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Regulation of Estrogen Receptor and Progesterone Receptor Messenger Ribonucleic Acid by Estrogen in the Brain of the Whiptail Lizard (*Cnemidophorus uniparens*)

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Abstract

Receptive behavior in females vertebrates is controlled by hormones, principally estrogen, secreted by the ovary. Estrogen influences behavior by interacting with a specific estrogen binding protein, or receptor, located in target cells in certain hypothalamic nuclei. To better understand the molecular mechanisms involved in the control of receptive behavior in whiptail lizards, we investigated the effects of exogenous estrogen on the regulation of estrogen receptor and progesterone receptor expression in several regions of the brains of *Cnemidophorus uniparens*. First we determined a dosage of 17β-estradiol 3-benzoate (0.5 μg) which reliably induced receptive behavior in ovariectomized *C. uniparens*. Then using *in situ* hybridization, we examined the effects of that dosage on the expression of estrogen receptor and progesterone receptor mRNA in the brain 24 h after injection. Estrogen treatment resulted in a significant up-regulation of estrogen receptor mRNA expression in the ventromedial nucleus of the hypothalamus and torus semicircularis, down-regulation of estrogen receptor mRNA expression in the lateral septum, and no change in the periventricular nuclei of the hypothalamus, the periventricular nucleus of the preoptic area, and the dorsal hypothalamus. The same dosage resulted in increased progesterone receptor mRNA expression in the ventromedial nucleus of the hypothalamus and the periventricular nucleus of the preoptic area; no significant changes in progesterone receptor mRNA expression were observed in the periventricular nuclei of the hypothalamus or the torus semicircularis, although the differences in progesterone receptor expression in the torus semicircularis approached statistical significance. The up-regulation of estrogen receptor gene expression by estrogen in the ventromedial nucleus of the hypothalamus of lizards is opposite to that reported in female rats in which estrogen down-regulates estrogen receptor expression in the ventromedial nucleus of the hypothalamus. We propose that this may be related to differences in reproductive physiology between vertebrates which have extended follicular phases and prolonged periods of estrus and rats, which have an abbreviated follicular phase and brief periods of estrus.

In most female vertebrates with internal fertilization, the onset of sexual behavior coincides with the time when fertilization is most likely to occur, around ovulation. The timing of female sexual behavior in fish (1), reptiles (2), birds (3) and mammals (4) is controlled by ovarian estrogen (E) secreted during the follicular phase of the ovarian cycle. Progesterone (P) is also involved in controlling sexual behavior in some mammals and the green anole lizard (4, 5). The general mechanisms by which E induces receptive behavior in female vertebrates appear to be highly conserved in vertebrates, although they are best understood in the rat (4). In rats, E and P act sequentially to promote receptive behavior (lordosis). Estrogen and P influence female sexual behavior in the rat by interacting with specific steroids binding proteins, or receptors, located within target neurons within certain hypothalamic nuclei, particularly the ventromedial nucleus of the hypothalamus (VMH). In several other vertebrate species as well, including whiptail lizards (6), the VMH has been identified as being an important site of estrogen action in the control of receptive behavior.

Steroid receptors are ligand-dependent transcription factors which exert their effects by modulating gene transcription in target cells (7). In the brain, E has been demonstrated to alter the expression of several genes which may be involved in the mediation of sexual behavior, including oxytocin, oxytocin receptor, progesterone receptor (PR) as well as regulating the expression of its own receptor (ER) (4). Although the neuroanatomical distribution of ER and PR has been established in a number of species representing diverse taxa (8–18), the regulation of ER and PR in the brain is less understood, particularly in non-mammalian species. The effects of E on ER expression in the brain have been established only in rats, in which E decreases the ER-mRNA content in VMH neurons within hours after exposure (19, 20). This down-regulation of ER expression is likely to decrease neural sensitivity to E following the E surge of proestrus

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(21). However, the reproductive physiology of the female rat, with its abbreviated follicular phase, is not representative of many vertebrates and information on the regulation of ER in the brains of vertebrates with prolonged follicular phases is not available. Estrogen exposure results in the opposite effect on PR, increasing PR expression and thus sensitivity to P in several hypothalamic and limbic regions. This phenomenon has been documented in a variety of vertebrate species, including rat (22), guinea pig (23), ferret (16), chicken (24), frog (9) and the green anole lizard (25).

Our research has focused on the reproductive physiology of whiptail lizards. *Cnemidophorus uniparens* is an all-female, parthenogenetic species of whiptail lizard inhabiting the desert-grasslands of Arizona and New Mexico. Although *C. uniparens* reproduce asexually, individuals regularly exhibit female-like receptive behavior during the follicular phase of the ovarian cycle (26). Unlike the female rat, which experiences elevated E for a single day of their four day ovarian cycle, with estrus occurring the following night when P levels surge, reptiles experience elevated E levels throughout vitellogenesis, which may last days, weeks or even months depending on the species. The vitellogenic phase of the ovarian cycle of *C. uniparens* averages nine days (27) during the latter stages of which individuals are receptive (26). In the green anole lizard, E acts synergistically with peri-ovulatory P to induce receptivity (28, 5); however the role of P in the control of female sexual behavior in whiptail lizards has yet to be established.

Recently fragments of the whiptail ER and PR genes have been cloned (see Fig. 1) and used in *in situ* hybridization analysis to demonstrate the expression of ER-mRNA and PR-mRNA in several limbic brain regions of *C. uniparens*, including the preoptic area, septum, amygdala, and hypothalamus (29). In the present investigation, we determined a dosage of 17β-estradiol 3-benzoate (EB) that is effective at inducing receptive behavior in ovariectomized *C. uniparens*. Then we used *in situ* hybridization analysis with radioactively labeled antisense RNA probes to determine the effects of this dosage of EB on ER- and PR-mRNA expression in the lizard brain.

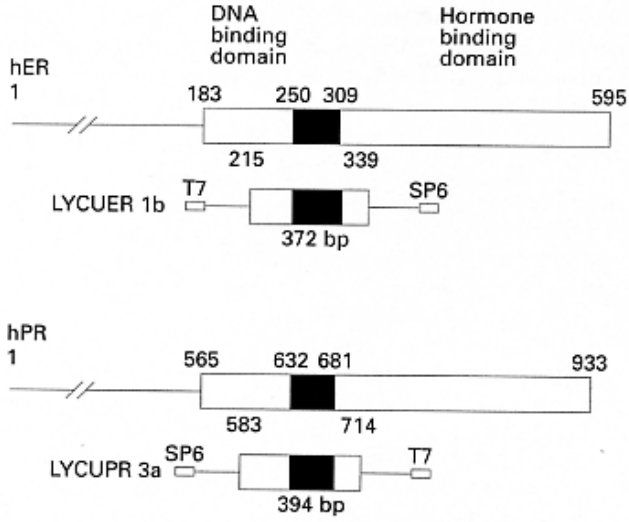

FIG. 1. Schematic diagrams showing the size and position of the *Cnemidophorus* estrogen receptor (LYCUER 1b), and progesterone receptor (LYCUPR 3a) clones relative to the full length human sequences. The structures of the human estrogen receptor (hER), and progesterone receptor (hPR) are illustrated 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 human estrogen receptor (38), and the human progesterone receptor (39).

Results

A single injection of 0.5 μg EB resulted in induction of sexual receptivity ($P<0.01$); five out the six animals were receptive after treatment, with four of the six being receptive within 24 h of the injection (Fig. 2).

In situ hybridization with antisense ER and PR probes resulted in accumulation of silver grains over cell somata in several forebrain areas as previously described (29), while hybridization with sense strands resulted in uniform background distribution of silver grains. The neuroanatomical positions of the brain nuclei in which ER-mRNA and PR-mRNA were quantified are illustrated in Fig. 3. Analysis of ER-mRNA expression as determined by silver grain density indicated that treatment with exogenous EB increased the ER-mRNA content in the VMH ($P<0.001$) (Fig. 4A, B) and the torus semicircularis (TS) ($P<0.01$) (Fig. 5A). ER-mRNA content was decreased in the nucleus septalis lateralis (NSL) ($P<0.05$) (Fig. 5A). No change in ER-mRNA expression was detected in the periventricular nucleus of the hypothalamus (PH) ($P>0.2$), dorsal hypothalamus (DH) ($P>0.5$), or the periventricular nucleus of the preoptic area (pvPOA) ($P>0.1$) (Fig. 5A).

The most notable change in PR-mRNA expression with EB treatment was observed in the VMH. Few silver grains were apparent in the VMH of untreated, ovariectomized animals. Hormone treatment resulted in a significant increase in silver grain accumulation over neurons the VMH ($P<0.001$) (Figs. 4C,

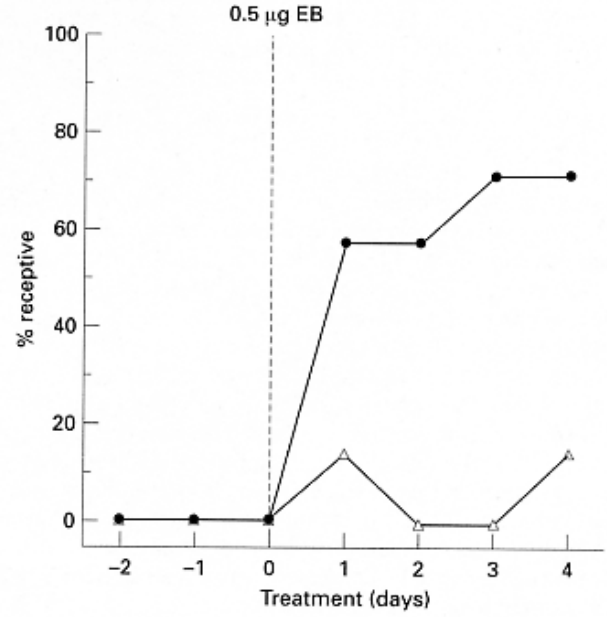

FIG. 2. The percentage of control (open triangle) and EB-injected (solid circle) lizards which were receptive on each day of behavior testing. The dashed line indicates the time of EB injection; testing days −2, −1 and 0 occurred prior to injection. △ control (n=6), ● EB (n=6).

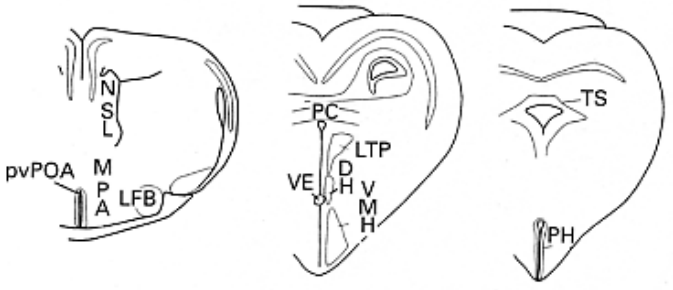

FIG. 3. Drawings of coronal sections of a *Cnemidophorus* brain illustrating the neuroanatomical positions of the brain nuclei in which estrogen receptor mRNA and progesterone receptor mRNA were quantified. For a more detailed atlas of the *Cnemidophorus* brain see (29). Abbreviations: pvPOA = periventricular nucleus of the preoptic area, MPA = medial preoptic area, NSL = nucleus septalis lateralis, LFB = lateral forebrain bundle, VE = ventricular ependymal organ, LTP = lentiformis thalami pars plicta, DH = dorsal hypothalamus, VMH = ventromedial nucleus of the hypothalamus, PH = periventricular nucleus of the hypothalamus, and TS = torus semicircularis.

D; 5B). Silver grain clusters of moderate density were located in the pvPOA in the absence of EB, indicating a relatively high level of constitutive expression of the PR gene in this area. In fact, this area had the highest concentration of PR-mRNA expression in the brains of ovariectomized animals. However, EB treatment resulted in a significant increase in silver grain density in this nucleus ($P<0.02$) (Fig. 5B). No change in PR expression was detected in the PH($P>0.2$) or TS ($P>0.1$) (Fig. 5B).

Discussion

In most vertebrates, female sexual behavior is regulated by ovarian hormones in such a way to synchronize sexual behavior with follicular maturation and ovulation. In reptiles, as in other vertebrates, E plays an essential role in the regulation of female sexual behavior (2). The reproductive cycle of female reptiles consists of a vitellogenic phase and a luteal phase. During the vitellogenic phase, which typically lasts several days, weeks, or even months depending on the species, the maturing follicle secretes E which stimulates oviducal proliferation and yolk secretion by the liver (vitellogenesis). During the mid to later stages of vitellogenesis and prior to ovulation, female whiptail lizards display receptive behavior (30). After ovulation, during the luteal phase, E levels decline and P titers increase (31), a pattern similar to that seen in birds and mammals. Receptivity gives way to aggressive sexual rejection behavior during the luteal phase (30).

The stimulation of ER-mRNA expression in the whiptail VMH by EB raises the possibility that E secreted early in the ovarian cycle sensitizes the neural circuits controlling the receptive behavior to E. The significance of the increase in PR-mRNA expression in the VMH of *C. uniparens* is not clear since the role of P in the regulation of female receptive behavior has not been determined in whiptail lizards. However, it should be noted that the increase in frequency of receptive behavior in the later stages of vitellogenesis is associated with rising plasma P levels. Thus, the EB-induced PR-mRNA expression in the VMH suggests that P may play some role in the induction of receptive behavior, although it has not been proven experimentally.

The behavioral significance of the estrogenic regulation of receptor mRNA expression in the TS, NSL and pvPOA is unknown. It is interesting to note that the regulation of steroid receptor gene expression in the brain is region specific. For instance, EB treatment increased ER-mRNA expression in the VMH and TS, had no effect in the pvPOA, and decreased ER expression in the septum. This complex regulation of steroid hormone receptor gene expression allows for region specific modulation of steroid sensitivity during the reproductive cycle.

Comparison with other species

Little is known about the hormonal regulation of brain steroid receptor gene expression in non-mammalian vertebrates. One study has demonstrated that E increases PR protein concentration in hypothalamic tissue blocks in the green anole lizard (25). However, the precise neuroanatomical distribution of these receptors was not determined. The present study provides the first detailed analysis of the regulation of steroid receptor gene expression in the reptilian brain. In most mammalian and avian species examined, the expression of PR in basal hypothalamic nuclei, including the arcuate nucleus and the VMH, is estrogen-dependent (16, 22, 23, 32). One notable exception is the cat in which PR-immunoreactivity in the VMH is reported to be barely detectable and, further, PR-immunoreactivity in the VMH does not appear to be significantly effected by EB treatment (13). It is perhaps significant that P does not play a significant role in the regulation of sexual behavior in the female cat, an induced ovulator (33).

The autoregulation of brain ER gene expression has only been studied in the rat. Estrogen decreases ER-mRNA expression in the VMH in the female rat (19, 20). Interestingly, similar EB treatment does not effect ER-mRNA expression in the VMH of the male rat, indicating that hormone-dependent regulation of steroid receptor gene expression can differ between sexes within the same species. In contrast to the female rat, we have demonstrated that EB treatment increases ER-mRNA expression in the VMH of the female whiptail lizards. The species differences in regulation of ER gene expression in the reproductive tract parallels that of the VMH; EB increases ER-mRNA expression in the lizard oviduct (L. Young, J. Godwin and D. Crews, unpublished data) while it decreases ER-mRNA in the rat uterus (34).

These differences may be due to the differences in reproductive physiology between rats and lizards, as well as other vertebrates with extended follicular phases. In contrast to female rats, which have a four day ovarian cycle during which estrus occurs on the night following the E surge of proestrus, many vertebrates experience elevated E levels and display behavioral estrus for several days. Elevated E and behavioral estrus in many carnivores, such as dogs, cats, bears, etc. lasts over one week (35). At the extreme are serial ovulators, such as the rabbit, in which females are in constant estrus, E being secreted by developing follicles throughout the breeding season (36).

Thus, while it may be adaptive for female rats to become behaviorally insensitive to E following the initial E surge, vertebrates with longer follicular phases and prolonged periods of estrus may not alter their sensitivity to E in the same manner. Rather, vertebrates without abbreviated follicular phases may actually require increased neural sensitivity to E in response to the first surges of E in the early stages of the follicular phase. If so, we predict that in other reptiles as well as in birds and certain

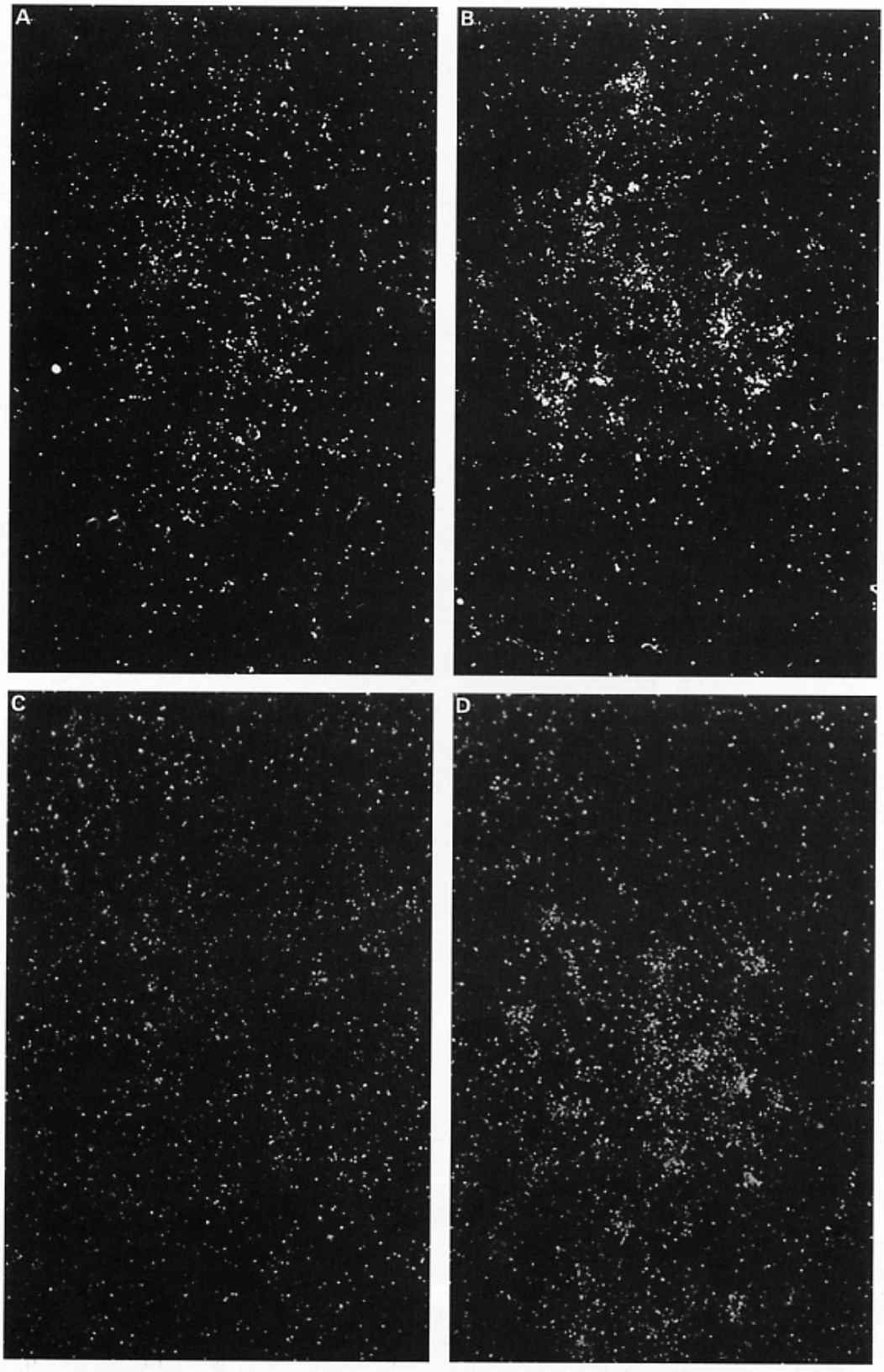

FIG. 4. Representative darkfield photomicrographs of silver grain distribution over cells in the VMH of control (4A,C) and EB injected (4B,D) lizards. Sections shown in the top panel were hybridized with radiolabeled antisense ER probe while sections shown in the bottom panel were hybridized with radiolabeled antisense PR probe. Scale bar = 10 μm.

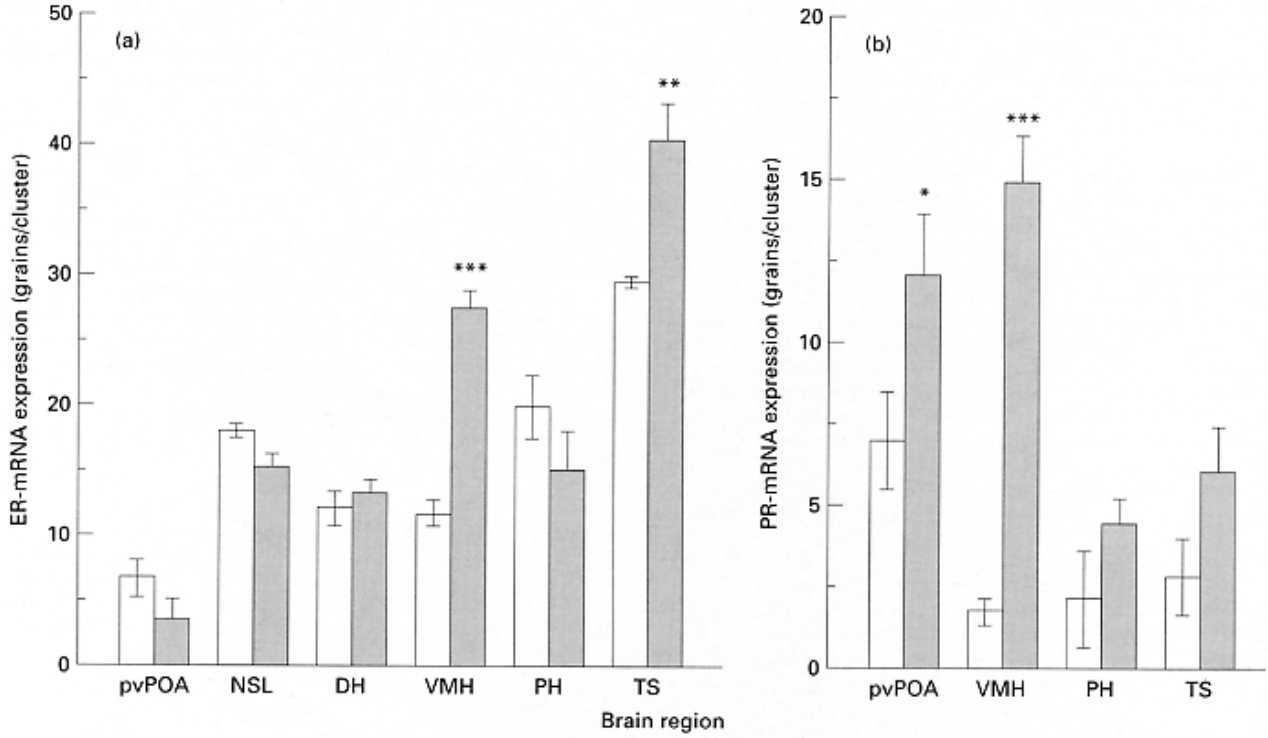

FIG. 5. Silver grain density over several lizard brain regions hybridized with radiolabeled antisense ER (5A) and PR (5B) probes. Mean silver grain density for control (open bars) and EB injected (toned bars) are plotted; error bars represent standard errors. Silver grain densities were calculated using a computerized image analysis system using darkfield microscopy. P-values were generated by performing *t*-test comparing control and EB-treated animals for each brain region. It should be noted that the differences in silver grain density between ER- and PR-hybridized sections do not necessarily reflect relative concentrations of ER- and PR-mRNA in the tissue since the *in situ* hybridization assays were not performed simultaneously and different probe preparations often produce different signal intensities. Abbreviations: pvPOA = periventricular nucleus of the preoptic area, NSL = nucleus septalis lateralis, DH = dorsal hypothalamus, VMH = ventromedial nucleus of the hypothalamus, PH = periventricular nucleus of the hypothalamus, and TS = torus semicircularis. *P < 0.05; **P < 0.01, ***P < 0.001; ▨ 0.5 μg EB; □ control.

mammals, E would regulate ER gene expression in the VMH in a manner similar to that observed in whiptail lizards.

General conclusion

The neuroanatomical distribution of sex steroid receptor gene expression has been remarkably conserved throughout evolution (8). In fact, the analysis of steroid receptor distribution in the brain is a useful method for determining neuroanatomical homologies across divergent taxa (29). However, the pattern of hormone-dependent steroid receptor regulation within specific brain nuclei appears to be less constrained. Therefore, steroid hormone regulation of steroid receptor gene expression is capable of evolving along with specific requirements dictated by the reproductive physiology of a particular species.

Materials and Methods

Animals

Adult *C. uniparens* were captured near Portal, Arizona, USA and, after transport to the University of Texas at Austin, were housed in environmental chambers as previously described (6). All animals were housed in groups of four in 29 gal aquaria prior to the onset of the experiment. Animals were ovariectomized as previously described (6) and housed three per 29 gal aquaria, with each of the three separated by a sheet of opaque Plexiglas. Each animal was supplied with a heat lamp, water dish, a small board for basking and cover and sand as substrate. Animals were fed crickets or meal worms three times per week.

Hormone treatment

A stock solution of 17β-estradiol 3-benzoate (EB) (Sigma) was prepared by dissolving it in 95% ethanol to a final concentration of 3 mg/ml. This stock solution was diluted with Steroid Suspension Vehicle (SSV) to create a suspension with a final concentration of 0.5 μg EB/10 μl. One week after ovariectomy, animals were given a single subcutaneous injection above the scapula of either vehicle only or 0.5 μg EB. This dosage was chosen since i) it approximates the minimum dosage of EB (0.4 μg) required to induce receptivity in female green anole lizards (28), and ii) it approximates the dosage of EB (0.8 μg) previously shown to increase PR concentration in the hypothalamus of female green anole lizards (37). Preliminary studies have indicated that dosages up to 3.0 μg of EB do not result in significantly greater levels of receptive behavior compared to the dosage used in this study (L. Young, unpublished data).

Fourteen animals were used in the behavior experiment (n = 7 per group). For the *in situ* hybridization, 12 similarly treated lizards were killed 24 h after injection (n = 6 per group). Stimulus animals used in behavior tests were ovariectomized *C. uniparens* given a subcutaneous Silastic capsule packed with crystalline testosterone (T) (Sigma) to stimulate mounting behavior as described previously (6).

Behavior testing

Behavior tests were performed as previously described (6). Briefly, receptive behavior was determined by placing the experimental animal into the cage of a stimulus animal. The experimental animal was classified as being receptive if it allowed the stimulus animal to mount and attempt copulation without resistance. Under these conditions, nonreceptive animals are typically aggressive to a courting stimulus animal and will roll if the stimulus animal attempts to mount it. The behavior tests were continued until the receptive status of the experimental animal was determined, typically within three minutes of testing.

Pre-tests began 5 days after ovariectomy and were performed on three successive days until seven days after ovariectomy. Immediately following the third pre-test, experimental animals were given hormone or vehicle treatment. Post-treatment behavior testing began 24 h after injection and continued daily for 4 days. The treatment of the experimental animal was unknown to the observer.

Tissue preparation

Animals were ovariectomized and injected with vehicle alone or 0.5 μg EB one week after surgery as described for the animals used in the behavior testing. Twenty-four h after hormone injection, the animals were killed by rapid decapitation, and the brains removed immediately, frozen on dry ice and stored at −80 °C until sectioning.

Twenty-micron thick coronal cryosections were melted on RNase free polylysine coated microscope slides and allowed to dry at room temperature before storing in slide boxes with desiccant at −80 °C. Sections were placed on a series of six slides so that adjacent sections could be hybridized to different probes. In order to determine the reproducibility of our technique, adjacent sections for each brain were placed on separate slides and included in the PR assay, serving as independent replicates. The replicates were randomized in the *in situ* hybridization procedure and coded separately prior to quantification to estimate intra-assay variation.

Probe Preparation

Probes for *in situ* hybridization were prepared from the whiptail ER clone LYCUER 1b (372 bp) and the whiptail PR clone LYCUPR3a (394 bp) as previously described (29). The structures of these clones relative to the human the human structures are illustrated in Fig. 1. Antisense and sense cRNA probes were synthesized using SP6 or T7 RNA polymerase incorporating ^{35}S-UTP (NEN) at a specific activity of 9×10^8 cpm/mg probe. Purified cRNA probes were heat-denatured and added to the hybridization solution (50% formamide, 10% dextran sulfate, 0.3 M NaCl, 10 mM Tris HCl (pH 8.0), 1 mM EDTA, 1 × Denhardt's solution, 10 mM dithiothreitol and 0.5 mg/ml tRNA) at a final concentration of 0.3 mg probe × length (kb)/ml.

In situ hybridization

At the time of the assay, the tissue was fixed in 4% paraformaldehyde (pH 7.2) at room temperature for 20 min, rinsed five min in 3 × phosphate buffered saline (PBS), followed by two five min washes in 1X PBS. The sections were then dipped in 0.1 M triethanolamine (TEA) (pH 8.0) followed by ten min in freshly prepared TEA/0.25% acetic anhydride, rinsed in 2 × SSC, dehydrated in ascending ethanols, delipidated in chloroform, rinsed in 95% ethanol, air-dried and prehybridized overnight (50 °C) in hybridization solution (see above) in which the probe was replaced with tRNA (2.5 mg/ml final concentration). Prehybridization and hybridization took place in air-tight chambers containing moistened paper towels. Prehybridization solution was rinsed off in 2 × SSC, and sections were dehydrated in ascending ethanols and air-dried prior to hybridization.

Hybridization solution was applied to the sections and hybridization proceeded overnight at 50 °C. The sections were then washed twice for 15 min in 1 × SSC, 50% formamide, 0.1% 2-mercaptoethanol (2-ME) at 50 °C, then RNase A digested (20 mg/ml) for 30 min at 37 °C, and washed 30 min in 1 × SSC, 0.1% 2-ME (50 °C). Final high stringency washes consisted of two 30 min washes in 0.1 × SSC, 1% 2-ME (50 °C). The sections were then dehydrated in alcohols and air-dried.

The slides were then dipped in Kodak NTB-2 autoradiographic emulsion, dried and exposed at 4 °C for 2 wks for PR and 2 wks for ER, developed in Kodak D-19 developer and fixed. These developing times were chosen based on optimum silver grain density for quantification. After fixation, the sections were washed in water, stained with cresyl violet and coverslipped using Permount.

Analysis

ER- and PR-mRNA in the hypothalamus of each animal were quantified using the Grains image analysis program (Personal communication, Donald K. Clifton, University of Washington). The slides were randomized and coded prior to analysis so that the identity of the slides were unknown to the investigator. The sections to be analyzed were first located using brightfield microscopy. Care was taken to insure that the same cell populations for a given region were analyzed for all brains. Analysis of each region began on an anatomically matched section for each brain. Due to the small size of the lizard brain, the area analyzed for most brain regions was localized to only one or two sections and could be encompassed in two to four fields of view. Silver grains clustered over cell somata were systematically counted using the computerized image analysis system utilizing darkfield microscopy with a 40 × objective. Each cluster appearing in the field of view was counted before changing the field of view. This was repeated until a minimum number of cells for each area was counted; 20 cells for the VMH, 10 cells for the pvPOA, NSL, DH, PH and TS. The minimum number of cells counted for a given area was chosen based on the typical number of labeled cells in that area and provides sufficient measurements to obtain an accurate estimate of the silver grain density of the labeled cell population in that area. Once the minimum number of cells were counted, the remainder of the clusters on that field of view were counted and the analysis was then complete. Background was calculated by counting silver grains over 10 cells in adjacent areas of the same sections. After decoding the mean number of silver grains per cluster for each animal was determined by substracting the mean background silver grains per cell from the mean grains per cell in the area of interest. For PR the average for the duplicate slides were used in the statistical analysis.

Statistical Analysis

To compare the effects of EB treatment on receptive behavior, the Mann-Whitney *U* test was performed comparing the total number tests that each animal was receptive in post treatment behavior tests. Two-tailed *t*-tests were used to compare the individual mean number of silver grains per cell in control and EB-treated groups for each brain region.

Acknowledgements

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