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Effect of Long-Term Castration and Long-Term Androgen Treatment on Sexually Dimorphic Estrogen-Inducible Progesterone Receptor mRNA Levels in the Ventromedial Hypothalamus of Whiptail Lizards

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In whiptail lizards, as in laboratory rodents, females will respond to exogenous estrogen by increasing progesterone receptor (PR) or PR mRNA in the ventromedial hypothalamus (VMH) while males show an attenuated response to the same treatment. In rodents, neonatal hormone manipulations affect the adult expression of this trait; however, few investigators have examined the effects of hormone treatment in adulthood. Therefore the current study was carried out to determine whether observed sex differences in the estrogen response in adulthood may be modified by steroid hormone manipulation. We castrated male whiptail lizards for 1 week (short term) or 6 weeks (long term). We also gonadectomized female whiptails and implanted them with either a Silastic capsule containing testosterone or an empty capsule. At the end of that time all implants were removed and the animals were injected with either estradiol benzoate (EB) or steroid suspension vehicle and their brains were assayed for PR mRNA expression using *in situ* hybridization. The results demonstrate that in male whiptail lizards, long-term castration increases sensitivity to estradiol as measured by induction of PR mRNA in the VMH; EB-injected long-term castrated males were not different from EB-injected females. However, long-term androgenization did not attenuate the estrogen response in females. This suggests that attenuation of the estrogen response in males requires activation by testicular secretions, but that females cannot be made to show a male phenotype via testosterone administration.

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One striking example of sexual dimorphism in the CNS is the phenomenon of estrogen-inducible progesterone receptors (PR) in the ventromedial hypothalamus (VMH). When given exogenous estrogens, females of many vertebrate species will increase PR (or PR mRNA) levels in that brain region. This response has been observed in birds (Gasc and Baulieu, 1988), mammals (Baum, Gerlach, Krey, and McEwen, 1986; Blaustein and Feder, 1979; Lauber, Romano, and Pfaff, 1991; Parsons, Rainbow, MacLusky, and Ewen, 1982), reptiles (Godwin and Crews, 1995; Tokarz, Crews, and McEwen, 1981; Young, Nag, and Crews, 1995a), and amphibians (Roy, Wilson, and Kelley, 1986). The apparent evolutionary conservation of this trait may be in part due to its behavioral relevance. The VMH plays an important role in the control of female-typical sexual behavior (Crews and Silver, 1985; Pfaff, Schwartz-Giblin, McCarthy, and Kow, 1994; Schwartz-Giblin, McEwen, and Pfaff, 1989), and in many species estrogens and progesterone act together in the VMH to modulate the expression of that behavior (see Pfaff *et al.*, 1994 for review). In female whiptails, administration of estradiol benzoate (EB) will elicit receptive behavior (Wade and Crews, 1991a; Young, Nag, and Crews, 1995a) and increase both estrogen receptor (ER) and PR mRNA levels in the VMH (Young, Nag, and Crews, 1995a, Godwin and Crews, 1995). Administration of progesterone (P) following EB terminates receptivity and decreases ER and PR levels (Godwin, Hartman, Grammer, and Crews, 1996). In contrast to the response in females, male laboratory rodents and

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whiptail lizards show either no response (Godwin and Crews, 1995) or an attenuated response (Lauber *et al.*, 1991; Blaustein, Ryer, and Feder, 1980).

The developmental basis for the observed sex difference in response to estrogen treatment has not been well investigated. Experiments in laboratory rodents suggest that the neonatal hormone environment affects adult estrogen responsiveness. Male rats given the aromatase inhibitor ATD during fetal and neonatal life readily display lordosis following estrogen priming and show an increase in PR in the VMH comparable to that of unmanipulated females (Parsons, Rainbow, and McEwen, 1984). However, few experiments have been conducted to determine whether this response might be modified after sexual maturity by manipulations of the hormonal environment. In hamsters, long-term castration appears to decrease male sensitivity to estradiol in the VMH (Fraile, Pfaff, and McEwen, 1987). A similar phenomenon has been described in rats, wherein long-term ovariectomy of females decreases both neural and behavioral sensitivity to estrogen (Clark, MacLusky, Parsons, and Naftolin, 1981; Beach and Orndoff, 1974). It has also been demonstrated that changing the way that estrogens are administered—by giving low-dose pulses rather than one large bolus—can alter the behavioral response of male rodents, inducing them to show levels of lordosis equivalent to that of females (Olster and Blaustein, 1988, 1990; Sodersten, Pettersson, and Eneroth, 1983).

Previous studies in whiptail lizards demonstrate that when treated with EB, hatchling whiptails of both sexes will increase PR mRNA in the VMH; full attenuation of the response in males does not occur until after the testes become spermatogenically active and the animals begin to court females (K. Wennstrom and D. Crews, unpublished data). Since changes in the male whiptails' response to EB are still occurring even after sexual maturity, it seems likely that the expression of this trait may be subject to modification later in adulthood. Therefore the current study was carried out to determine whether the observed sex difference in estrogen responsiveness in adulthood is permanent. Specifically, does long-term castration of adult males or long-term androgenization of adult females alter the sex difference in estrogen-inducible PR mRNA?

MATERIALS AND METHODS

Animals. Adult male and female animals were collected in and around Sanderson, Texas, and transported to the University of Texas at Austin. They were maintained in the laboratory as described previously (Grassman and Crews, 1990) for 7 to 8 months before the study.

Surgery and hormone treatment. Both male and female animals were gonadectomized as described previously (Wade and Crews, 1991a). At the time of surgery, females were implanted intraperitoneally with either Silastic implants containing testosterone (T) (10 mm packed hormone, o.d. 1.96 mm, i.d. 1.47 mm) or with identical empty implants (BL). Males did not receive implants. Following surgery, all animals were housed individually. One week (short term) or 6 weeks (long term) following castration, males received an intraperitoneal injection of either 0.5 μg EB in steroid suspension vehicle (SSV) or SSV only. This dose of EB has been shown to increase PR mRNA in the VMH of adult females but not males (Godwin and Crews, 1995; Young, Nag, and Crews, 1995a). The implants were removed from females at 6 weeks; the animals were allowed to recover for 1 week and were then similarly injected with EB or SSV. This mimics the castration/recovery/injection paradigm used for males in previous studies (Godwin and Crews, 1995) and for short-term castrate males in this study.

In order to assess the effectiveness of the testosterone implants, females' ventral coloration and degree of femoral pore activity were examined at the time of sacrifice. Blue ventral coloration and waxy secretions from a row of enlarged pores on the ventral side of the upper hindlimb are androgen-dependent secondary sexual characteristics in male whiptails. Their presence or absence in the females was used to confirm peripheral T stimulation.

Tissue preparation. Twenty-four hours after injection, animals were sacrificed by rapid decapitation and their brains were removed and frozen in isopentane over dry ice. They were then stored at −74°C until cryostat sectioning. Twenty-micrometer-thick sections were collected in series of six on RNase-free poly-L-lysine-coated slides, which were stored in slide boxes at −74°C.

In situ hybridization and quantification. Slides containing brain sections were processed by *in situ* hybridization as described previously (Young, Lopreato, Horan, and Crews, 1994; Godwin and Crews, 1995). After hybridization to an antisense riboprobe specific for whiptail PR mRNA, slides were dipped in Kodak NTB-2 autoradiographic emulsion and were exposed at 4°C for 11 days. They were then developed using Kodak D-19 developer, stained lightly with cresyl violet, and coverslipped.

The brain sections were analyzed using dark-field

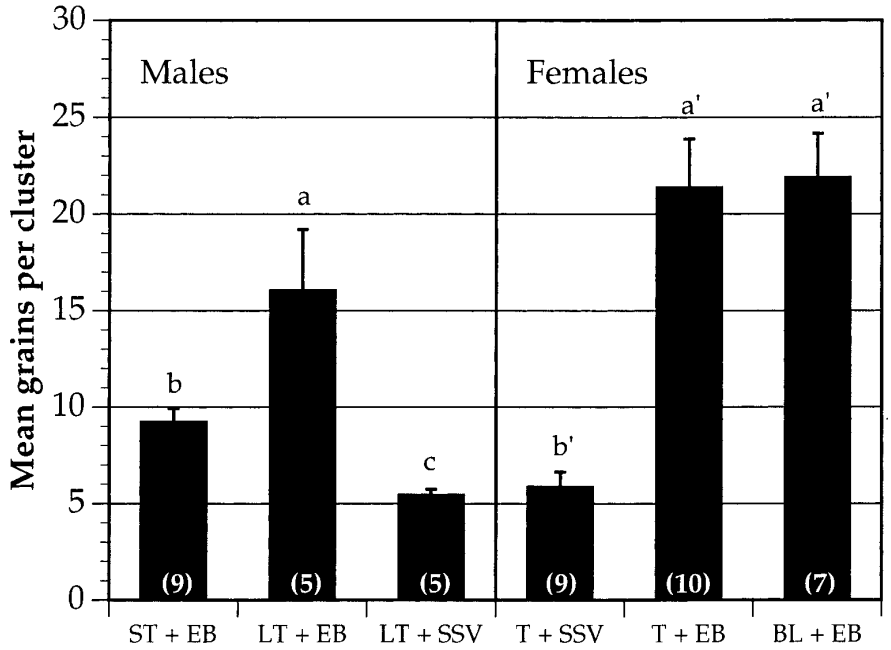

FIG. 1. Progesterone receptor mRNA abundance in the ventromedial hypothalamus of male and female whiptail lizards (*Cremidophorus inornatus*). Males were castrated for 1 week (ST) or 6 weeks (LT) and then injected with either 0.5 μg of estradiol benzoate (EB) or steroid suspension vehicle (SSV) alone. Females were castrated and given Silastic implants of testosterone (T) or an empty capsule (BL). Six weeks later they were deimplanted and after 1 week of recovery were given an injection of either 0.5 μg of EB or SSV. LT + EB males were not significantly different from BL + EB females (two-sample *t* test, $P = 0.17$). Within each sex, groups with different letters were significantly different from one another in a post hoc Tukey's test. Error bars are + 1 SEM.

microscopy and the University of Washington grain-counting software, Grains. Slides were coded to prevent bias during measurement. The quantification method was similar to that previously described (Young, Nag, and Crews, 1995b; Godwin and Crews, 1995). Briefly, in specifically labeled cells, silver grains cluster over the cell nucleus. To quantify the intensity of labeling in the VMH, the 20 densest silver grain clusters in that brain area (10 on each side of the brain) were selected automatically within the Grains program. The grains within the selected clusters were counted. Background measurements of silver grain number were taken over nonspecifically labeled cells near the VMH. The average background silver grains per cluster for each animal was subtracted from the average VMH silver grains per cluster. This corrected value is reported as the mean grains per cluster.

Statistical analysis. Mean grains per cluster were compared between treatment groups within each sex using a one-way analysis of variance. Where necessary, values were log-transformed to preserve homogeneity of variance. Post hoc Tukey's tests were used to further examine significant effects. EB-injected males and blank-implanted, EB-injected females were compared using two-sample *t* tests. All statistics were performed on an Apple Macintosh computer using Systat 5.1. *P* values of ≤ 0.05 were considered statistically significant.

RESULTS

There was a significant effect of time since castration on EB induction of PR mRNA in the VMH (treatment effect $P = 0.0001$, $F = 18.3$, $df = 2$). Long-term castrated males injected with EB showed more than two times as much PR mRNA as SSV-injected males ($P = 0.008$) or short-term castrated EB-injected males ($P = 0.01$) (Fig. 1). In fact, in this study the EB-injected long-term castrated males were not different from BL-implanted, EB-injected females ($P = 0.17$, $T = 1.5$, $df = 7.8$).

Six-week exposure to testosterone did not alter the female animals' response to estrogen (treatment effect $P < 0.0001$, $F = 25.4$, $df = 2$). Regardless of implant type, EB-injected females showed three to four times more PR mRNA in the VMH than SSV-injected females (T + EB vs T + SSV, $P = 0.0001$; BL + EB vs T + SSV, $P = 0.0001$) (Fig. 1). Testosterone treatment did

affect peripheral structures in the females, stimulating secondary sexual characteristics typical of males. Of the 19 T-implanted animals, 17 showed blue ventral coloration and 17 had active femoral pore secretion (one animal had blue coloration but inactive femoral pores, and one animal had active femoral pores but a white vent; only a single animal showed neither androgen-dependent characteristic). In contrast, the BL-implanted animals all had white vents and showed no secretion from the femoral pores.

DISCUSSION

We have demonstrated that in male whiptail lizards, long-term castration causes an increase in sensitivity to estradiol as measured by induction of PR mRNA in the VMH. Although a long-term absence of testicular secretions causes males to show a female-like VMH phenotype in this respect, females did not show a male phenotype following testosterone administration. Long-term androgenization of females had no effect on estrogen-inducible PR mRNA.

The breeding season for little striped whiptail lizards occupies at most a few months during the summer, during which time males have elevated androgen levels and fully recrudescent testes (Moore and Crews, 1986). Toward the end of June as animals prepare for hibernation, the males' testes begin to regress and androgen levels fall. The heightened responsiveness to estrogenic stimulation observed in the long-term castrates in this study would thus presumably be experienced by animals in the field as well. However, some evidence suggests that while long-term castrated males are neurochemically responsive to EB, they are not behaviorally responsive; i.e., they do not show female-typical receptivity (Godwin and Crews, 1995). Thus it is unclear what role a seasonal increase in estrogen sensitivity might play in the natural reproductive cycle of these animals. Further research is necessary to examine the link between changes in the regulation of steroid hormone receptors and changes in the likelihood of sexual behavior.

One interesting aspect of the current results is the difference in the response of 1-week castrated males from the response of 6-week castrates. What makes the long-term castrates more sensitive to estradiol? One hypothesis is that the long-term castrates may have more ER in the VMH than short-term castrates. While this has not been examined directly, previous studies in whiptails using 1-week castrates suggest that increased sensitivity to sex steroids need not imply higher expression of their receptors. In whiptails, the sexes have nearly identical baseline levels of ER mRNA in the VMH, but males and females respond to EB treatment very differently. Females increase both PR and ER mRNA levels in that brain region while males do not (Godwin and Crews, 1995; Young, Nag, and Crews, 1995a).

The presence of equal amounts of ER mRNA in a particular brain region does not, of course, rule out the possibility of differences in the amount of ER protein. However, the fact that males require more than a week-long deprivation of gonadal secretions before changes are observed in their sensitivity to EB suggests that the nature of testicular influence on estrogen-induced PR mRNA involves more than simply a change in receptor abundance. Hormone-induced changes in steroid receptor transcription and in an individual's reponse to sex steroids can frequently be observed within hours of hormone administration (Young, Nag, and Crews, 1995a; Jones, Pfaff, and McEwen, 1985; Parsons, McEwen, and Pfaff, 1981).

It also seems unlikely that much T is still present in the circulation a week after castration, since previous experiments in this laboratory have shown that T will upregulate ER mRNA in the VMH of male whiptails (J. Godwin, V. Hartman, P. Nag, D. Crews, unpublished data). The SSV-injected males in this study had very low levels of PR mRNA, comparable to those of the SSV-injected females. Anecdotal evidence from the current study is in agreement with these data. At the time of sacrifice, two males (both SSV-injected) were found to have regrown testes. Although the data were not included in the statistical analyses, these two animals had higher PR mRNA levels than any other male in the SSV-injected group. Studies in rats have also demonstrated that intact males have higher PR mRNA levels in the VMH than do castrates; in fact the levels in intact males are not different from those in estradiol-treated females (Lauber *et al.,* 1991).

Since the attenuating effect of the testis on EB-induced PR mRNA takes more than a week to dissipate, yet the hormone itself disappears more rapidly, it is likely that the effect is taking place downstream of the androgen receptor itself. It is possible that T may trigger alterations in the interaction of the estrogen receptor with DNA. Studies measuring protein–DNA interactions with a putative estrogen response element (ERE) from preproenkephalin, an estrogen-inducible gene in rat hypothalamus, demonstrate that males show attenuated ERE binding in comparison to females (Zhu and Pfaff, 1995). Testosterone can also alter the morphology of brain nuclei and the arrange-

ment of neural circuits in adulthood (Wade, Huang, and Crews, 1993; Commins and Yahr, 1984; Nottebohm, 1980, 1981); reorganization following removal of testosterone may take time to occur.

The findings presented here for PR mRNA responsiveness agree closely with the results of volumetric analyses of sexually dimorphic limbic nuclei in the whiptails. Adult male whiptails have a larger POAH and a smaller VMH than do adult females (Crews, Wade, and Wilczynski, 1990; Wade *et al.*, 1993). Circulating androgens play a role in maintaining the sex difference in nucleus size; when males are castrated or are outside the breeding season, their brains assume a female phenotype. However, females are unresponsive to androgen treatment (Wade *et al.*, 1993).

Taken together, these data suggest that sensitivity to androgens is greater in male than in female whiptails; in the case of limbic morphology, long-term castration does not alter that difference in sensitivity (Wade and Crews, 1991b). In contrast, both the size of the POAH and the reponse of the VMH to EB are plastic in males and depend on the individual's hormonal milieu. Further studies will be necessary to uncover the basis for the attenuation of the estrogen response by androgens, as well as the timing and mechanism by which sensitivity to androgens is sexually differentiated during development.

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