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Progesterone Inhibits Female-Typical Receptive Behavior and Decreases Hypothalamic Estrogen and Progesterone Receptor Messenger Ribonucleic Acid Levels in Whiptail Lizards (Genus *Cnemidophorus*)

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Female-typical sexual behavior in tetrapods is mediated primarily by estrogen and progesterone acting through intracellular receptors at specific sites in the mediobasal hypothalamus. Progesterone exerts both faciliatory and inhibitory actions on female sexual behavior and in well-studied rodent models, the inhibitory actions are exerted through downregulation of progesterone and estrogen receptors. This study examined progesterone effects on both female-typical sexual behavior and hypothalamic estrogen and progesterone receptor mRNA expression (ER- and PR-mRNA) in a sexual and parthenogenetic species of whiptail lizard. Progesterone capsules administered to ovariectomized female *Cnemidophorus inornatus* and *Cnemidophorus uniparens* following a receptivity-inducing dosage of estradiol benzoate (EB) strongly inhibited receptive behavior as compared to blank implanted controls. Progesterone capsules administered either before or after an EB injection also strongly downregulated ER- and PR-mRNA abundance in the ventromedial nucleus of the hypothalamus relative to blank implanted controls. The correlated decrease in both EB-induced receptive behavior and ER- and PR-mRNAs following progesterone administration are similar to findings in rats and guinea pigs, suggesting that this is an evolutionarily conserved mechanism in the regulation of female sexual behavior.

We are interested in the neuroendocrine mechanisms controlling sexual and pseudosexual behavior in an ancestor/descendant pair of whiptail lizards (genus *Cnemidophorus*) native to the desert grasslands of Southwestern North America. *Cnemidophorus uniparens* is an all-female, obligately parthenogenic species descended from the bisexual, sexually reproducing *Cnemidophorus inornatus* (Wright, 1993). Despite consisting entirely of female individuals, *C. uniparens* regularly exhibits male-like and female-like sexual behaviors which are indistinguishable from those shown by male and female *C. inornatus,* respectively (Crews and Fitzgerald, 1980). As in other female lizards, female *Cnemidophorus* exhibit sexual receptivity during the preovulatory phase of the follicular cycle when circulating estrogen levels are high (Moore, Whittier, and Crews, 1985; Moore and Crews, 1985; Whittier and Tokarz, 1992). Receptive behavior is not exhibited by ovariectomized female *C. uniparens* and *C. inornatus,* but can be induced by exogenous estrogen. This estrogen-induced receptive behavior and that observed in intact animals during the preovulatory phase of the ovarian cycle are correlated with increased abundance of estrogen receptor (ER)- and progesterone receptor (PR)-mRNA in the ventromedial nucleus of the hypothalamus (VMH) (Young, Nag, and Crews, 1995a, 1995c). The VMH of *Cnemidophorus* is known to control female-typical receptive behavior based on studies employing electrolytic lesions and intracranial implantation of estradiol (Kendrick, Rand, and Crews, 1995; Rand and Crews, 1994; Wade and Crews, 1991). Behavioral and steroid receptor mRNA responses to exogenous estrogen in the Cnemidophorus VMH are similar to rats in some respects. The strength of the receptivity response is positively correlated with

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PR-mRNA abundance in the VMH both intraspecifically between estrogen dosages and interspecifically at a given estrogen dose (Young, Nag, and Crews, 1995b). Also, castrated male *C. inornatus* lack either a behavioral (receptivity) or VMH PR-mRNA response to an estrogen dose that is behaviorally effective in females (Godwin and Crews, 1995). Despite this correlation of receptive behavior with PR-mRNA in the VMH, the exact role of progesterone and PR in controlling receptive behavior in *Cnemidophorus* and other lizards is not well understood. In females of another lizard species, *Anolis carolinensis,* estrogen upregulates progestin binding sites in the mediobasal hypothalamus and progesterone can synergize with estrogen to induce female-typical receptive behavior (Tokarz, Crews, and McEwen, 1981; McNicol and Crews, 1979; Wu, Whittier, and Crews, 1985). In contrast, progesterone has been correlated with sexual rejection behavior in other lizard species and with specific gravid colorations, which appear to signal this nonreceptivity to courting males (Cooper and Crews, 1987, 1988; Cooper and Greenberg, 1992). *Cnemidophorus* females do not display receptive behavior during the postovulatory period when progesterone levels are elevated and typically respond aggressively to courtship attempts (McNicol and Crews, 1979; Moore *et al.,* 1985; Moore and Crews, 1986).

This study examines the effect of progesterone on female-typical sexual behavior and neuroendocrine correlates of this behavior in *Cnemidophorus* lizards. Two specific questions are addressed. First, what is the effect of progesterone on estrogen-induced receptive behavior in ovariectomized female *Cnemidophorus?* Second, what is the effect of progesterone on PR- and ER-mRNA abundance in the VMH, the brain area which controls receptive behavior? The general objective is to explore the possible evolutionary conservation of hormonal and molecular mechanisms which regulate female-typical reproductive behavior in tetrapods.

METHODS

Animals

Male and female *C. inornatus* were captured near Sanderson, Texas (*C. inornatus*) or Portal, Arizona (*C. uniparens*), transported to the University of Texas, and maintained in environmental chambers under breeding season conditions as described previously (Wade and Crews, 1991).

Gonadectomy and Hormone Treatment

Experimental females were taken from group-housed conditions, ovariectomized, and held in social isolation as described previously (Wade and Crews, 1991) for 1 week to allow metabolic clearance of endogenous gonadal steroids. Five different hormonal treatments (Table 1) were employed which consisted of different orders of (i) a single subcutaneous injection of 0.5 μg estradiol benzoate (EB; Sigma) or vehicle alone (control) combined with (ii) subcutaneous implantation of silastic capsules containing either crystalline progesterone or empty capsules which served as controls [dimensions: 13 mm outside length, 10 mm inside length (packed hormone), 1.47 mm inner diameter, 1.96 mm outer diameter; prepared as described in Lindzey and Crews, 1986]. In previous experiments, this progesterone administration protocol has produced circulating progesterone concentrations which approximate those of wild-caught female *C. uniparens* and *C. inornatus* during the postovulatory part of the follicular cycle (Moore *et al.,* 1985; Moore and Crews, 1986; D. Crews, unpublished data). The administration procedures for EB injections and subcutaneous silastic capsule implantation have been described (Godwin and Crews, 1995; Young *et al.,* 1995a,b). Treatments 1 and 2 (1, EB injection then blank capsule; 2, EB injection then progesterone capsule) were intended (i) to mimic the natural sequential exposure to estrogen then progesterone during the follicular cycle (Moore *et al.,* 1985; Moore and Crews, 1986) and (ii) test for behavioral effects of progesterone when administered following an EB dose known to effectively stimulate receptive behavior based on previous work (Young *et al.,* 1995a,b). Treatments 1 and 2 consisted of a single 0.5-μg EB injection at 1 week after ovariectomy, followed by blank or progesterone capsule implantation 24 hr later, and tissue harvesting at 24 hr following capsule implantation. Treatment 3 was intended as a measure of baseline levels of ER- and PR-mRNA in the VMH in the absence of EB-stimulation. Treatments 4 and 5 were intended to characterize the effect of P on EB-induced levels of ER- and PR-mRNA at a time point (24 hr postinjection) well-characterized for EB effects on these mRNA species in the VMH (Godwin and Crews, 1995; Young *et al.,* 1995a,b). Capsules were administered in treatments 3–5 at 24 hr prior to injection, followed 24 hr later by a blank (SSV) or EB injection, and brains were harvested 24 hr after the injection. Capsules were given 24 hr prior to injection rather than simultaneously with the injection in treatments 3–5 so that females in these treatments would receive injections in a normal alert state as in the first two treatments

TABLE 1
Experimental Design

Treatment	Days since ovariectomy				Interval (hr)
	6	7	8	9	
1—EB injection, blank capsule	—	EB injection	b1 capsule	Behavior test and sacrifice	48
2—EB injection, P capsule	—	EB injection	P capsule	Behavior test and sacrifice	48
3—blank capsule, blank injection	b1 capsule	b1 injection	Sacrifice	—	24
4—blank capsule, EB injection	b1 capsule	EB injection	Sacrifice	—	24
5—P capsule, EB injection	P capsule	EB injection	Sacrifice	—	24

Note. Interval refers to time between EB injection and brain removal.

(implantation requires anesthesia by hypothermia). It is important to note the difference in time elapsed between receiving the EB injection and tissue harvesting in the two sets of treatments: 48 hr in treatments 1 and 2, 24 hr in treatments 3–5. Hormone injection presumably produces a ''pulse'' of estrogen exposure, rather than a tonic stimulus, and the interval between injection and sacrifice could therefore affect observed mRNA levels. Fourteen *C. uniparens* were used for treatment 1, 22 *C. uniparens* were used in treatment 2, and 6 female *C. inornatus* were used in each of treatments 1–5. All the *C. inornatus* were sacrificed and their brains taken at the termination of experiments; the *C. uniparens* used were not dissected at the termination of experiments.

Behavior Tests

Experimental females were tested for sexual receptivity in treatments 1 and 2 at 24 hr after progesterone implantation. Individual females were placed into the home cages of ovariectomized, long-term testosterone-implanted *C. uniparens.* Testosterone-implanted *C. uniparens* display strong male-typical courtship and copulatory behavior. This is an advantage for this type of study because potential intromission and intromission-induced effects on VMH gene transcription in steroid receptor-containing neurons cannot occur. These effects are observed in rats exposed to vaginocervical stimulation (Blaustein, Tetel, Nielsen, Ricciardi, Delville, and Turcotte, 1994). Following placement of an experimental female into the cage of a stimulus testosterone-implanted *C. uniparens,* tests lasted until the test female allowed either (i) the stimulus animal to at least mount and assume a riding posture on the test female's back (classed as sexually receptive), or (ii) rolled along the longitudinal axis of the body in response to a mount attempt (classed as sexually nonreceptive). Rolling behavior in sexually nonreceptive females is also typically accompanied by aggressive behaviors such as head bobbing, charging, and biting (Lindzey and Crews, 1988).

Tissue Preparation, in Situ Hybridization, and Silver Grain Quantification

Experimental animals were sacrificed 24 hr after either EB injection or capsule implantation depending on treatment (Table 1) and between 1200 and 1500 hr. The *in situ* hybridization and silver grain quantification procedures were identical to those described previously (Godwin and Crews, 1995; Young *et al.,* 1995a,b). The slides are briefly exposed with this method to achieve an approximate minimum hybridization signal of three times background silver grain density and simultaneously also prevent overexposure. Overexposure can obscure differences between cells through a ''ceiling effect'' on silver grain density. The light exposure makes quantification of absolute numbers of cells positive for a given mRNA species unreliable in comparison to immunocytochemical methods.

Statistical Analysis

Proportions of animals classed as receptive were compared among treatment groups with Fisher's exact test (Zar, 1984). Mean silver grain densities (grains/cluster) were compared across the EB-injected treatment groups by two-way analysis of variance to assess the effect of progesterone and order of capsule administration with respect to the injection. Homogeneity of variance among treatment groups was tested using Bartlett's test. Capsule type by administration order interaction terms were examined to determine whether the timing of P administration had a significant effect on ER- and PR-mRNA abundance. Effects of progesterone were also compared to blank capsule treatments

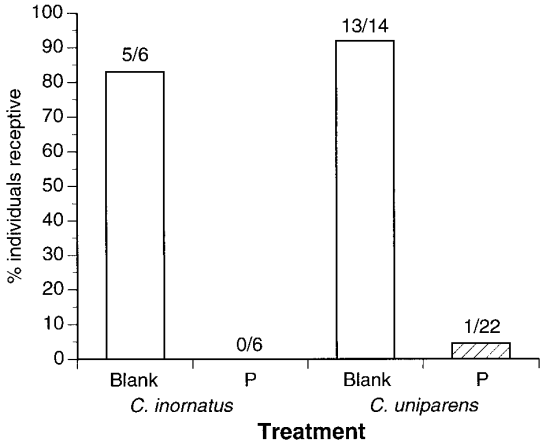

FIG. 1. Results of receptivity behavior tests in ovariectomized, EB-injected females following either blank or progesterone capsule implantation.

within both administration order groups with preplanned two-sample *t* test contrasts. Two-sample *t* tests were also used for direct comparisons of blank-capsule, blank-injection control groups with the EB-injected, blank-implanted experimental groups. A significant difference refers to rejection of the null hypothesis $\alpha = 0.05$ unless otherwise noted.

RESULTS

Behavior Testing

Exogenous progesterone abolished EB-induced receptive behavior within 24 hr of capsule implantation in both *C. uniparens* and *C. inornatus* (Fig. 1). Thirteen of 14 *C. uniparens* receiving blank capsules after an EB injection were sexually receptive at 24 hr postimplantation, while only 1 of 22 receiving progesterone capsules was receptive (Fisher's exact test: $P < 0.0001$). The same pattern was seen with *C. inornatus.* Five of 6 female *C. inornatus* receiving a blank capsule were receptive, but no females of 6 tested 24 hr after progesterone implantation were receptive (Fisher's exact test: $P < 0.01$).

ER- and PR-mRNA in the VMH

EB-injected, blank implanted females had higher PR-mRNA levels than blank-injected, blank-implanted females (*t* test: $P < 0.01$), but ER-mRNA abundance did not differ significantly (*t* test: $P = 0.226$) (Fig. 2). Both ER- and PR-mRNA abundance were significantly lower in females receiving progesterone implants either before or after an EB injection than in females receiving blank implants (two-way ANOVA comparisons: $P < 0.001$ for effect of implant type on both ER- and PR-mRNA). No significant effects of administration order (ER-mRNA; $P = 0.640$; PR-mRNA; $P = 0.386$) or significant interactions between administration order and implant type were found (ER-mRNA; $P = 0.649$; PR-mRNA; $P = 0.132$), indicating that the effects of progesterone on ER- and PR-mRNA abundance did not differ by administration order. The effects of progesterone on ER- and PR-mRNA abundance are also significant if comparisons are made within the implantation before EB-injection (treatment 4 vs 5) and implantation after

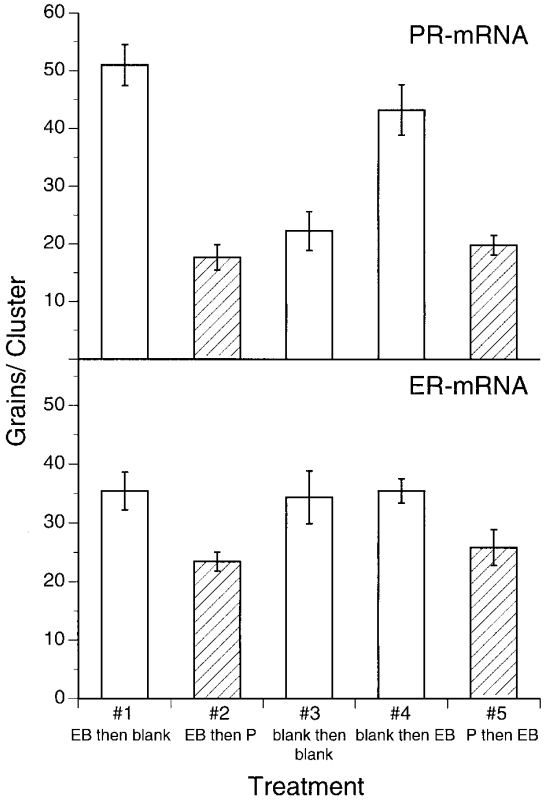

FIG. 2. PR-mRNA and ER-mRNA relative abundance measured as silver grains per cluster in the VMH of female *C. inornatus* in various treatments groups (see treatment key in Table 1, $n = 6$ per group except treatment 3 for ER-mRNA where $n = 5$, error bars are 1 SEM). Statistical comparisons: PR-mRNA, $1 > 2$, $4 > 3, 5$ (*t* tests, $P < 0.01$); ER-mRNA, $1 > 2$, $4 > 5$, $3 = 4$ (*t* tests, $P < 0.05$). Two-way ANOVAs for implant type and administration order: $P < 0.001$ for implant type and $P > 0.386$ for adminstration order for both PR- and ER-mRNA abundances.

EB-injection treatments (treatment 1 vs 2; *t* tests: $P < 0.05$ for ER-mRNA comparisons, $P < 0.01$ for PR-mRNA comparisons).

DISCUSSION

Progesterone Effects on Receptive Behavior

Exogenous progesterone can abolish female-typical receptive behavior in ovariectomized female *Cnemidophorus* given a behaviorally effective dosage of EB. This effect of exogenous progesterone supports previous work in this laboratory on *Cnemidophorus,* showing that receptivity is not observed in either intact females during the postovulatory period when progesterone levels are elevated (Lindzey and Crews, 1988; Moore *et al.,* 1985; Moore and Crews, 1986) or in ovariectomized, progesterone-implanted *C. uniparens* in group-housed conditions (these animals do exhibit male-like pseudosexual behavior; Grassman and Crews, 1986). In other lizard species with similar ovarian cycles to *Cnemidophorus* (an extended vitellogenic, preovulatory phase followed by a postovulatory, gravid phase), progesterone can also block sexual receptivity and also induce active rejection behaviors in some species (e.g., *Holbrookia propinqua*—Cooper and Crews, 1987; Cooper and Greenberg, 1992). However, progesterone has variable effects on receptivity in female *A. carolinensis* depending on both the estrogen and progesterone dosages administered. This species is an asymmetric ovulator which typically has a postovulatory egg in one oviduct and elevated progesterone levels while simultaneously undergoing vitellogenesis in the contralateral ovary and exhibiting receptive behavior (Jones, Guillette, Summers, Tokarz, and Crews, 1983). Receptive behavior in ovariectomized *A. carolinensis* can be induced by either high dosages of EB or a synergism of subthreshold EB and progesterone (McNicol and Crews, 1979). Low progesterone dosages following high, behaviorally effective dosages of EB do not inhibit receptivity (Wu *et al.,* 1985), but higher progesterone dosages do (Valenstein and Crews, 1977).

PR- and ER-mRNA Abundance in the VMH

The abundance differences in steroid receptor mRNAs in the VMH between the blank implant, blank injected (treatment 3), and blank implant, EB-injected (treatment 4) groups agree well with previous studies for PR-mRNA abundance, but less well for ER-mRNA abundance (Godwin and Crews, 1995; Young *et al.,* 1995b). These studies found increases in PR-mRNA in the VMH which were comparable to those seen here at the same EB dosage (0.5 μg). Both studies also found that ER-mRNA abundance in the VMH was increased approximately 40–50% by EB; this difference was statistically significant in the study by Godwin and Crews (1995). The lack of a difference in VMH ER-mRNA abundance between the EB- and blank-injected treatments here may be due to some effect of implantation surgery 24 hr before injection (implants were not given in the previous studies).

Progesterone had strong effects on both ER- and PR-mRNA abundance in the *C. inornatus* VMH, causing apparent decreases of roughly 33 and 66% (respectively) relative to blank-implanted controls. The levels of both mRNA species were strikingly similar between analogous hormone treatments (treatment 1 vs 4, 2 vs 5), indicating that the effect of progesterone on PR- and ER-mRNAs in the VMH was dependent on hormone treatment, but did not differ with differing order of progesterone implantation and timing of tissue harvesting relative to EB injection. It is interesting that at least PR-mRNA abundance in the VMH of progesterone-treated animals is similar to that of blank-implanted, non-estrogen-stimulated animals. This pattern suggests that progesterone treatment may return these cells to a baseline level of PR-mRNA expression or that distinct populations of cells which differ in estrogen response properties are present in the VMH. Blaustein and Turcotte (1989) found that estrogen-induced PR-immunoreactivity was restricted to cells which also expressed estrogen receptor-immunoreactivity in the guinea pig brain. The presence of different estrogen-sensitive neuron phenotypes has also been suggested with respect to progesterone effects on estrogen receptors in the rat VMn (Brown and MacLusky, 1994). While measurements were made blindly and in the same anatomical area of the VMH between individuals, it is nevertheless possible that cells which respond to estrogen with increased PR-mRNA and cells which express PR-mRNA but do not respond to estrogen in the same way are present in the same regions. If this is true, we may have measured stimulated cells of a responsive population in the estrogen-injected, blank-implanted animals and of a nonresponsive population in the blank-implanted, blank-injected, and progesterone-implanted animals. We presently have no information regarding cellular colocalization of ER- and PR-mRNAs in the *Cnemidophorus* brain.

The lower relative abundance of PR- and ER-mRNA in the VMH of progesterone-implanted animals is similar to differences found between intact animals at differ-

ent stages of the follicular cycle (Young *et al.,* 1995c). Both ER- and PR-mRNA are relatively more abundant in the VMH of preovulatory, vitellogenic females (elevated circulating estrogen levels, low progesterone levels) than in the postovulatory stage (low estrogen levels, elevated progesterone levels). These differences could be due to differences in positive estrogenic stimulation of these mRNAs (Young *et al.,* 1995a, 1995b), downregulation by progesterone, or both. The simultaneous variation of both estrogen and progesterone levels over the ovarian cycle prevents separation of these possibilities in intact females, but the present results suggest that progesterone-mediated downregulation may be important in producing these ER- and PR-mRNA variations.

The progesterone effects on both receptive behavior and ER- and PR-mRNA expression described here for *Cnemidophorus* are similar to those described in rats and guinea pigs (Pfaff, Schwartz-Giblin, McCarthy, and Kow, 1994). In these rodent species, progesterone exerts a biphasic effect on lordosis consisting initially of stimulation followed by a later inhibition. Progesterone downregulates numbers of PR-immunoreactive neurons in the ventrolateral nucleus of the mediobasal hypothalamus (guinea pigs, VLH is homologous to the VMn of rats) (Blaustein and Turcotte, 1990; Blaustein *et al.,* 1994) and progestin-binding sites in the VMn of rats (Blaustein and Feder, 1980). This decrease in PR following progesterone administration is thought to account for the behavioral refractoriness of female rodents to a second progesterone administration shortly following initial exposure (Blaustein and Olster, 1989). Downregulation of estrogen receptors in the mediobasal hypothalamus by progesterone is also known from rats and a decrease in estrogen-induced PR levels could therefore also play a role in decreasing behavioral responsiveness to progesterone (Brown and MacLusky, 1994).

This study demonstrates that progesterone can inhibit receptivity in female *Cnemidophorus* lizards and that this behavioral effect is correlated with decreases in the abundance of PR- and ER-mRNAs in the brain area controlling this behavior. The similarity of these progesterone effects to those in well-studied rodent models suggests they are part of an evolutionarily conserved behavior-controlling mechanism in female reproduction.

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