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Research report

Sexually dimorphic regulation of estrogen receptor α mRNA in the ventromedial hypothalamus of adult whiptail lizards is testosterone dependent

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Abstract

Female, but not male, whiptail lizards will respond to exogenous estrogen with an increase in estrogen receptor α (ERα) mRNA in the ventromedial hypothalamus (VMH). The current study tested whether differences in long-term testosterone exposure may be responsible for this sex difference. In the first experiment, female whiptails were gonadectomized and implanted for 6 weeks with either a Silastic capsule containing testosterone or an empty capsule after which the implant was removed. In a second experiment, male whiptail lizards were castrated for 1 week or for 6 weeks. In both experiments animals were then injected with either estradiol benzoate or steroid suspension vehicle and the brains collected 24 h later. Brain mRNA expression was assayed using in situ hybridization. Chronic testosterone treatment reduced estrogen-induced ERα mRNA levels (number of grains per cell) in the VMH in females. In contrast, there were no significant effects of time after castration or estrogen treatment on induction of ERα mRNA in the VMH in males. The abundance of estrogen-induced ERα mRNA in the VMH, therefore, appears to be responsive to testosterone environment in the female but not in the male, suggesting a difference in this species between the sexes in the mechanism of steroid receptor regulation.

1. Introduction

Male and female vertebrates in adulthood differ both in their sexual behavior and their sensitivity to exogenous hormones [1,16,18]. This difference is particularly evident in how adult males and females differ in their sensitivity to estrogen at the neuroendocrine level. The sexual dimorphism in estrogen sensitivity is postulated to be due to differences in estrogen receptor α (ERα) expression and regulation in specific brain areas, with the most pronounced difference in the ventromedial nucleus of the hypothalamus (VMH). The VMH is a brain area critical for the estrogen-regulated expression of sexual receptivity in female vertebrates. In both mammals and lizards ablation of the VMH eliminates receptive behavior whereas implantation of estrogen into the VMH restores receptive behavior in ovariectomized females; similar manipulations in males do not produce these effects.

Though brains of both males and females contain ERα in the VMH, the amount of ERα mRNA and protein in the VMH is higher in females than males in the rat [3,21,30,33–35], mouse [19] and guinea pig [4]. Estrogen decreases the levels of ERα mRNA and protein in female rats [20,28] and guinea pigs [4,26]. However, in the whiptail lizard (*Cnemidophorus inornatus*), adult males and females have nearly identical baseline levels of ERα mRNA in the VMH and exogenous estrogen increases ERα mRNA abundance in the VMH in females while males show no change in ERα mRNA abundance [13,14,44].

Hatchling male and female whiptail lizards show a significant increase in ERα mRNA after estrogen treatment [41]. As adults, however, males fail to respond to

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exogenous estrogen with an increase in ERα mRNA [13]. It is possible that the long-term exposure to androgen that males experience after sexual maturation may be responsible for this sex difference in adulthood. Indeed, adult sexually active males have plasma levels of testosterone and dihydrotestosterone of 6 and 17 ng/ml, respectively, during the breeding season while females have circulating concentrations at or below the level of detectability of the assay (0.05–0.20 ng/ml depending on sample volume and recovery); estrogen is undetectable in breeding males and on average 0.8 ng/ml in breeding females [27]. In this study, we examined the effect of exogenous estrogen on ERα mRNA levels (number of grains per cell) in the VMH to determine whether the observed sexually dimorphic pattern of ERα regulation by estrogen is a consequence of differences between adult female and male whiptail lizards in testosterone exposure.

2. General materials and methods

2.1. Animals

Animals were treated according to a research protocol approved by the university's institutional animal care and use committee. Adult breeding male and female whiptail lizards (*C. inornatus*) were collected in and around Sanderson, TX and transported to the University of Texas at Austin 7–8 months before the study. Animals were housed in 30 gal glass terraria in large walk-in environmental chambers controlled for photoperiod (14L:10D), temperature (32 °C during day: 27 °C during night), and humidity (35–40%). Each terrarium had a sand bottom layer, wood and plastic objects for cover and basking, a heat lamp and a Vita-light (Durotest) broad spectrum light. Animals were provided water ad libitum and fed mealworms and vitamin-dusted crickets three times a week.

2.2. In situ hybridization and quantification

Following the appropriate experimental manipulation (see below), animals were sacrificed by rapid decapitation and their brains were removed and frozen in isopentane over dry ice. Brain tissue was stored at − 80 °C until cryostat sectioning. Twenty micrometers thick sections in the coronal plane were collected in a series of six onto RNAse free poly-L-lysine coated slides that were then stored in slide boxes at − 80 °C.

Slides containing brain sections were processed by in situ hybridization as described previously [11,13,15,17,41–44]. After hybridization to an antisense and sense control riboprobes specific for whiptail ERα mRNA, slides were dipped in Kodak NTB-2 autoradiographic emulsion and allowed to expose at 4 °C for 17 days. They were then developed using Kodak D-19 developer, stained lightly with cresyl violet, and coverslipped.

The brain sections were analyzed using darkfield microscopy and the University of Washington grain-counting software, Grains. Slides were coded so that the person measuring grain labeling was unaware of the sex or treatment of an animal. The quantification method was as described previously [11,13,15,17,41–44]. Specific labeling was defined as clusters of silver grains lying over individual cells at a density of at least three times that of background [42]. Briefly, in specifically labeled cells, silver grains cluster over the cell nucleus. Within the VMH, the twenty most dense silver grain clusters (ten on each on each side of the brain) were selected and the grains within the selected clusters counted automatically by the Grains program. 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 silver grains per cluster in the VMH. This corrected value is reported as the mean grains per cluster. 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. 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 ERα mRNA is unreliable compared to immunocytochemical methods.

2.3. Statistical analysis

Mean grains per cluster were compared between treatment groups within each sex using a one-way analysis 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. *P*-values of < 0.05 were considered statistically significant.

3. Specific methods and results

3.1. Experiment 1. Effect of long-term androgen treatment on responsiveness to exogenous estrogen in the female whiptail lizard

Female lizards were ovariectomized as described previously [37,43,44] and, at the time of surgery, received an intraperitoneal Silastic implant (o.d. 1.96 mm, i.d. 1.47 mm) that was empty (BL) or contained testosterone (T) (10 mm packed hormone). This size of implant results in circulating concentrations of testosterone in the physiological range of sexually active male whiptail lizards [22–24,39]. The im-

plant was removed 6 weeks later and the females allowed to recover for 1 week before receiving an intraperitoneal injection of either 0.5μg estradiol benzoate (EB) in steroid suspension vehicle (VEH) or VEH only; 24 h later brains were harvested. Due to limitations in the number of available animals from field collection, it was not possible to have a group that was ovariectomized and receiving a BL implant and a VEH injection.

In order to assess the effectiveness of the testosterone implants, the female's ventral coloration and degree of femoral pore activity were examined at the time of sacrifice. Blue ventral coloration and waxy secretions from the femoral pores on the ventral side of the upper hindlimb are androgen-dependent secondary sexual characteristics in male whiptails [9,24,39]. We required that both traits be expressed to confirm peripheral testosterone stimulation in the females. One female that had only blue ventral coloration and one female that had only femoral pore secretion were excluded from the analysis.

Six weeks of exposure to testosterone suppressed the increase in ERα mRNA in response to exogenous EB in females ($F_{2,20} = 10.91$, $p < 0.001$; BL-implanted>EB and VEH-treated, T-implanted, $p < 0.05$ for each) (Fig. 1). Testosterone treatment also affected peripheral structures in the females, stimulating secondary sexual characteristics typical of males. All of the BL-implanted females had white ventral coloration and no femoral pore secretion.

3.2. Experiment 2. Effect of exogenous estrogen on ERα mRNA abundance in the brain of castrated male whiptail lizards

Male lizards were castrated as described previously [7,22] and 1 week (short-term) or 6 weeks (long-term) following
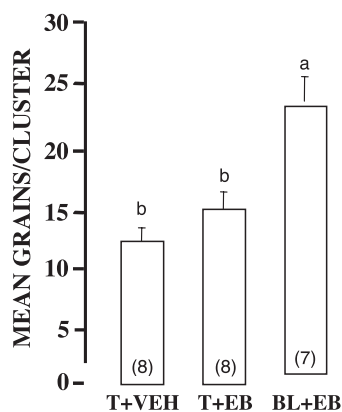

Fig. 1. Estrogen receptor mRNA abundance in the ventromedial hypothalamus in female whiptail lizards (*Cnemidophorus inornatus*). Females were exposed for six weeks to a testosterone (T) or a blank (BL) implant before the implant was removed and individuals given a single injection of 0.5 μg estradiol benzoate (EB) or steroid suspension vehicle (VEH). Group means (+/− 1 standard error) depicted with different letters indicating significantly different groups (α = 0.05). Sample sizes for each group are shown at the base of each bar.

castration, received an intraperitoneal injection of 0.5 μg EB; a third group received an injection of VEH only 6 weeks following castration. This dose of EB has been shown to elicit receptive behavior [43,40] and increase ERα mRNA in the VMH [13] in female and not male whiptails. In all three groups brains were harvested 24 h later. Due to the number of available animals from field collection, it was not possible to have a group that was castrated for 1 week and then received a VEH injection; however, a previous study has shown that VEH has no effect on short-term castrate males [13].

There were no significant effects of time since castration or EB treatment on induction of ERα mRNA in the VMH in males ($F_{2,20} = 0.92$, $p = 0.41$). Following EB injection, the short-term castrate males had less ERα mRNA in the VMH than did BL-implanted females ($p < 0.04$); long-term castrated males had levels comparable to BL-implanted females ($p = 0.36$).

4. Discussion

Though the behavioral effects of estrogen and the distribution of ERα in specific brain areas are highly conserved, sexual dimorphism in neural ER expression has been examined extensively in only a few species. Female and male whiptail lizards differ in the regulation of ERα in the VMH. A single injection of estrogen increases ERα mRNA in the VMH of female, but not male, whiptail lizards [13,14]. In this study, we demonstrated that there is also a sex difference in the effect of testosterone exposure on the sensitivity of the VMH to estrogen. Long-term exposure to testosterone results in a loss of the ability for estrogen to induce ERα mRNA in the VMH in females. However, long-term absence of testosterone after castration did not cause males to gain estrogen-inducible ERα mRNA expression. These results suggest that there is a permanent neural difference between female and male whiptail lizards in ERα mRNA regulation in the VMH.

As indicated, the control group of BL + VEH was missing in the present design. A persistent problem with investigation of rare and/or unconventional animal models is how to weigh sample sizes against parametric experimental designs. In the present instance too few animals were collected to enable us to conduct a fully balanced design. Thus, it is difficult to discern whether the significantly higher grain count in BL + EB lizards (compared to T + VEH and T + EB groups) is caused by the EB injection rather than the long-term gonadectomy. However, an increase associated solely with gonadectomy would imply estrogen suppression of ER transcription in the VMH. This is unlikely as all effects described to date in this and other species is that estrogen is stimulatory. In addition, using the same design as the present study we have found that PR responsiveness does not differ with a T vs. BL implant when

both groups receive EB [40], suggesting a similar response to exogenous estrogen; PR mRNA levels are very low without EB stimulation.

These results are particularly intriguing when compared to results from a previous study examining PR mRNA regulation in the VMH of the same individuals. In situ hybridization for PR mRNA on adjacent tissue sections to those used here showed that estrogen-inducible PR expression in the VMH is sensitive to long-term testosterone exposure in males, but not in females [40]. Long-term castrated male whiptails show a significant increase in PR mRNA in the VMH in response to exogenous estrogen. In contrast, 6 weeks of testosterone exposure did not attenuate the increase in PR mRNA in the VMH of females given estrogen. The sexes thus differ in which receptor is sensitive to adult testosterone exposure. That is, regulation of the ERα is sensitive in females, but regulation of the PR is sensitive in males. Related to this point, we have found that newborn individuals differ from adults in that males exhibit an increase in ERα mRNA levels in the VMH in response to exogenous estrogen [41]. A wealth of studies with other animals indicates that the change to the adult phenotype is associated with the secretion of androgen at sexual maturity.

It was previously postulated that the increased PR mRNA levels in long-term male castrates given estrogen might have been the result of a concurrent increase in ERα in the VMH [40]. The present results suggest that this is not the case; ERα mRNA levels are not increased in long-term castrate males. The presence of equal amounts of ERα mRNA in a particular brain region does not, of course, rule out the possibility of differences in the amount of ERα protein. However, the fact that males require more than a week of deprivation of gonadal secretions before changes are observed in PR mRNA sensitivity to EB suggests that the nature of testicular influence on estrogen-induced PR mRNA involves more than simply a change in receptor protein abundance. Changes in both steroid receptor transcription and sex behavior in response to sex steroids have been observed within hours of hormone administration in the whiptail lizard [44].

Because we used testosterone rather than a non-aromatizable androgen, it can be argued that the differences we observe result from a difference between males and females in the amount of neural aromatization of testosterone to estrogen, as has been demonstated in the rat [12,31]. Long-term testosterone exposure would increase local neural concentrations of estrogen in the sex having greater aromatase activity. Differences in neural estrogen levels could then have varying effects on the estrogen feedback regulation of ERα. As noted above, however, all of the animals in these studies had no testosterone at least 1 week prior to the estrogen injection and measurement of receptor levels, hence no immediate differences in aromatized estrogen availability between groups were expected. In addition, differences in the ability to aromatize testosterone would not explain why the two receptors respond oppositely within

one sex to changes in testosterone levels unless long-term estrogen exposure supports the regulation of only one receptor and its effects require more than one week to dissipate. Also, aromatization may not have the same role in lizards as in mammalian species studied to date. Male whiptail lizard sexual behavior is restored by non-aromatizable androgen [32] and, in some individuals, by progesterone (reviewed in Ref. [8]); in the brown anole blockade of the androgen receptor is sufficient to disrupt male sexual behavior [36].

The patterns of hormone sensitivity we observe relate in interesting ways to those of other sexually dimorphic traits in the whiptail. The VMH is larger in whiptail females than in males [10,14,38]. In the absence of testosterone, the VMH will become larger in males. The VMH will not become smaller in females, however, under the influence of testosterone [39]. Thus, this morphological difference has the same direction of sensitivity to testosterone as PR regulation in the VMH; VMH size and PR expression up-regulation are suppressed in males, but not females, by testosterone.

In contrast, the regulation of ERα sensitivity correlates more closely with sex differences in behavioral sensitivity. In response to exogenous estrogen treatment, female whiptails will display sexually receptive behavior along with the increase in ERα and PR mRNA in the VMH, but male whiptails show neither receptor up-regulation nor receptive behavior [13,40] (D. Crews, unpublished data). Males do not show female-typical receptivity even with long-term castration even though they have female-like PR expression [13] and VMH size [10,39]. Thus, the degree of responsiveness of ERα, but not PR, to estrogenic stimulation correlates with the likelihood of female-typical sexual behavior. Progesterone and PR expression are more closely implicated in regulation of male-typical behavior in whiptails [8,11].

Estrogen receptor α and its action in the VMH/VMH are critical for the expression of sexual behavior in all female vertebrates studied to date, but there are notable species differences in receptor regulation [16]. Estrogen receptor α mRNA and protein levels are higher in the VMH of female rats and guinea pigs than in the VMH of males but in these species estrogen down-regulates ERα mRNA in the VMH [4,20,25,34] and, to a lesser extent, protein [5]. This difference mirrors the difference between female whiptails and rats in the duration of sexual behavior. Female rats engage in sexual behavior for less than 24 h around the periovulatory period. When follicular secretions of estrogen levels peak, sex behavior begins to decline soon after, perhaps as a consequence of decreased ERα in the VMH. Female whiptails, on the other hand, have reproductive cycles of weeks and engage in sexual behavior over many days. Immediate suppression of ERα and sexual behavior by high circulating concentrations of estrogen would be a disadvantage in this species. In female rats and guinea pigs, the level of PR rather than ERα in the preoptic area (POA),

VMH and ventrolateral hypothalamus correlates with receptive sexual behavior [2,6,29], though clearly estrogen and progesterone act together in the VMH to modulate sexually receptive behavior in females [1].

As with our results in whiptails, ERα but not PR, regulation in the VMH of male rats may be insensitive to testosterone. This could indicate similar mechanisms of organization of ERα regulation in the VMH or a genetically determined sensitivity in males of both species. If ERα and PR regulation is organized in the whiptail, our results suggest that the mechanism for estrogen regulation of ERα is organized prior to adulthood in the *male*, but not *female* whiptail and the mechanism for estrogen regulation of PR is organized prior to adulthood in the *female*, but not *male*. The testosterone sensitivity we observe in adult whiptails reflects the influence of long-term testosterone (or aromatized estrogen) availability. There is ample evidence of long-term effects of both testosterone and estrogen on neuron survival, dendritic growth, synaptic connectivity and neurotransmitter and protein availability [1], each of which could be involved differently in females and males. Our results add support to the idea that males and females differ in the patterns of regulation of sex hormone receptors.

Acknowledgements

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