Sex Differences in Estrogen and Progesterone Receptor Messenger Ribonucleic Acid Regulation in the Brain of Little Striped Whiptail Lizards

Key Words
Gonadal steroids
Gonadal steroid receptors
Sex behavior
Sexual dimorphism
Reptiles

Abstract
Sex differences in the regulation of steroid hormone receptors in brain areas controlling female- and male-typical sexual behavior may be important in determining sex differences in the display of these behaviors. This study examined sex differences in estrogenic effects on the relative abundance of messenger RNA for estrogen receptor (ER) and progesterone receptor (PR) in discrete brain areas of whiptail lizards, *Cnemidophorus inornatus*, by in situ hybridization with radiolabeled riboprobes. Gonadectomized females and males received an estradiol benzoate (EB) injection (0.5 µg) which effectively induces receptive behavior in females; controls received vehicle alone. Sex and regional differences in estrogenic effects on ER- and PR-mRNA abundance were found. Females responded to EB treatment with increases in ER- and PR-mRNA relative abundance in the ventromedial nucleus of the hypothalamus (VMH). Males had similar relative mRNA abundances to females in gonadectomized controls, but did not exhibit increases with EB treatment. EB treatment increased ER-mRNA abundance in the dorsal hypothalamus of females, but not males. ER-mRNA decreases in the lateral septum and PR-mRNA increases in the posterior hypothalamus with hormone treatment were also found, but did not differ by sex. Neither sex nor treatment effects were definitively shown for ER- or PR-mRNA abundance in the anterior hypothalamus-preoptic area. The VMH controls female-typical receptive behavior in this species. Sex differences in the response to estrogen in this nucleus may therefore underlie sex differences in the display of receptive behavior. The VMH sex differences described are similar to those in rats in that females exhibit estrogenic regulation of ER- and PR-mRNA while males do not, suggesting that this pattern is evolutionarily conserved.

Introduction
Characterization of steroid hormone receptor regulation in brain areas important for sex-typical reproductive behaviors is an important step in identifying the neural bases of these behaviors and sex differences in their occurrence. The distribution of intracellular steroid hormone receptors in the brain has been described for species from all the major vertebrate groups and is strikingly conserved [1, see 2 for review]. Sex differences in steroid
receptor distribution have not been found. However, sex differences in measured levels of steroid receptor proteins and messenger RNA encoding these proteins have been described in the rat [3–8]. These sex differences in receptor regulation and their behavioral correlates are especially well studied in the ventromedial nucleus (VMH) of the mediobasal hypothalamus. This tuberal area of the hypothalamus has been implicated as critical to female-typical sexual behavior in a broad range of vertebrates [2].

A continuing focus of this laboratory is the identification and characterization of the neural substrates of male- and female-typical behavior in an ancestor-descendant species pair of lizards. These Cnemidophorus species are native to the desert grasslands of Southwestern North America. The ancestral species, Cnemidophorus inornatus, is sexual while the descendant species, Cnemidophorus unipares, consists entirely of females and reproduces by parthenogenesis. Despite being female in morphology of the body and size of nuclei in the limbic system, individuals of C. unipares naturally display both female-like receptive behavior and male-like mounting behavior [9]. This is in contrast to C. inornatus where only females display receptive behavior and only males display mounting behavior.

Radiofrequency lesion, intracranial steroid implantation, and brain metabolic capacity studies have established that, similar to other vertebrates, the VMH of the mediobasal hypothalamus is a primary area controlling sexual receptivity and the anterior hypothalamus-preoptic area (AH-POA) is a primary area controlling mounting and intromission behavior in both C. inornatus and C. unipares [10–15]. Previous work has demonstrated sexual dimorphisms in the size of the nucleus and the soma of neurons in the VMH and AH-POA of C. inornatus [16, 17], as well as in the steroid regulation of these dimorphisms [18, 19]. Portions of the estrogen (ER), progesterone (PR) and androgen receptors (AR) have been cloned and used in in situ hybridization studies to map the distribution of cells expressing these mRNAs in the brain of both C. inornatus and C. unipares [1]. Additionally, variations in the abundance of ER- and PR-mRNA in discrete limbic nuclei have been compared between female C. inornatus and C. unipares both over the course of the ovarian cycle and in response to exogenous estradiol benzoate [20, 21]. As in rats, the expression of estrogen-induced PR-mRNA is strongly correlated to the expression of female-typical receptive behavior in both C. inornatus and C. unipares [22].

This study compares the relative expression of ER- and PR-mRNAs between female and male C. inornatus in two conditions: gonadectomized and following injection of the dosage of estradiol benzoate which effectively induces receptive behavior in females. We do not attempt a general characterization of sex differences in the control of steroid receptor mRNAs in Cnemidophorus here. This is the first characterization of sex differences in regulation of these receptors outside the mammals generally and lizards in particular, and the first study at the level of steroid receptor mRNA regulation in any animal besides the rat. The specific question addressed is whether there are differences between females and males in ER- and PR-mRNA abundance in discrete limbic nuclei either basal or in response to estrogen stimulation. More generally, how do sex differences in estrogenic regulation of mRNAs and/or PR-mRNA abundance correlate with the display of female-typical receptive behavior?

Materials and Methods

Animals
Male and female C. inornatus were captured near Sanderson, Texas, USA, transported to the University of Texas, and maintained in environmental chambers under breeding season conditions described previously [13].

Gonadectomy and Hormone Treatment
Male and female experimental animals were taken from presumably wild housed conditions, gonadectomized, and held in social isolation described previously [13] for 1 week to allow metabolic clearance of endogenous gonadal steroids. Hormone treatment consisted of a single injection of either (a) 0.5 μg estradiol benzoate (EB, Sigma) dissolved in 10 μl Steroid Suspending Vehicle (SSV; supplied by National Cancer Institute, NIH, USA; ingredients per 500 μl: 9 mg sodium chloride, 5 mg sodium carboxymethylcellulose, 0.004 ml polysorbate 80, 0.009 ml benzyl alcohol) or (b) 10 μl alone as a control. This EB-injection protocol reliably induces receptive behavior in female C. inornatus [21]. Subcutaneous injections were given between 12.00 and 14.00 h at 1 week following gonadectomy. A Hamilton glass syringe with a fixed needle was inserted tangentially approximately 2–3 cm caudal to the scapula on the surface of the animal and 0.5 cm lateral to the midline. The needle was moved directly anterior between the skin and body masses to the area of the scapula and the injection delivered.

Tissue Preparation
Experimental animals were killed by quick decapitation after injection. Their brains were quickly removed, frozen on dry ice and stored at −80°C until sectioning. 20-μm coronal cryosections were taken on a cryostat in series of six and melted onto ribon-free poly-L-lysine-coated microscope slides. Placing sections on different slides in the series of six slides allowed control of adjacent sections hybridized to either sense strand control or probes for a different mRNA species in the in situ hybridization.
In situ Hybridization and Silver Grain Quantification

The protocols and validation of the in situ hybridization, autoradiography, and grain quantification procedures used in this study have been described [1, 20]. Figure 1 illustrates the LYCUER1b and LYCUSPR3a clones used to generate riboprobes and their structures relative to human receptor structures. All slides in all treatment groups were processed in the in situ hybridization procedure at the same time. Following hybridization, slides were dipped in Kodak NTB-2 emulsion and allowed to expose at 4°C for 11 days for quantification of PR-mRNA and 3 weeks for ER-mRNA, developed in Kodak D-19 developer, and fixed. Silver grain density was defined as number of grains per cluster where clusters were groups of silver grains lying over cell somata in discrete, cresyl violet defined brain nuclei on sections which were anatomically matched between individual lizards (see Young et al. [1] and figure 2 for anatomical maps of the Cnemidophorus brain). Silver grain density was quantified using the 'Grains' program [D.K. Clifton, University of Washington, pers. commun.] on a Macintosh IIci computer equipped with an image-capture system exactly as previously described [20]. Control slides hybridized to sense strand control probes exhibited uniform background densities of silver grains and no specific labeling of cells. Sample sizes differ for different nuclei because sections were sometimes lost in cryosectioning.

Statistical Analysis

Mean silver grain densities (grains/cluster) measured in given nuclei were compared across treatment groups by two-way analysis of variance. The primary effects of interest here were those of treat-

Fig. 1. Schematic diagrams showing the size and position of the Cnemidophorus estrogen receptor (LYCUER1b), and progesterone receptor (LYCUSPR3a) clones relative to the full-length human sequence. The structures of the human estrogen receptor (hER) and progesterone receptor (hPR) are illustrated above the lizard clones to demonstrate the relative position of the sequences used to generate the radioactive probes used in the present study. The numbers positioned above and below the human receptor structures are amino acid sequence positions for the hER [33] and the hPR [34].

Fig. 2. Drawings of coronal sections of a Cnemidophorus brain illustrating the neuroanatomical positions of the brain nuclei in which estrogen receptor mRNA and progesterone receptor mRNA were quantified. For a more detailed atlas of the Cnemidophorus brain, see Young et al. [1]. pvPOA = Periventricular nucleus of the preoptic area; MPA = medial preoptic area; LS = lateral septum; LFB = lateral forebrain bundle; VE = ventricular ependymal organ; LTP = lentiformis thalami pars plicata; DH = dorsal hypothalamus; VMH = ventromedial nucleus of the hypothalamus; PH = periventricular nucleus of the hypothalamus; TS = torus semicircularis. Anterior hypothalamus (AH) not shown.
Table 1. Silver grain density measured as number of grains per cluster in various diencephalic nuclei of C. inornatus females and males and in either EB- or vehicle-injected treatments

<table>
<thead>
<tr>
<th></th>
<th>Females</th>
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<th>Males</th>
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<td></td>
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<td>p</td>
<td>0.5 µg EB</td>
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<td>Ventromedial</td>
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<td>31.30±1.89 (6)</td>
<td>**</td>
<td>19.07±1.89 (6)</td>
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<td>hypothalamus</td>
<td>9.35±1.55 (5)</td>
<td>13.75±0.68 (6)</td>
<td>*</td>
<td>15.29±1.74 (6)</td>
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<td>Dorsal hypothalamus</td>
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<td>20.46±1.69 (4)</td>
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<td>23.28±2.56 (6)</td>
</tr>
<tr>
<td>Posterior</td>
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<td>17.32±2.12 (6)</td>
<td>ns</td>
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<td>hypothalamus</td>
<td>20.76±3.11 (4)</td>
<td>20.51±0.84 (5)</td>
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<td>21.40±1.80 (6)</td>
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<td>Anterior</td>
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<td>16.98±1.67 (6)</td>
<td>ns</td>
<td>18.10±0.89 (5)</td>
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<td>33.59±4.11 (5)</td>
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<td>hypothalamus</td>
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<td>12.29±1.63 (5)</td>
<td>*</td>
<td>7.82±1.08 (6)</td>
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<td>hypothalamus</td>
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<td>32.77±5.42 (5)</td>
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<td>33.78±3.97 (6)</td>
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<td>Medial preoptic area</td>
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<td>12.16±1.64 (5)</td>
<td>ns</td>
<td>12.34±1.22 (4)</td>
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Values are means ± 1 SEM, samples sizes shown in parentheses; t test probability values: * 0.10 > p > 0.05; ** p < 0.05; *** p < 0.01.

Discussion and treatment by sex interactions. To better characterize treatment differences within sex, preplanned contrasts of the control and EB treatments were performed with two-sample t tests. Data were log10 transformed to reduce heterogeneity of variance among treatment groups where necessary [23]. All analyses were performed using SYSTAT 5.1.2 on an Apple Macintosh computer.

**Results**

**ER-mRNA Expression**

ER-mRNA abundance was significantly higher in the VMH of EB-injected female C. inornatus than in vehicle-injected controls, but no difference between treatments was seen in males (table 1, fig. 3; 2-way ANOVA: sex effect p < 0.01, treatment effect p < 0.01, sex by treatment interaction p < 0.05; t test comparisons: female vehicle control vs. female EB p < 0.01; male vehicle control vs. male EB p = 0.54). ER-mRNA abundance was also higher in the DH of EB-injected females than controls (fig. 3, t test female control vs. female EB p < 0.03; male control vs. male EB p = 0.66). This sex difference in the DH was supported by a near significant sex by treatment interaction in ER-mRNA abundance (2-way ANOVA p = 0.05). Male and females did not show a difference in DH ER-mRNA with hormone treatment, DH ER-mRNA was more abundant in male than female controls (a posteriori t test comparison: male vehicle control vs. female vehicle control, p < 0.05). ER-mRNA abundance was significantly lower in the lateral septum of EB-injected animals than controls (fig. 3; treatment effect p = 0.01), but there was no apparent sex difference in response (sex by treatment interaction p = 0.84). Within-sex comparisons suggested that the effect was stronger in males (t test: p < 0.04) than in females (t test: p = 0.12). No sex or treatment differences were found in ER-mRNA abundance in the AH, PH, pvPOA, MPOA, or TS. There was a trend suggesting lower ER-mRNA abundance in the pvPOA of males (t test: p = 0.07). One control female had a particularly high grain density in the TS (grains/cluster = 70) that was almost 3 times higher than the group mean for the remaining 40 individuals (26.6 ± 5.6 grains/cluster) and was therefore dropped from the analysis.

**PR-mRNA Expression**

As found for ER-mRNA, PR-mRNA abundance was significantly higher in the VMH of EB-injected females than controls, but no difference was seen in males (table 1, fig. 4; 2-way ANOVA: treatment effect, p < 0.001; sex by treatment interaction, p < 0.01; sex effect, p = 0.20; t test comparisons: female control vs. female EB, p < 0.01; male control vs. male EB, p = 0.30). PR-mRNA abundance in the PH was also significantly higher in the EB treatment.
(fig. 4; treatment effect, p < 0.02), but the effect did not appear to differ by sex (sex effect, p = 0.47; sex by treatment interaction, p = 0.56). Within-sex comparisons showed female, but not male, PR-mRNA to be significantly elevated by EB (t test comparisons: female control vs. female EB, p < 0.05; male control vs. male EB, p = 0.19). No sex or treatment effects on PR-mRNA levels were found in the MPOA, pvPOA, or TS. This was a weak comparison for the MPOA as only 2 individuals were measured in the female vehicle control group. Silver grain intensities in the DH, AH, and lentiformis thalami plicta were very low and not measured.
Discussion

Receptor Regulation

The findings for regulation of ER-mRNA and PR-mRNA in this study are in good agreement with previous work in Cnemidophorus. Both the ophideren C. unipares and female C. inornatus exhibit more ER-mRNA and PR-mRNA in the VMH and a stimulation of receptive behavior in response to a 0.5-μg EB injection [20]. Because these effects of EB were replicated for female C. inornatus here, the finding of a lack of such effects in male C. inornatus is strengthened. It might be argued that although this EB dose and administration protocol are effective both behaviorally and in increasing ER- and PR-mRNAs in female C. inornatus, male C. inornatus are not physiologically responsive to it for some reason. This was not the case. Male C. inornatus did respond to EB treatment with a decrease in ER-mRNA levels in the lateral septum and a trend towards a similar decrease in the periventricular preoptic area. Moreover, the pattern of change in ER-mRNA with treatment (significant treatment effect) in the lateral septum is similar between male and female C. inornatus, and supports a previous finding of lower ER-mRNA abundance in the lateral septum of C. unipares with this EB administration protocol [20]. The possibility that male C. inornatus possess ER-mRNA but not ER protein in the nuclei examined also appears unlikely based on LR-like immunoreactivity demonstrated in these nuclei in male C. inornatus with a mammalian ER antibody [Gahr and Crews, unpubl. data].

The sex differences in receptor mRNA abundance with estrogen treatment in the VMH of C. inornatus agree with findings for both ER-mRNA and PR-mRNA in rats, except with respect to the direction of ER-mRNA regulation in females by estrogen. Acute estrogen treatment decreases ER-mRNA abundance in the ventromedial nucleus of the mediobasal hypothalamus (VMn) in female rats, but not in males [6]. Testosterone did decrease VMn ER-mRNA in male rats [24]. In contrast, exogenous estradiol increased ER-mRNA abundance in female C. inornatus as described previously [21] but had no discernible effect in males. The possibility of ER-mRNA regulation in the male C. inornatus VMH by testosterone is under investigation.

The greater abundance of ER-mRNA in the DH of EB-injected females than controls, higher abundance in control males than control females, and similar, but not statistically significant, patterns in the PH are difficult to interpret. No data are available for comparison from other species and little is known of the functions of these nuclei. Farragher and Crews [25] found that lesions in the basal hypothalamus area (in the region of the dorsal and posterior hypothalamic nuclei) of male green anole lizards, Anolis carolinensis, caused decreases in both courtship and agonistic behaviors, but not the gonadal collapse which was observed in more anteriorly placed lesions. In slight contrast to this study, a previous study on C. unipares [20] found that EB injection produced a nonsignificant increase in ER-mRNA in the DH and significant increase in TS ER-mRNA. The means for TS ER-mRNA did not differ with EB treatment here for either females or males, but the control mean for females is nonsignificantly lower than that for EB-injected animals and the proportional difference between groups is similar to that found in C. unipares.

PR-mRNA abundances in the VMH and the pattern of sex differences in C. inornatus is similar to that described in rats. PR-mRNA is increased in the VMn of ovarioctomized female rats by exogenous EB, but neither EB nor testosterone propionate alters PR-mRNA abundance in the VMn of gondactomized male rats [7, 8]. The sex difference in VMn PR-mRNA regulation correlates well with a sex difference in VMn PR protein, although these studies show male rats are not completely unresponsive to estrogen stimulation at the receptor protein level [3, 4]. Various mammalian monoclonal and polyclonal antibodies do not give PR-like immunoreactivity in Cnemidophorus [Gahr and Crews, unpubl. data], and no data are available regarding progestin binding sites in Cnemidophorus. In the only study of hypothalamic PR protein in reptiles, estradiol treatment increased hypothalamic progestin binding sites roughly twofold in female A. carolinensis [26]. Interestingly, intact male rats have similar VMn PR-mRNA levels to those seen in ovarioctomized EB-treated females, suggesting that either longer-term exposure to steroid hormones or some nonsteroidal influence may stimulate PR-mRNA expression in the intact condition [7, 8]. Receptor mRNA abundances were not examined in intact male C. inornatus.

Definite sex differences were not found in either ERmRNA or PR-mRNA levels or regulation in nuclei of the AH-POA (AH, MPOA, pvPOA) in C. inornatus. This brain area is known to exert primary control over the display of male-typical sexual behavior in Cnemidophorus and likely also control of gonadotropin release through effects on gonadotropin-releasing hormone cells as in other vertebrates [22, 27]. The pattern of PR-mRNA abundance suggested an increase in the pvPOA of EB-injected females (fig. 2), but large variances precluded a powerful test. This EB dose did significantly up-regulate PR.
mRNA in the pvPOA of *C. uniparens* [20], and that finding was replicated with a group of *C. uniparens* included in this study [J. Godwin and D. Crews, unpubl. data]. However, *C. inornatus* females are less sensitive to EB in the VMH [21] and also in the pvPOA with respect to PR-mRNA abundance [J. Godwin and D. Crews, unpubl. data]. A lower relative sensitivity could account for the lack of a statistically significant effect of EB in the pvPOA observed here.

The lack of sex differences otherwise in the AH-POA should be regarded with caution at present as interpretation is limited by several factors including the small sample size in the female control ER-mRNA MPOA group. Also, this EB dose has not been characterized for possible effects on male-typical sexual behavior in *C. inornatus* and the correspondence of ER- and PR-mRNA to actual receptor protein levels is not known.

**Behavioral Correlates of VMH Receptor mRNA Regulation**

This study demonstrates that ER- and PR-mRNA regulation in the VMH of *C. inornatus* in response to a behaviorally effective dose of EB differs between males and females. As noted above, the VMH is critical for the display of female-typical sexual receptivity in *Cnemidophorus* species. A high percentage of female *C. inornatus* reliably exhibit receptivity in response to a 0.5-μg EB injection. This behavioral expression is correlated with increased ER- and PR-mRNA abundance in the VMH [21]. In contrast, male *C. inornatus* do not exhibit increases in either ER-mRNA or PR-mRNA abundance in the VMH and receptive behavior has never been observed in intact males. In a preliminary experiment with long-term castrate (3 months) males, female-typical sexual behavior was not elicited in response to 0.5 μg EB. This might be attributable to a general hyposensitivity to hormonal stimulation related to long-term castration, however, and the observed lack of receptivity in males needs to be tested with short-term castrates.

The precise roles of estrogen and progesterone in the control of receptive behavior in female *Cnemidophorus* are not known. As in other female tetrapods, female *Cnemidophorus* have high estrogen levels and low progesterone levels during the vitellogenic, preovulatory phase of the follicular cycle followed by a decrease in estrogen and increase in progesterone during the postovulatory phase [28, 29]. These circulating steroid changes are associated with the display of receptive behavior prior to ovulation and aggressive behavior following ovulation [30]. Receptive behavior can be stimulated by estrogen alone in ovariectomized females [21], while progesterone can abolish this estrogen-induced receptive behavior by 24 h after administration in *C. uniparens* [J. Godwin and D. Crews, unpubl. data]. It is not known whether this receptivity-abolishing effect of progesterone is preceded by progesterone facilitation of receptive behavior as seen in some species [31, 32]. Further work elucidating the precise roles of estrogen and progesterone in the control of female receptive behavior will be necessary to fully address the molecular basis of female/male differences in the capacity to express this behavior.

Both female rats and female *C. inornatus* exhibit estrogenic regulation of ER- and PR-mRNA in the VMH; males do not. This similarity suggests that while the pattern of regulation of these receptor mRNAs in females shows differences between rats and *Cnemidophorus* [20], sex differences in this regulation are conserved. As with steroid hormone receptor distribution in the brain of vertebrates, sex differences in steroid receptor regulation may have arisen early in the evolution of the neural circuits underlying sexual behavior and continued to be important in sexually differentiating their function.

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