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Research report

Sex differences in estrogen-induced progesterone and estrogen receptor mRNA in the ventromedial hypothalamus of hatchling whiptail lizards

Kira L. Wennstrom[a,*], Cynthia J. Gill[b], David Crews[b]

[a]*Department of Biology, University of Arkansas at Little Rock, 2801 S. University, Little Rock, AR 72204, USA*
[b]*Section of Integrative Biology, University of Texas at Austin, Austin, TX, USA*

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Abstract

The ventromedial hypothalamus (VMH) is an important neural locus for the control of female-typical sexual behavior in vertebrates, and exogenous estrogen stimulates a strong increase in progesterone receptor (PR) in the VMH of adult females. Estrogen also regulates its own receptor (ER), though the direction of the response varies from species to species. In rodents and whiptail lizards, males either lack estrogen regulation of PR and ER mRNA in the VMH or display a greatly attenuated response. We examined hatchlings of two closely related species of whiptail lizards, one of which is parthenogenetic. Though normally all female, the parthenogens can be made to develop as gonadal males by treating with aromatase inhibitor early in development. Thus, we were able to ask whether the brain sex of these 'created male' parthenogens corresponded to their gonadal sex or their genetic sex. We injected 1- and 30-day-old animals of both species and sexes with estradiol benzoate (EB) and assayed for PR and ER mRNA using in situ hybridization. All animals given EB responded with a strong increase in PR mRNA in the VMH. However, females of the sexual species had higher EB-induced PR mRNA levels than did conspecific males; there was no sex difference between the normal parthenogens and the created males of the parthenogenetic species. EB also stimulated an increase in ER mRNA in the VMH, but the pattern of response was more complex. Normal parthenogens did not increase ER mRNA in response to EB in either age group, in contrast to the strong response of 1-day-old males and females of the sexual species and 30-day-old created males. The results indicate that hatchling whiptails show striking species and sexual differences in the regulation of sex steroid receptor mRNAs in an area of the brain important for adult sexual behavior. This variation may play a role in the development of species and sexual differences in the adult neuroendocrine phenotype.

Theme: Neural basis of behavior

Topic: Hormonal control of reproductive behavior

Keywords: Gonadal steroid; Steroid receptor; Brain; Organization; Reptile

1. Introduction

The ventromedial hypothalamus (VMH) plays an important role in the control of female-typical sexual behavior [8,30,27]. Estrogens, whether delivered systemically or implanted in the VMH, stimulate receptive behavior in many species. Progesterone (P), too, modulates the expression of receptivity, but it acts most powerfully when administered following a dose of estrogen. This can be explained mechanistically by the fact that estrogen triggers an increase in the abundance of progesterone receptors (PR) in the VMH, making it more sensitive to a subsequent dose of P [28]. The similarity of this response in birds [11], mammals [5,26,3,17,18], reptiles [32,12,43], and amphibians [29] suggests that it has been evolutionarily conserved. In species where both sexes have been examined (primarily laboratory rodents and whiptail lizards), these estrogen-inducible PR are sexually differentiated; females respond much more strongly than males [17,18,12,6]. In addition to regulating PR expression, estrogens also regulate the expression of estrogen receptors (ER) in the VMH, although the magnitude and direction of this response varies from species to species, depending on the pattern of the reproductive cycle [39]. As is the case

*Corresponding author. Tel.: +1-501-569-3502; fax: +1-501-569-3271.
E-mail address: klwennstrom@ualr.edu (K.L. Wennstrom).

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with PR, the effect of estrogen on ER expression is typically greater in females than in males [17,18,7], but see [31].

The parthenogenetic whiptail lizard *Cnemidophorus uniparens* and its sexually reproducing ancestor *C. inornatus* provide useful animal models for the study of sexual differentiation of the brain in an evolutionary context. Extensive information is available about sexual behavior, reproductive cycles, and the distribution and regulation of steroid hormone receptors in the brains of both species. Work in this laboratory has focused on the fact that the parthenogens naturally display both female-like and male-like pseudosexual behavior. The frequencies of the behaviors alternate depending on the stage of the animal's reproductive cycle [23,22]. Despite these striking behavioral differences, the parthenogens are in many ways remarkably similar to females of the ancestral species. The ovarian cycle of each species is nearly identical, though females of the sexual species have much higher circulating levels of estradiol [23,22]. Both parthenogens and females of the sexual species have a smaller preoptic area–anterior hypothalamus (POAH) volume and a larger VMH volume than do males of the sexual species [9,34]. In addition, females of both species show a strong increase in PR and ER mRNA in the VMH in response to estrogen treatment, while the sexual males do not [12,43]. This robust response can thus serve as a marker for a female-like brain in studies of sexual differentiation.

Another advantage of the *Cnemidophorus* model system is the ability to manipulate the sex of developing embryos. Application of fadrozole, a non-steroidal aromatase inhibitor, to eggs from the all-female parthenogen soon after they are laid causes the treated embryos to develop as gonadal males [38]. These 'created males' have fully developed hemipenes and vasa deferentia, and their testes are capable of producing motile sperm [35]. As adults, they closely resemble genetic males of the sexually reproducing species, with blue ventral coloration and secretory femoral pores. While the created males appear very similar to genetic males in many ways, we know little about their neural phenotype. The volumes of their preoptic area–anterior hypothalamus (POAH) and VMH are not significantly different from that of normal females [37]. Despite this finding, intact created males court and mount females and do not show female-typical receptive behavior when presented with other males [35]. It is possible that this behavior represents a purely activational effect of testosterone; females of both species given exogenous androgen behave similarly [34,15]. One goal of the current research is to discover whether the neuroendocrine response of the created males matches their gonadal sex or their genetic sex. Such a finding would provide insight into the role of the testis in sexual differentiation of the brain in these reptiles.

This paper represents the first attempt to examine the ontogeny of PR and ER gene expression in a reptilian model and the first in which gonadal sex is manipulated independent of genetic sex. The experiments described here were designed to determine whether young whiptail lizards are capable of increasing PR and ER mRNA in the VMH in response to exogenous estrogen, and whether that response is sexually differentiated.

2. Materials and methods

2.1. Animals

We collected adult animals in and around Portal, AZ (*C. uniparens*) and Sanderson, TX (*C. inornatus*) and transported them to the University of Texas at Austin and maintained them in aquaria in groups of four to six as described previously [14].

2.2. Egg collection and treatment

To determine the degree of ovarian development, we palpated the abdomens of the adult females weekly and checked those with eggs in the oviduct daily until they laid their eggs. We collected the eggs within 24 h after they were laid and placed each clutch in a 30-ml plastic cup containing equal amounts of vermiculite and water by weight, sealed with plastic sandwich bags secured by a rubber band. The eggs developed in an incubator at 28.5 °C. At this temperature, the hatchlings emerged in an average of 57 days. To produce males in the parthenogenetic species, we treated half the *C. uniparens* eggs on day 5 of incubation by placing 20 μg of fadrozole dissolved in 1 μl of 95% ethanol directly onto the eggshell. Drug administration by this method provides a sustained transfer of the administered compound across the eggshell [10]. Control eggs received ethanol only. In a previous study, it was established that fadrozole doses of 1 μg and higher per egg produce 100% gonadal male hatchlings with no detectable ovarian tissue. All animals in the present study were opened and examined under a dissection microscope at the time of sacrifice in order to confirm their gonadal sex.

2.3. Hatchling maintenance

The hatchlings lived in 10-gallon aquaria under the same environmental conditions as the adults, with up to 10 hatchlings per aquarium (mixed species and sexes). The cages contained 3–4 cm of sand, a water bowl, and a small pine board placed on the sand as a refuge. A 60-W bulb with a reflector lit at one end of the cage to provide a thermal gradient. We fed the hatchlings 2-week-old crickets, dusted weekly with vitamin powder containing calcium and vitamin D_3.

2.4. Hormone treatment

We divided animals into two groups based on their age at the time of hormone treatment: 1 and 30 days of age. Each age group contained four subgroups: females of the sexual species, males of the sexual species, females of the parthenogenetic species and created males of the parthenogenetic species. At the appropriate age, half of the animals in each subgroup received a subcutaneous injection of 0.2 μg of estradiol benzoate in 4 μl of steroid suspension vehicle (SSV). The other half received vehicle only. The animals were returned to their home cages following injections.

2.5. Brain tissue preparation

Twenty-four hours after hormone injection, we sacrificed the animals by rapid decapitation, removed the brains, and stored them at −74 °C until they were sectioned on a cryostat. We collected 20-μm sections in a series of four on RNAse-free poly-L-lysine coated slides and stored the slides in slide boxes at −74 °C.

2.6. In situ hybridization and quantification

The protocol and validation of the in situ hybridization has been described previously [42]. After hybridizing the brain sections to an antisense riboprobe for whiptail lizard PR or ER mRNA, we dipped the slides in Kodak NTB-2 autoradiographic emulsion and exposed them for 21 (PR) or 17 days (ER). We chose these exposure times to prevent overexposure, which can obscure differences between cells through a 'ceiling effect' on silver grain density. Following the prescribed exposure period, we developed the slides using Kodak D-19 developer, stained them lightly with cresyl violet, and coverslipped them. In order to minimize variation in probe binding, all the samples hybridized to each probe were treated together in the same assay; the ER and PR assays were run separately from each other.

We analyzed the brain sections using darkfield microscopy and grain-counting software, Grains (University of Washington). To prevent bias during measurement, each slide was given a unique, randomly generated three-letter code. The method of quantification was similar to that previously described [12,43,40]. On the developed brain sections, silver grains appear in clusters centered over individual cells. The Grains program automatically detects a user-defined number of the most densely labeled silver grain clusters in a given field and counts the silver grains that overlie them. We have used this method of silver grain quantification extensively and find it ideal for measuring group differences in response to hormone treatment [12,40,41]. This method of silver grain quantification more accurately portrays differences in mRNA abundance between individuals than might densitometric analysis of the entire VMH, because background grains tend to obscure very lightly labeled cells. This bias could introduce a 'floor effect' complementary to the 'ceiling effect' described earlier. For similar reasons, quantification of absolute numbers of cells positive for PR or ER mRNA is unreliable compared to immunocytochemical methods.

We counted ten clusters per hatchling (half the number typically counted for the much larger adult whiptails), equally distributed on left and right side of each animal. A similar number of measurements from nearby, non-specifically labeled tissue provided a value for background silver grain density. We averaged the values for both VMH and background for each animal and subtracted the background value from the VMH value to obtain the corrected mean grains per cluster.

Though the number of cells counted may seem small, the VMH in hatchling whiptails is tiny (<0.02 mm^3 [34]) and only a small portion of the nucleus in both adults and hatchlings (the dorsolateral region) responds to estrogen treatment by increasing PR and ER mRNA. This estrogen-sensitive region typically appears on only one or two brain sections per series for each animal, and we feel that our methodology adequately captures group differences in receptor regulation.

2.7. Statistical analysis

We log-transformed the data to reduce heterogeneity of variance where a Levene median test indicated this was necessary and performed a two-way analysis of variance followed by a post-hoc Tukey's test on significant factors or interactions. Since our primary interest was in looking for the effects of treatment and sex, species and age groups were analyzed separately from one another. All statistics were performed using SigmaStat for PC, and P values of ≤ 0.05 were considered statistically significant.

3. Results

A total of 94 animals were successfully hatched, of which 54 were examined at 1 day of age and 40 were maintained until 30 days of age. Despite this success, less than one-third of the animals represented in this study were from the sexual species (63 parthenogens, 31 sexuals). In captivity, whiptails of the sexual species lay fewer fertile eggs, have a poorer hatching success, and are less hardy as hatchlings than the parthenogens. As a result, sample sizes for the sexual species are smaller than for the parthenogenetic species.

3.1. EB regulation of PR mRNA

For all groups tested, EB treatment increased PR mRNA expression in the VMH (Figs. 1 and 2). In the sexual species, the 1-day-old group also showed a significant treatment-by-sex interaction ($P=0.01$) indicating a differ-

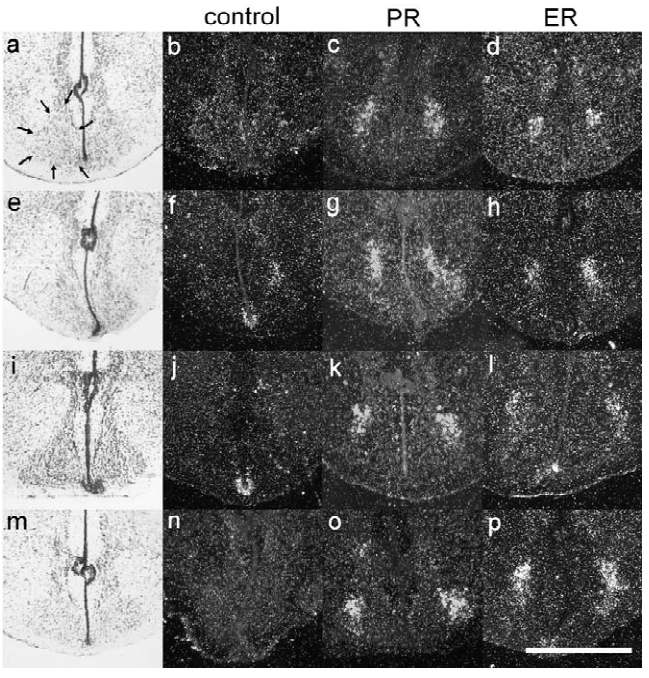

Fig. 1. Photomicrographs showing coronal sections through the ventromedial hypothalamus (VMH) of male and female *Cnemidophorus* hatchlings. Lightfield images (panels a, e, i, m) illustrate the appearance of this brain region; black arrows (panel a) indicate the borders of the VMH. Only the dorsolateral portion of this nucleus responds to estradiol treatment. Darkfield images (panels b–d, f–h, j–l, n–p) illustrate brain sections from 30-day-old animals that were hybridized in situ to radioactive riboprobes complementary to progesterone receptor (PR, third panel of each row) or estrogen receptor (ER, fourth panel of each row) mRNA. Animals in the third and fourth panels of each row were treated with 0.2 μg of estradiol benzoate in steroid suspension vehicle, while animals in the second panel of each row received vehicle only. From top to bottom, the rows show sexual females (a–d), sexual males (e–h), parthenogens (i–l), and created males (m–p). Scale bar=500 μm.

ence in the way the sexes respond to EB. A post-hoc Tukey's test indicated that the EB-treated sexual females had significantly higher PR mRNA levels than the EB-treated sexual males ($P<0.001$), but there was no difference between the control groups ($P=0.472$). The 30-day-old sexual group was excluded from statistical analysis because of small sample size, but we present the data for the purpose of comparison; the pattern of response in the females looks similar but one of the three males receiving estrogen failed to show an induction/up regulation. In the parthenogenetic species, the interaction between treatment and sex was non-significant; created males and normal parthenogens responded equally strongly to EB.

3.2. EB regulation of ER mRNA

We found a significant increase in ER mRNA after EB treatment in the sexual 1-day-old group and a trend toward a treatment effect in both the parthenogenetic age groups (Fig. 3). Again, the 30-day-old sexual group was excluded from statistical analysis due to small sample size. In the

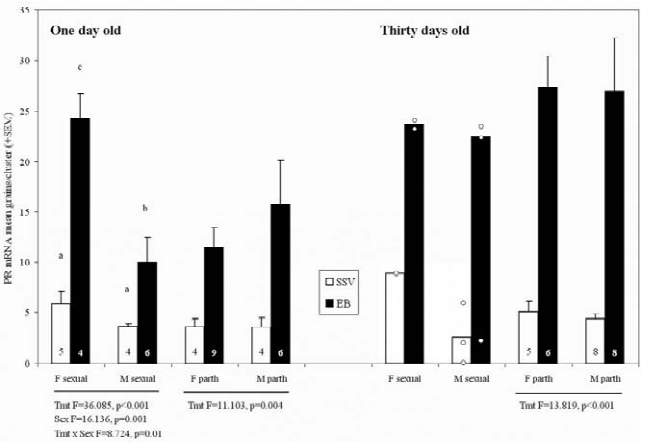

Fig. 2. Progesterone receptor mRNA abundance in the ventromedial hypothalamus of 1- and 30-day-old whiptail lizards. Animals were treated with either 0.2 μg of estradiol benzoate in steroid suspension vehicle (EB) or with vehicle only (SSV). Numbers at the base of each bar indicate sample size for that group. Species and age groups were analyzed separately. The sexual species was excluded from analysis in the 30-day-old group due to low sample size, and individual values for these animals are plotted along with group means. Statistically significant factors and/or interactions are indicated below each group. Different letters above the bars indicate significant differences between sex and treatment groups (post-hoc Tukey's test, $P\leq0.05$). Error bars are ±1 S.E.M.

30-day-old parthenogenetic group, we found a significant treatment-by-sex interaction, indicating a difference in the way the sexes respond to EB. Post-hoc tests indicated that while there was no difference between the female SSV and EB groups, the EB-treated created males showed a statistically significant increase in ER mRNA. Note that the

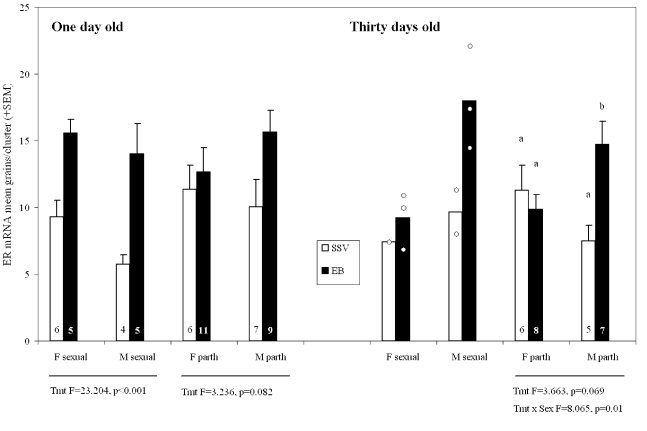

Fig. 3. Estrogen receptor mRNA abundance in the ventromedial hypothalamus of 1- and 30-day-old whiptail lizards. Animals were treated with either 0.2 μg of estradiol benzoate in steroid suspension vehicle (EB) or with vehicle only (SSV). Numbers at the base of each bar indicate sample size for that group. Species and age groups were analyzed separately. The sexual species was excluded from analysis in the 30-day-old group due to low sample size, and individual values for these animals are plotted along with group means. Statistically significant factors and/or interactions are indicated below each group. Different letters above the bars indicate significant differences between sex and treatment groups (post-hoc Tukey's test, $P\leq0.05$). Error bars are ±1 S.E.M.

30-day-olds of the sexual species appear to show a similar pattern of response, though we emphasize the preliminary nature of the data from this group.

4. Discussion

We have demonstrated that both male and female hatchling whiptail lizards respond to exogenous estrogen with a substantial increase in PR mRNA in the VMH. The pattern of regulation of ER mRNA was more complex. In 1-day-olds of the sexual species, both males and females up-regulated ER mRNA in response to estrogen. In the parthenogenetic species, there was a trend toward an EB-induced increase in ER mRNA, but this response did not reach statistical significance. In the 30-day-old animals, only males responded to EB treatment by increasing ER mRNA.

We asked whether the pattern of regulation of PR and ER mRNA in hatchling whiptails was similar to that in adults. In adults, males do not respond to estrogen treatment by up-regulating either ER or PR mRNA in this brain area [12]. In the hatchlings, however, both sexes showed a strong increase in PR mRNA in response to estrogen treatment. Clearly, the brains of the hatchlings have not reached their adult phenotype; there must be developmental events that induce sexual differentiation after the hatchling stage. One candidate for such an event is exposure to androgens. Previously, we have demonstrated that long-term castration of adult male whiptails results in their assuming a female-like VMH phenotype, both in terms of PR regulation [36] and in morphology [34,33], indicating that exposure to androgens is an important trigger in the development of the masculine neural phenotype. In contrast, long-term exposure of adult female whiptails to testosterone (via Silastic capsule implant) does not masculinize their PR response to estrogen treatment [36] or VMH volume [34]. Therefore, there appears to be a sex difference in sensitivity to androgens, with females less responsive to testosterone than males. Similar findings have been described in other model systems, including regulation of preoptic area aromatase activity in rats and doves [44], the size of neurons in the preoptic area of quail [24], and sexual behavior in ferrets [4,2].With regard to estrogen induction of ER mRNA in the VMH, we observed an interesting species difference. In hatchling animals of the sexual species, both males and females responded to estrogen treatment by up-regulating ER mRNA. In the parthenogenetic species, however, this was not the case. The response to estrogen treatment in 1-day-old animals was non-significant, and in the 30-day-old animals only created males responded. As was the case for PR mRNA regulation, it is clear that ER mRNA regulation in the brains of the hatchling whiptails is very unlike that observed in adults. In this case, however, the females of the two species appear to be on different developmental paths, characterized by differences in each species' responsiveness to estrogen. Prenatal estrogen has been implicated in the development of sex differences in hormone sensitivity in other species [16,21], and further investigation into this possibility is warranted in whiptails.

We also asked whether the created males would respond to EB treatment in a way that reflected their gonadal or their genetic sex. There is a sex difference in the PR response of 1-day-old individuals of the sexual species, and none between the normal and created male parthenogens. Looked at in one light, this might mean that the created males have female-like brains, in accord with their female genetic sex. However, there is another possible interpretation. Both phenotypes in the parthenogenetic species closely resemble males of the sexual species in the intensity of PR mRNA induction. It could be that in this respect the hatchling parthenogens have male-like brains despite being gonadal females. In any case the created males appear more similar to males of the sexual species in their response to estrogen than do the parthenogens to sexual females. In this regard it is interesting that the regulation of PR in males shows a female-like pattern of PR mRNA regulation in response to exogenous estrogen at a time when the adult sexually dimorphic response may be developing in the sexual species

In terms of ER expression, normal parthenogens do not appear to respond to estrogen at either age group examined. This pattern is distinct from that of any other group in the study, regardless of sex or species. As suggested above, perhaps it is the parthenogens who are different, responding to estrogen in ways that are male-like in some aspects and female-like in others. This interpretation fits well with observations of adult behavior in the parthenogenetic species. Unlike females of the sexual species, adult *C. uniparens* normally display both male-like and female-like pseudosexual activity, with male-like activity more likely during the luteal phase of the ovarian cycle, and female-like behavior more likely during the follicular phase [23,22]. There are also more subtle differences between the two species in steroid receptor regulation: in the medial and periventricular preoptic area, parthenogens regulate ER more strongly in response to plasma estradiol levels than do females of the sexual species. The same is true for regulation of PR in the medial preoptic area and dorsal hypothalamus [40,41,13]. It is well established that exposure to gonadal steroids early in life can lead to permanent changes in adult behavior and brain phenotype [1,19]. The difference in sensitivity to estrogen stimulation that we observed in females of the two whiptail species could be the basis of the above-mentioned differences we see between these two species in adulthood. A more thorough examination of hormone receptor expression and regulation, as well as the long-term effects of hormone manipulations, at the particular time points when parthenogens differ from females of the sexual species in their response to estrogen may prove

fruitful in explaining their distinct behavioral and neuroendocrinological phenotypes.

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