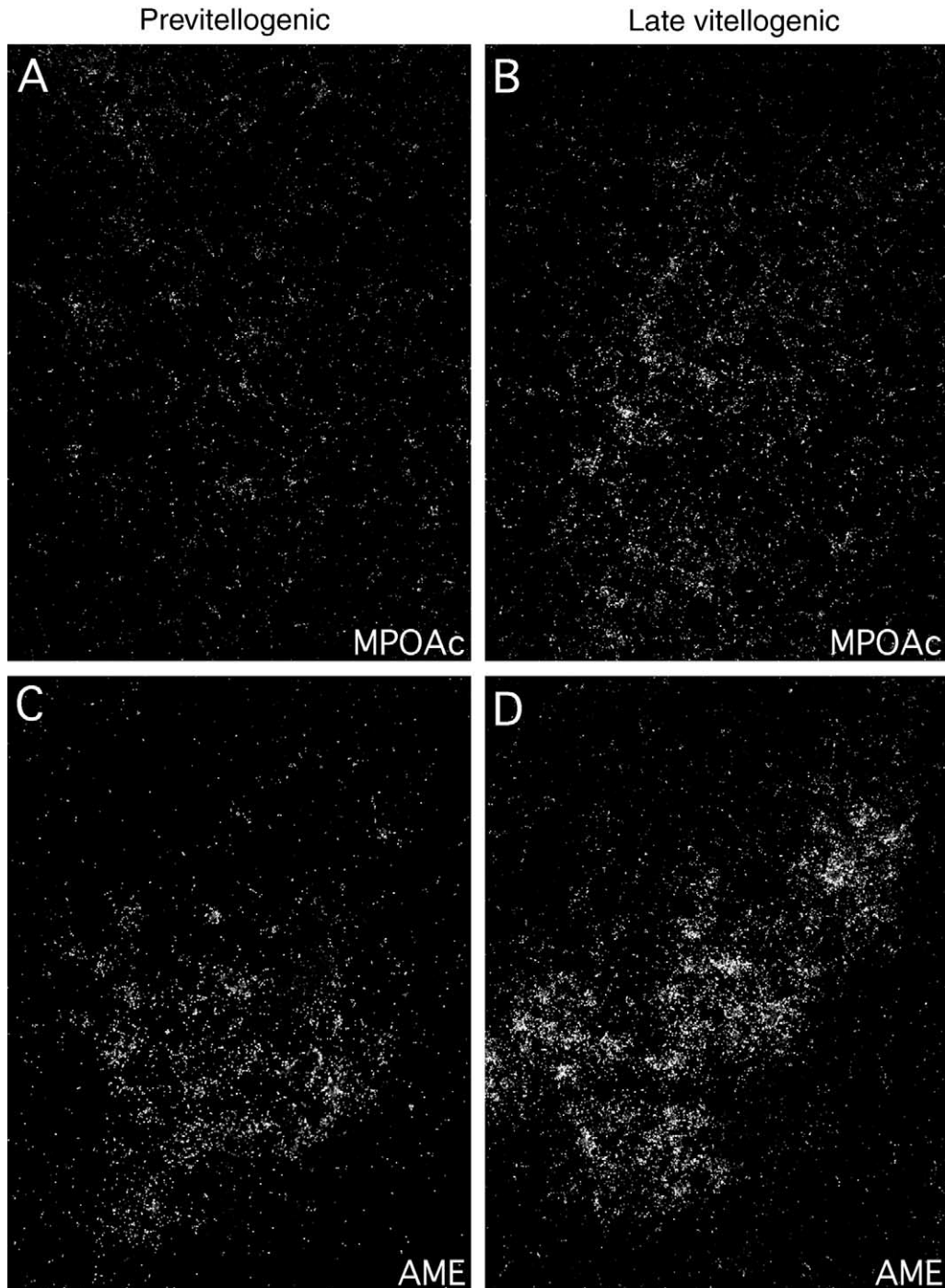


Fig. 1. Darkfield photomicrographs of silver grain distribution over cells in the brains of female leopard geckos hybridized to AR-mRNA antisense probe. Images are representative of the mean difference in AR-mRNA expression between previtellogenic and late vitellogenic females. (A) AR-mRNA in the MPOAc of a previtellogenic female. (B) AR-mRNA in the MPOAc of a late vitellogenic female. (C) AR-mRNA in the AME of a previtellogenic female. (D) AR-mRNA in the AME of a late vitellogenic female.

and MPOAc), the periventricular preoptic area (PP), the anterior hypothalamus (AH), the medial amygdala (AME), the ventromedial and dorsolateral aspect of the ventromedial hypothalamus (VMHm and VMHdl, respectively), the lateral septum (LS), the periventricular hypothalamus (PVH). Nuclei were identified using the descriptions provided by Young et al. (1994). Androgen receptor mRNA expression in the oviduct was not quantified using computer software because the Grains image analysis software had difficulty in quantifying the extremely high expression observed in the previtellogenic females.

2.3. Statistical analysis

Patterns of AR-mRNA expression (i.e., number of grains per cluster) were analyzed using multivariate analysis of variance (MANOVA). This method was employed because multiple measures of steroid receptor mRNA abundance in various nuclei of one individual are not independent variables. Moreover, MANOVA is a robust statistical method that does not make stringent assumptions about the structure of the covariance matrices among variables (Crowder and Hand, 1990). In addition, MANOVA is a powerful method to discern small but reliable differences across a suite of traits. We used a repeated measures MANOVA design to test for overall differences between previtellogenic and vitellogenic females, overall differences among brain areas, and interactions between reproductive state and brain area. The latter effect, if significant, would represent region specific differences in steroid receptor mRNA abundance between previtellogenic and vitellogenic females. We used Pillai's trace as our multivariate test statistic because it is robust to deviations in multivariate normality and group differences in the variance-covariance matrix. To determine how AR-mRNA expression covaried among nuclei, we also examined the data using correlation analysis. All statistics were done using JMP 3.2 software (SAS Institute).

3. Results

3.1. AR-mRNA in the brain

Reproductive status had a significant effect on expression of AR-mRNA (F -statistic = 6.7, p = 0.024). The relative amount of AR-mRNA across various brain areas was greater in late vitellogenic than in previtellogenic females. Darkfield photomicrographs shown in Fig. 1 illustrate the mean difference in AR-mRNA expression in the MPOAc and AME as a function of reproductive status. This same pattern was observed in the MPOAr, MPOAc, AH, AME, VMHdl, LS, and PVH (Fig. 2). In contrast, this difference was not apparent in

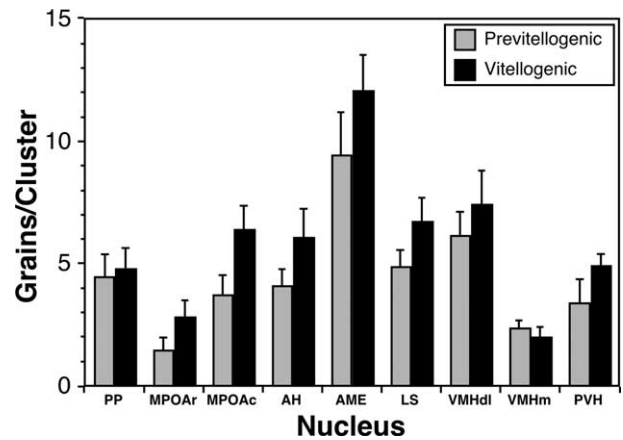


Fig. 2. Androgen receptor mRNA expression in the brains of previtellogenic and late vitellogenic female leopard geckos. Presented are the mean numbers of silver grains per cluster in each nucleus after in situ hybridization with the antisense probe. Abbreviations are as noted in the text. Bars represent standard errors.

the PP or VMHm. There were significant differences in abundance of AR-mRNA among brain areas (F -statistic = 17.4, p = 0.003). AR-mRNA expression was highest in the AME, and moderate levels were found in the PP, MPOAc, AH, LS, VMHdl, and PVH. The lowest levels were found in the MPOAr and VMHm. Although the effect of reproductive status on AR-mRNA abundance appeared to differ among brain regions, there was not a significant interaction between these factors (F -statistic = 1.43, p = 0.36).

There were strong positive correlations for AR-mRNA expression between the AH and AME (ρ = 0.67, p = 0.009), between the MPOAr and AME (ρ = 0.83, p = 0.0003), and between the LS and PVH (ρ = 0.83, p = 0.0003). A weaker positive correlation occurred between the AH and MPOAr (ρ = 0.58, p = 0.03). In

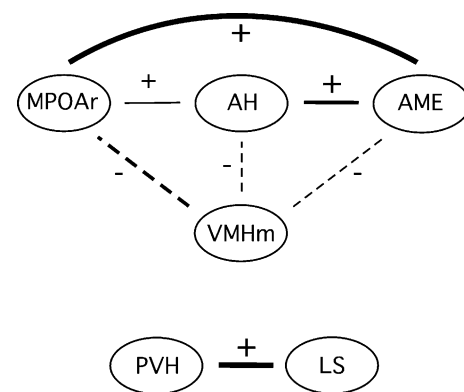


Fig. 3. Line drawing of select nuclei in the leopard gecko brain illustrating significant correlations for AR-mRNA expression among the MPOAr, AH, AME, VMHm, PVH, and LS. Positive correlations are represented by solid lines and negative correlations by dashed lines. The strength of a given correlation is proportional to the thickness of the lines connecting the nuclei. Abbreviations are as noted in the text.

contrast, there was a negative correlation between the VMHm and MPOAr ($\rho = -0.62$, $p = 0.019$) and weak negative correlations between the VMHm and AME ($\rho = -0.52$, $p = 0.057$) and between the VMHm and AH ($\rho = -0.48$, $p = 0.08$). Correlations among other pairs of nuclei did not reach statistical significance. These relationships are summarized in Fig. 3.

3.2. AR-mRNA in the oviduct

Reproductive status had a dramatic influence on the morphology of the oviduct (Figs. 4A,B). The overall size of the oviduct increased greatly during late vitellogenesis in association with the development of shell glands. Expression of AR-mRNA was very high in both the luminal epithelium and stroma of the oviduct in previtellogenic females (Fig. 4C). In contrast, expression of AR-mRNA was prominent in the luminal epithelium of late vitellogenic females, but was much lower within the stroma (Fig. 4D).

4. Discussion

The main finding in the current study was that the relative abundance of androgen receptor (AR) mRNA

across a number of limbic nuclei was greater in late vitellogenic than in previtellogenic female leopard geckos, *E. macularius*. This pattern was observed in the rostral and caudal medial preoptic area (MPOAr and MPOAc), the anterior hypothalamus (AH), the external nucleus of the amygdala (AME), the dorsolateral aspect of the ventromedial hypothalamus (VMHdl), the lateral septum (LS), and the periventricular hypothalamus (PVH), but not in the periventricular preoptic area (PP) or the medial part of the ventromedial hypothalamus (VMHm). Regional differences in AR-mRNA expression were also evident, with the highest expression in the AME and VMHdl and the lowest expression in the MPOAr and VMHm. Levels of AR-mRNA were regulated differently among cell types in the oviduct. Whereas AR-mRNA expression was high in the stroma and luminal epithelium of previtellogenic females, levels of AR-mRNA decreased in the stroma of late vitellogenic females.

Changes in AR-mRNA expression during the reproductive cycle raise questions about the potential function of androgens and the mechanisms regulating AR-mRNA levels in different tissues and cell types. Testosterone (T) concentrations increase by two orders of magnitude from the previtellogenic stage to the late vitellogenic stage, which is when females are most receptive to courting

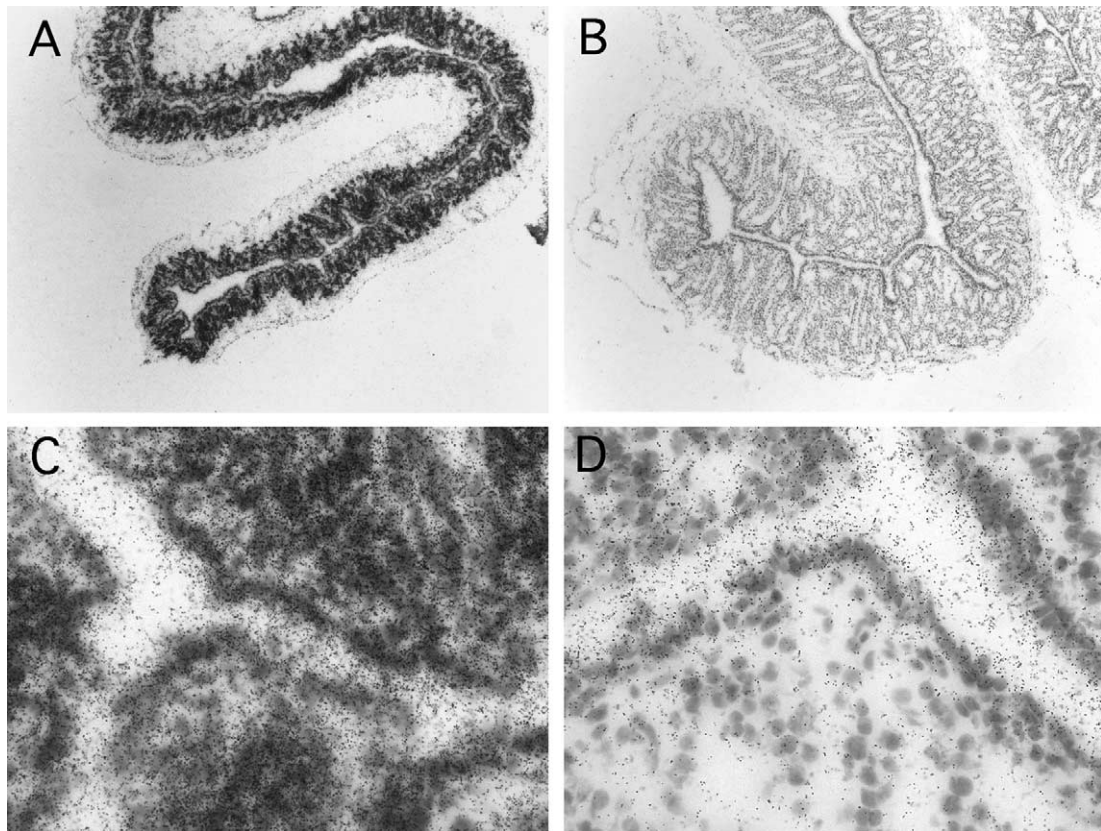


Fig. 4. Low and high magnification photomicrographs of oviducts from previtellogenic and late vitellogenic female leopard geckos. Images in the upper panels illustrate gross differences in oviduct morphology between (A) previtellogenic and (B) late vitellogenic females. Images in the lower panels show differences in AR-mRNA expression (i.e., silver grain distribution) between (C) previtellogenic and (D) late vitellogenic females.

males (Rhen et al., 2000). Short-term treatment with T increases receptivity without affecting circulating concentrations of 17β -estradiol (E2) (Rhen et al., 1999). Moreover, brain nuclei with elevated levels of AR-mRNA in late vitellogenic leopard geckos are involved in the control of sociosexual behaviors in other vertebrates (Flanagan et al., 1993; Freeman and Rissman, 1996; Gill et al., 1998; Kato and Sakuma, 2000; Pfaus et al., 1993; Veney and Rissman, 1997; Veney and Rissman, 2000; Yang et al., 1999). In particular, the VMHdl has been implicated in the control of estrogen-dependent receptive behavior in whiptail lizards. Lesions of this area, which contains abundant estrogen receptor (ER) mRNA in whiptails, abolish E2-induced receptive behavior (Kendrick et al., 1995; Young et al., 1994). Whereas AR-mRNA was especially abundant in the VMHdl of female leopard geckos (this study), ER-mRNA was not detected (Rhen and Crews, 2001). Androgens and AR in the VMHdl might therefore play a role in the regulation of receptive behavior in female leopard geckos. Alternatively, aromatization of T into E2 in other regions of the brain that contain ER may be critical because receptivity can be induced by exogenous E2 but not by DHT (Rhen and Crews, 2000; Rhen et al., 1999). Another possibility is that both hormones are important in females. Ultimately, it will be vital to characterize AR, ER, and aromatase function to identify nuclei that control receptive behavior in the leopard gecko.

The pattern of AR-mRNA expression was different in the oviduct. Abundance of AR-mRNA in the luminal epithelium did not change with reproductive stage, but AR-mRNA levels were dramatically reduced in stromal cells in late vitellogenic compared to previtellogenic females. This finding indicates that AR-mRNA is specifically down-regulated in the stroma. Shell glands differentiate in parallel with the increase in circulating levels of androgens and the decrease in AR-mRNA expression. These results suggest that androgens regulate at least some aspects of oviduct morphology and function in the leopard gecko. Androgen action in female vertebrates is in general poorly understood. However, observations in various species indicate that androgens are important. For example, female knockout mice for 5α -reductase, which converts T into DHT, have problems with parturition (Kimura et al., 1999). Female knockout mice for AR have smaller litter sizes than wild type littermates (Yeh et al., 2002). In the rat uterus, AR appears to modulate E2-induced cell proliferation (Weihua et al., 2002). Most tissue types in the chicken oviduct express AR, and experimental evidence indicates that androgens also play a direct role in growth and differentiation of this tissue (Joensuu et al., 1992; Tokarz et al., 1979; Yu and Marquardt, 1973a,b). Taken together, these and other studies suggest that androgens could have a conserved physiological function in the female reproductive tract.

Changes in AR-mRNA expression during the reproductive cycle of female leopard geckos may reflect the fact that AR is an auto-regulated gene. Androgenic regulation of AR-mRNA is cell-type dependent in other vertebrates: androgens can act to down-regulate AR-mRNA and protein abundance in some cell types or up-regulate AR-mRNA and protein expression in others (Burnstein et al., 1995; Grad et al., 1999). For instance, gonadectomy of mice decreases AR protein levels in the hypothalamus (Lu et al., 1998; Lu et al., 1999). Conversely, androgen replacement elevates AR protein levels in a dose-dependent manner in the brains of both male and female mice (Lu et al., 1998). Testosterone has also been shown to up regulate AR in the brain of female Atlantic croaker, *Micropogonias undulatus* (Larsson et al., 2002). Autologous up-regulation of human AR-mRNA by androgens in cell culture depends upon androgen responsive elements in AR-mRNA (Grad et al., 1999). It is interesting to note that these androgen responsive elements are perfectly conserved in the nucleotide sequence for the gecko AR-mRNA (compare Fig. 1 in Rhen and Crews, 2001 to Fig. 6 in Grad et al., 1999). These observations suggest that the mechanism responsible for androgen up-regulation of AR-mRNA in the vertebrate brain may be conserved from fish to reptiles to mammals. Down-regulation of AR-mRNA is dependent upon the same intragenic androgen responsive elements, which confer cell type specific regulation of AR-mRNA levels (Burnstein et al., 1995). However, the mechanisms responsible for tissue and cell-specific patterns of AR-mRNA regulation during the reproductive cycle in the female leopard gecko remain to be determined.

In summary, elevated expression of AR-mRNA in the brain during late vitellogenesis is correlated with higher circulating concentrations of androgens and greater propensity for receptive behavior in this species (Rhen et al., 2000). Previtellogenic and late vitellogenic females also differ in oviduct morphology and AR-mRNA expression. Although cyclical changes in concentrations of androgens and abundance of AR-mRNA in the oviduct and forebrain suggest that AR might play a role in regulating reproductive physiology and behavior, further studies are required to determine the specific role of T acting as an androgen versus T that is aromatized to E2.

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