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Steroid Feedback on Gonadotropin Release and Pituitary Gonadotropin Subunit mRNA in Mice Lacking a Functional Estrogen Receptor α

Scott R. Wersinger,[1] Daniel J. Haisenleder,[2] Dennis B. Lubahn,[3] and Emilie F. Rissman[1,4]

[1]Department of Biology and [2]Department of Medicine, University of Virginia, Charlottesville, VA; and [3]Departments of Biochemistry and Child Health, University of Missouri, Columbia, MO

Steroid hormones regulate levels of gonadotropin mRNA in the pituitary, and gonadotropic hormones in plasma. To determine whether estrogen receptor α (ERα) mediates steroid negative feedback, wild type (WT) and estrogen receptor α knockout (ERαKO) mice of both sexes were gonadectomized and implanted with a Silastic capsule containing either estradiol (E_2), dihydrotestosterone (DHT), testosterone, or a blank capsule. Ten days later, plasma luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels were measured. Pituitary mRNA levels of gonadotropin subunit (α, LHβ, FSHβ) and prolactin (PRL) were quantified. LH levels in gonad-intact ERαKO females were elevated, similar to values seen following gonadectomy. By contrast, serum LH concentrations in gonad-intact ERαKO males were low and rose following gonadectomy, suggesting androgen feedback. Estradiol treatment significantly decreased plasma LH in WT animals, but not in ERαKOs. In fact, in female ERαKOs, our dose of E_2 increased plasma levels of LH as compared with untreated, ovariectomized ERαKOs. All the steroid treatments suppressed LH in WT animals whereas only DHT consistently suppressed LH concentrations in ERαKO mice. The postgonadectomy rise in plasma FSH was prevented by steroid treatments in WT females, but not in any of the other groups. Gonadotropin subunit and PRL mRNA responses to E_2 treatment (both inhibitory and stimulatory) were absent in ERαKO mice, suggesting a critical role for ERα. Although E_2 can exert negative feedback effects on LH release in both males and females by actions at the ERα, the androgen receptor plays the primary physiological role in the male mouse.

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Author to whom all correspondence and reprint requests should be addressed: Emilie F. Rissman, Department of Biology, University of Virginia, Gilmer Hall, McCormick Road, Charlottesville, VA 22903. E-mail: efr2f@virginia.edu

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Introduction

The neuroendocrine regulation of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) release is complex, involving positive and negative feedback loops *(1–4)*. Sex steroids exert negative feedback effects on gonadotropin release in many species. Gonadectomy results in a steady rise in plasma LH and FSH in both sexes, an effect prevented by treatment with exogenous steroids *(1,5,6)*. Similarly, pituitary levels of gonadotropin subunit mRNA increase after gonadectomy; steroid replacement blocks this effect *(6–8)*. Estrogen suppresses LH release in mice of both sexes *(5)*, suggesting a role for an estrogen receptor (ER) in steroid negative feedback.

Estrogen can act via a number of pathways. Thus, the question is no longer just, Does estrogen exert an effect? but also By which pathway does estrogen act? For example, a second ER, the ERβ, has been recently cloned and characterized *(9,10)* and shown to have several isoforms *(11–13)*. The ERβ has similar binding characteristics *(9,10,14,15)* and has a similar, but not identical, neuronal distribution to the ERα *(16,17)*. In addition, there are alternative isoforms of the ERα *(18,19)*, membrane ERs *(20)*, and the newly described ara70 protein, which allows estrogen to activate the androgen receptor (AR) *(21)*. The physiological significance of these different proteins has not yet been determined, but researchers are beginning to characterize of the independent and dependent actions of different ER proteins *(22)*.

Since steroids regulate the hypothalamic-pituitary axis, one might predict that animals lacking a functional ER would have several neuroendocrine anomalies. Indeed, female ERα knockout (ERαKO) mice have higher levels of LH *(23–25)* as well as elevated pituitary α-subunit and

LHβ mRNA as compared to their wild-type (WT) littermates *(26)*. Thus, steroid negative feedback is disrupted in female ERαKOs. Likewise, in adult male ERαKOs, testosterone and LH are elevated in plasma, compared with WT littermates *(23,27)*. While treatment with E_2, testosterone, or DHT lowers postcastration elevations in LH in WT males, only DHT appears to reduce LH levels in castrated ERαKOs *(27)*.

Testosterone can also suppress LH release in mice of both sexes. However, the interaction between androgens and estrogens in steroid negative feedback has not been well characterized. These two steroids do not appear to act synergistically *(1)*, although there is a sex difference in sensitivity of the hypothalamic-pituitary-gonadal (HPG) axis to steroid negative feedback *(27–30)*. In vivo, testosterone is aromatized to estrogen or reduced to DHT *(31)* and can therefore act at the ER, or the AR, or both.

In this experiment, WT and ERαKO mice of both sexes were gonadectomized and treated with exogenous steroids to determine whether the ERα mediates estrogen negative feedback on the HPG axis. In addition, we wished to determine the relative contribution of the ERα, the AR, or both in the negative feedback effects of testosterone.

Results

Plasma LH

In females, there was a significant effect of genotype ($F[1,63] = 36.26$, $p < 0.001$), steroid treatment ($F[4,63] = 7.23$, $p < 0.001$), and an interaction between the two factors ($F[4,63] = 12.30$, $p < 0.001$) on plasma LH concentrations. In males, there was a significant effect of steroid treatment ($F[4,63] = 9.28$, $p < 0.001$) on plasma LH levels, no effect of genotype, and no interaction between these two factors.

In females, but not in males, gonad-intact ERαKOs had significantly higher plasma LH than intact WT mice (Fig. 1). Gonadectomy resulted in a significant increase in plasma LH in WT males and females as well as in ERαKO males, but not in ERαKO females. As expected, E_2 replacement suppressed LH release in WT mice. In the ERαKO males, E_2 was without effect, yet, surprisingly, E_2 significantly increased plasma LH in ERαKO females. Both DHT and testosterone prevented the postgonadectomy rise of LH in WT mice and ERαKO males, but only DHT suppressed LH release in ERαKO females.

Plasma FSH

In females, there was an effect of genotype ($F[1,63] = 16.20$, $p < 0.001$), steroid treatment ($F[4,63] = 6.11$, $p < 0.001$), and an interaction between these factors ($F[4,63] = 11.21$, $p < 0.001$) on plasma FSH concentrations. In males, there was a significant effect of steroid treatment ($F[4,63]$) $= 3.28$, $p < 0.01$) on plasma FSH levels, no effect of genotype, and no interaction between these two factors.

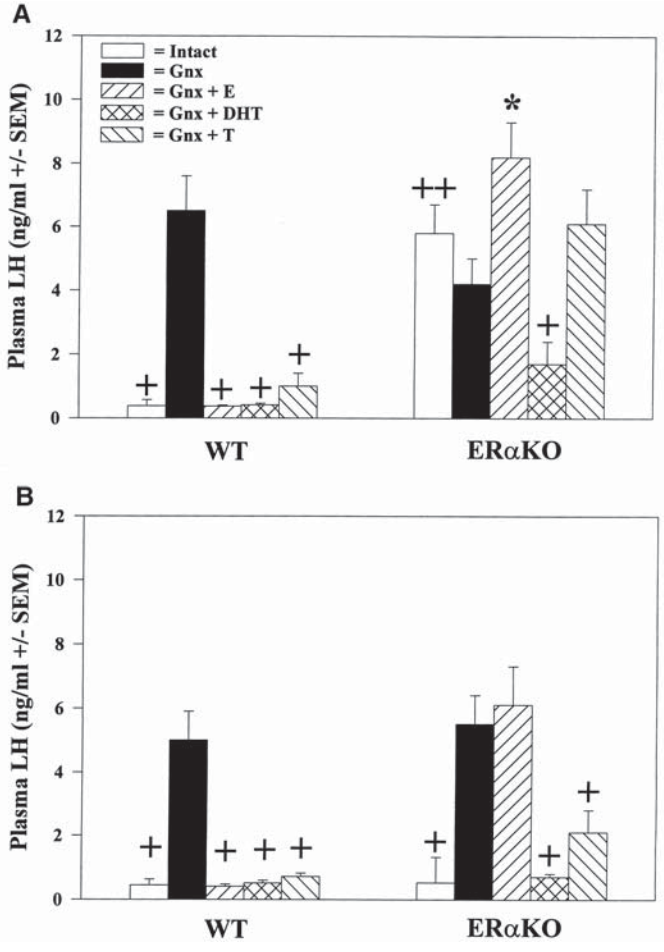

Fig. 1. The mean (±SEM) level of plasma LH of intact (□); gonadectomized (■); gonadectomized, estradiol-treated ( ); gonadectomized, DHT ( ); or gonadectomized, testosterone-treated ( ) female **(A)** and male **(B)** WT and ERαKO mice. + = significantly less than gonadectomized of same sex and genotype ($p < 0.01$); * = significantly greater than gonadectomized of same sex and genotype ($p < 0.01$); ++ = significantly greater than intact WT female ($p < 0.01$).

Figure 2A shows the concentration of plasma FSH in females. Plasma FSH levels were equivalent in gonad-intact animals of both genotypes, and gonadectomy resulted in a significant increase in FSH concentrations. All three steroids prevented the FSH rise in WT animals, but none did so in ERαKOs. Figure 2B shows plasma FSH in male mice. As with LH, gonadectomy resulted in higher FSH levels. However, steroid treatment did not reduce FSH levels in male mice of either genotype.

Pituitary mRNAs

To determine the effect of steroid negative feedback, a second study was conducted in gonadectomized animals with and without E_2, testosterone, or DHT replacement.

Gonadotropin α-Subunit Expression

There was an effect of genotype ($F[1,25] = 9.45$, $p < 0.05$), no effect of hormone treatment, and a significant interaction between the two factors ($F[2,25] = 4.23$, $p < 0.02$) on

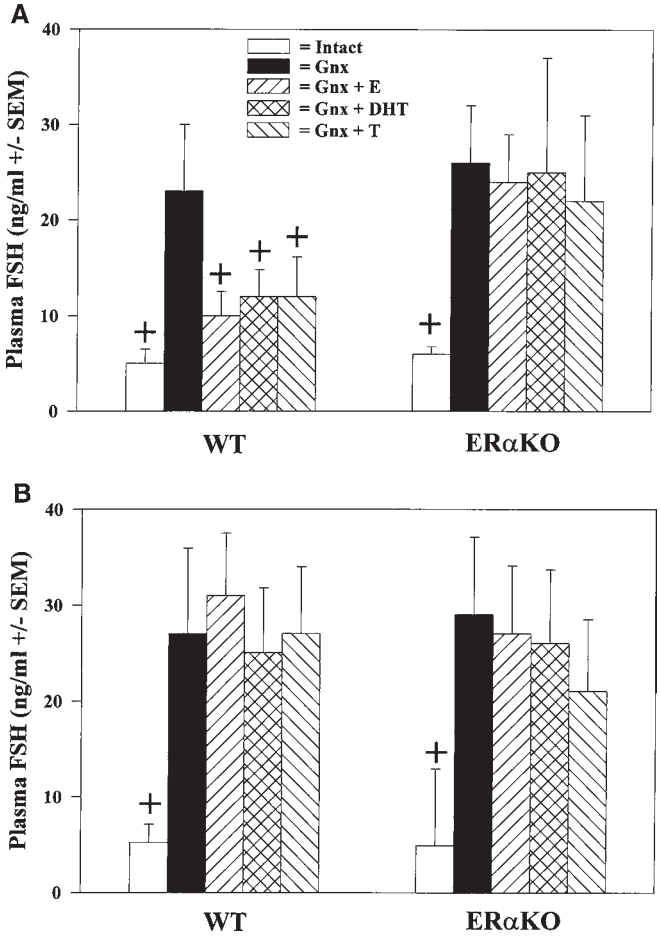

Fig. 2. The mean (±SEM) level of plasma FSH of intact (□); gonadectomized (■); gonadectomized, estradiol-treated ( ); gonadectomized, DHT ( ); or gonadectomized, testosterone-treated ( ) female **(A)** and male **(B)** WT and ERαKO mice. + = significantly less than gonadectomized of same sex and genotype ($p < 0.01$).

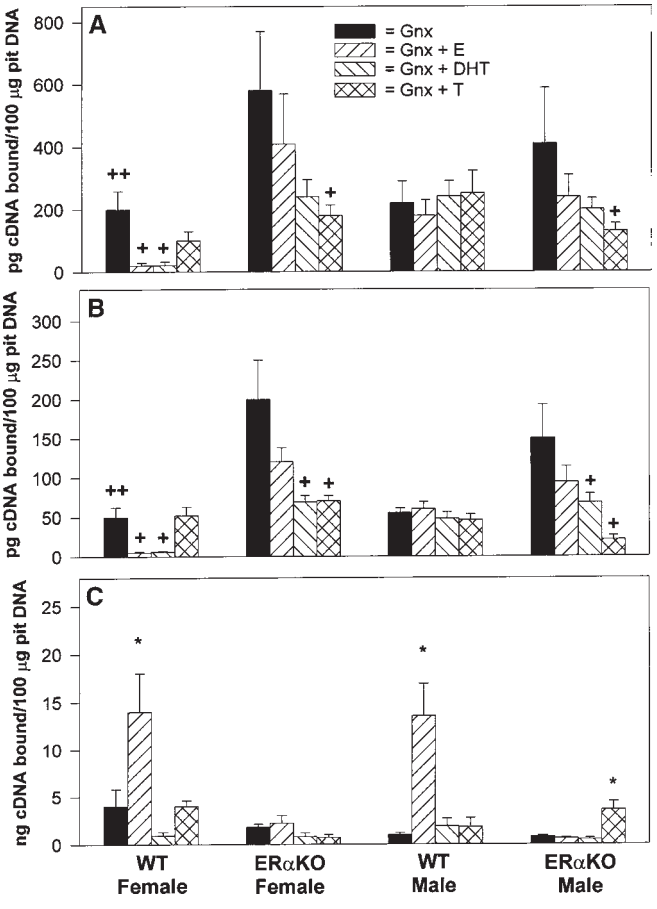

Fig. 3. The mean (±SEM) quantity of gonadotropin α-subunit **(A)**, LHβ **(B)**, and PRL **(C)** mRNA of gonadectomized (■); gonadectomized, estradiol-treated ( ); gonadectomized, DHT ( ); or gonadectomized, testosterone-treated ( ) WT and ERαKO mice of both sexes. + = significantly less than gonadectomized of same sex and genotype ($p < 0.01$); ++ = significantly less than gonadectomized ERαKO of same sex ($p < 0.01$); * = significantly greater than gonadectomized of same sex and genotype ($p < 0.01$)

pituitary α-subunit mRNA levels. Female ERαKOs had higher amounts of mRNA for gonadotropin α-subunit than WT females ($p < 0.05$). As shown in Fig. 3A, α-subunit mRNA levels were significantly lower in WT females treated with E and DHT than untreated, gonadectomized WT females ($p < 0.05$). Testosterone suppressed α-subunit expression in ERαKO, but not WT mice of both sexes ($p < 0.05$).

Lutenizing Hormone β

There was an effect of genotype ($F[1,25] = 8.32$, $p < 0.05$), but not of hormone treatment, and a significant interaction between treatment and genotype was noted ($F[2,25] = 5.75$, $p < 0.02$) on LHβ mRNA. Gonadectomized ERαKOs that received a blank capsule had higher levels of LHβ mRNA than similarly treated WT mice (Fig. 3B; $p < 0.05$). Estradiol and DHT suppressed LHβ mRNA expression in WT females ($p < 0.05$). None of the steroid treatments affected LHβ mRNA in WT males. Androgens (both testosterone and DHT) significantly decreased LHβ expression in male and female ERαKOs, compared with gonadectomized WT mice of the same sex ($p < 0.05$).

*PRL and FSH*β

There was a significant interaction between genotype and treatment ($F[2,25] = 15.75$, $p < 0.01$) on PRL mRNA, but no significant effect of either factor alone. The level of PRL mRNA was increased by estrogen treatment in WT mice (Fig. 3C; $p < 0.01$). Estradiol had no effect on PRL mRNA in ERαKO pituitaries. In addition, male ERαKOs treated with testosterone had a small but significant increase in the level of prolactin mRNA ($p < 0.05$). FSHβ gene expression was unaffected by steroid treatment in WT or ERαKO mice of either sex (data not presented).

Discussion

The present data reveal that ERα plays a major role in negative feedback regulation of LH release in the female

mouse, as demonstrated by elevated serum LH levels in intact ERαKOs. These findings also suggest that the AR may play a critical negative feedback role on LH secretion in the male, based on the finding that LH was similar in intact WT and ERαKO males.

It is also interesting that although female ERαKOs lack a functional ERα, their HPG axis can respond to exogenous E_2. Plasma LH concentrations are higher in E_2-treated female ERαKOs than in either intact or gonadectomized ERαKOs. This effect of E_2 in mice lacking the ERα suggests that another ER mediates this response. One candidate is the ERβ. This estrogen-responsive protein has been localized to the brain *(16,17)*, has similar binding characteristics to the ERα *(10–12)*, and has been reported in rat pituitary *(32)*. The existence of an ER, other than the classical ERα, that stimulates LH release could provide a mechanism for the paradoxical effects of E_2 during the ovarian cycle. Female mice lacking functional copies of the ERβ gene have smaller litters and, when superovulated, shed significantly fewer ova than their WT littermates *(33)*. This suggests that both ERα and ERβ are required for the regulation of ovulation.

Estradiol's effects on the HPG axis are not necessarily mediated by a classical ER (ERα or β). Das et al. *(22)* showed that catecholestrogen increased uterine lactoferrin mRNA levels independent of either the ERα or the ERβ. Since E_2 can be converted to metabolites such as catecholestrogen in vivo, the effects of E_2 that we observed in ERαKO mice could be a result of indirect action of a metabolite of E_2.

In the present study, the concentration of plasma LH was elevated in ovary-intact ERαKO females but normal in gonad-intact ERαKO males as compared to WT mice of the same sex. This lack of a difference in LH levels in male ERαKO mice has been reported before *(24–26)*, but higher levels of LH in ERαKO males, compared with WT males, have also been reported *(27)*. Androgens (testosterone or DHT) suppress LH after gonadectomy in both male and female WT mice. However, in the female ERαKOs, testosterone was not as effective at suppressing LH as was DHT, even though the same dose of testosterone fully suppressed LH in WT females. In males, we found that DHT and testosterone were both capable of suppressing LH in both genotypes. These results are in contrast with those reported by Lindzey et al. *(27)*. In that study LH concentrations were not reduced by DHT in WT males, yet DHT, but not testosterone, had a significant effect on LH levels in ERαKOs. Perhaps their DHT treatment was ineffective in WT males because they waited 3 wk before collecting blood for assay. During that time DHT could have been metabolized. In our study, only 10 d elapsed between castration/hormone treatment and blood collection. If our hypothesis is correct, it suggests that steroid metabolism is altered by the lack of ERα. Both studies, and our data in females, suggest that part of testosterone's effects on LH occurs after its aromatization to E_2 *(31)*. Thus, testosterone may promote negative feedback on LH via its actions on both ERs and AR in WT mice, but only via AR in ERαKOs. If this is the case, then testosterone cannot exert its full negative feedback effects in ERαKOs. Another possibility is that ERαKOs are less sensitive to androgens. This hypothesis is supported by the observation that the area of AR immunoreactivity in the bed nucleus of the stria terminalis is significantly smaller in ERαKOs of both sexes than in WT mice *(34)*.

In contrast to LH, plasma FSH levels were equivalent in gonad-intact mice of both sexes and genotypes (present results; *23,24*). As expected, gonadectomy led to an increase in plasma FSH in both sexes and genotypes. Ovary-intact and steroid-treated WT females had significantly lower plasma FSH concentrations than gonadectomized WT females. On the other hand, steroids had no effect on FSH concentrations in WT male mice, or in ERαKOs. Rodin et al. *(35)* also found that androgen failed to suppress serum FSH in male mice 10 d postcastration. These findings are not consistent with those of Lindzey et al. *(27)*. They *(27)* reported that E_2 and T, but not DHT suppressed FSH in WT males. Again, the different time courses used may explain the differences. More than 10 d of treatment may be required for steroids to exert negative feedback of FSH in males. This sexually dimorphic response is consistent with previous reports showing that females are more sensitive to steroid negative feedback on LH release *(28–30)* or, perhaps, that inhibin plays a greater role in the regulation of FSH in males than in females *(36)*.

In WT females, plasma FSH concentrations are suppressed by steroid treatment. By contrast, steroid treatment fails to suppress FSH release in ERαKOs, even though they have normal ARs *(34)*. One possible explanation is that the AR system is compromised in ERαKOs brains. Studies have shown that E_2 lengthens the time androgen is bound to the AR in rat brain *(37)* and upregulates AR gene transcription *(38,39)*. As mentioned, ERαKO mice have less AR immunoreactivity in at least one neural region as compared to their WT littermates of the same sex *(34)*. Thus, it seems that the ERα plays a role in both estrogen and androgen negative feedback.

The present data suggest that gonadotropin α-subunit and LHβ gene expression are regulated by steroid negative feedback in a similar manner. Gonadectomized ERαKO females have higher levels of α-subunit and LHβ mRNA than gonadectomized WT females. Ovary-intact ERαKO females also have higher gonadotropin subunit mRNA levels than WTs *(26)*. Although α-subunit and LHβ mRNA levels are higher in gonadectomized ERαKOs than in gonadectomized WT mice, plasma LH levels are similar between these groups. Thus, the elevation in α-subunit and LHβ mRNA levels observed in ERαKOs is not correlated with higher levels of plasma LH. This dissociation between subunit

mRNA and plasma gonadotropin levels has been reported to occur at the time of the preovulatory LH surge *(40,41)*.

Interestingly, DHT, but not testosterone, suppressed α-subunit and LHβ expression in WT females. This could be because DHT has a higher affinity for AR than testosterone *(42,43)* and perhaps a higher dose of testosterone is required to suppress α-subunit and LHβ expression. Both DHT and testosterone suppress α-subunit and LHβ expression in ERαKOs of both sexes. We hypothesize that androgen action in the pituitary may normally be masked by activation of the ERα in WT mice. In the absence of the ERα, however, androgen's effects are revealed. This hypothesis is supported by the observation that testosterone selectively increased PRL mRNA in ERαKO males.

Our results also show that pituitary PRL mRNA is regulated by E_2 acting via the ERα since E_2 increased PRL mRNA levels in WT, but not ERαKO, mice of either sex. These data complement and extend those reported earlier showing a reduction in PRL mRNA in pituitaries collected from gonad-intact ERαKO vs WT mice *(26)*. This effect is likely to be a direct action on the PRL gene, since E_2 has been shown to stimulate PRL transcription by binding to estrogen-responsive regions *(44)*.

In summary, our data show that E_2 stimulates the pituitary release of LH in female mice even though they lack a functional ERα. The data also show that ERα plays the primary role in E_2 regulation of gonadotropin secretion and gonadotropin and PRL mRNAs. These data also suggest that E_2 (acting via the ERα during development, during adulthood, or both) affects responses to androgen, either indirectly (via aromatization) or directly by modification of AR function.

Materials and Methods

Animals

The ERαKO colony was maintained at the University of Virginia, from progeny provided by one of the coauthors (DBL). The ERαKOs were from a mixed C57BL/6J/129SVJ background and have been previously described *(45)*. All animal procedures and care were in accordance with university and National Institutes of Health guidelines on care and use of laboratory animals. Heterozygous breeding pairs were used to produce homozygous (+/+, WT, and –/– ERαKO) animals. Each mouse was screened by polymerase chain reaction (PCR) analysis of DNA from tail clips using a modified version of a protocol described in detail elsewhere *(45)*. After weaning (18–20 d of age), male and female mice of both genotypes were group housed by sex and genotype on a 12-h light:12-h dark photoperiod (lights out at 1:00 PM EDT). Mice were housed singly for at least 2 wk prior to the experiment.

Gonadectomy and Implantation

At 60 d of age each adult mouse (n = 12 per group) was gonadectomized under xylazine (100 mg/kg)/ketamine anesthesia (10 mg/kg) injected intraperitoneally between 8:00 AM and 11:00 AM. A 250-μL blood sample was taken at this time. The plasma was frozen at –70°C. until assay. At the time of gonadectomy, animals were implanted with a Silastic capsule (1.02 mm id, 2.16 mm od) containing either no steroid (10-mm empty capsule), testosterone (5 mm of crystalline testosterone), E_2 (5 mm of 17β-estradiol diluted 1:1 with cholesterol), or DHT (10 mm of 5α-androstan-17β-ol-3-one). At the time of perfusion, each implant was located and inspected to confirm that it was still intact and in the subject. Owing to the limited volume of plasma, we were unable to measure serum steroid hormone levels in our experimental groups. However, we have measured testosterone and E_2 in other mice implanted with these same-sized capsules. Testosterone was in the high physiological range (approx 7.80 ng/mL). Estradiol was in the vicinity of 80 pg/mL, which is slightly lower than the level observed during estrus (about 150 pg/mL; Rissman, E. F., unpublished data). According to another report in mice *(24)*, a Silastic capsule containing 15 mm of DHT resulted in serum DHT levels of 3.6 ± 0.4 and 3.2 ± 0.3 ng/mL in WT and ERαKO males, respectively. Thus, our Silastic capsules likely resulted in lower levels, and all three of our steroid treatments probably resulted in serum hormone levels within the physiological range.

Tissue Collection

Ten days after surgery, between 8:00 AM and 11:00 AM, the animals were anesthetized and a 750-μL blood sample taken via cardiac puncture. While deeply anesthetized, the animals were decapitated and the brains removed. The pituitaries were removed (within 1 min of taking the blood sample), frozen, and stored at –70°C until analysis. The blood was spun and the plasma stored at –70°C until assay.

LH and FSH

Assays for LH and FSH were performed using radioimmunoassay (RIA) for LH and FSH at the University of Virginia Center for Research in Reproduction Ligand and Assay Core Lab (National Institutes of Health U54 HD-36199). Plasma LH was measured by a modified supersensitive two-site sandwich immunoassay using monoclonal antibodies MAB1 (no. 58187) against bovine LH *(46)* and TMA (no. 5303, Medix Kaunianinen, Finland) against the human LH as described previously *(47)*. The assay has a sensitivity of 7 pg/tube and the intraassay coefficient of variations (CVs) of the quality controls ranged from 3.10 to 5.47%. All samples were run in duplicate in a single assay using a rat standard (National Institutes of Health reference prep RP-3). This assay has been previously validated for mouse LH *(48)*. LH specificity was demonstrated by showing appropriate physiological responses to castration and administration of a gonadotropin-releasing hormone antagonist. Parallelism was demonstrated by comparing serial dilutions of castrate male rat serum vs castrate mouse serum. The profiles were similar in slope between the species.

Plasma FSH was determined by RIA using reagents provided by the National Hormone and Pituitary Program and procedures validated earlier *(49)*. FSH reference prep RP-3 was used for assay standards, and antirat FSH *(S–11)* diluted to a final concentration of 1:125,000 was used as primary antibody. The assay has a sensitivity of 0.3 ng/mL and <0.5% crossreactivity with other pituitary hormones. The intraassay CVs ranged from 2.47 to 8.43%. All samples were run in duplicate in a single assay.

Gonadotropin mRNA

Gonadotropin α-subunit, LHβ, FSHβ, and PRL mRNAs were measured using dot-blot analysis as previously described *(50)*. The cDNA inserts for α-subunit *(51)*, LHβ *(52)*, FSHβ *(35)*, and PRL *(53)* have been characterized previously. Briefly, two pituitaries were pooled to obtain adequate mRNA for analysis, resulting in six data points per treatment group. The pituitaries were homogenized in 10 *mM* Tris, 0.5% Nonidet P-40, 1 *mM* EDTA, placental RNase inhibitor (20 U/mL), pH 7.4, and total RNA was extracted from the cytosol using phenol/chloroform/isoamyl alcohol (100:100:1). Total RNA was measured by absorbance at 260 nm. DNA was measured in the nuclear pellet by fluorometric assay. Samples were spotted on nitrocellulose filters then fixed by heating at 80°C under reduced pressure for 90 min. Rat α-subunit, LHβ, FSHβ, and prolactin cDNA inserts were labeled with ^{32}P-CTP by random primer method (specific activity of $2–5 \times 10^8$ cpm/μg). Following hybridization, the nitrocellulose filters were exposed to film, the spots removed, and their radioactivities measured. The results are expressed as picograms (α, LHβ, FSHβ) or nanograms (PRL) cDNA bound per 100 μg of pituitary DNA. The intraassay CVs were 15.1% for α, 18.6% for LHβ, and 18.8% for FSHβ. CV was determined during the characterization of dot-blot assays for each mRNA. Four replicate dots were spotted from a rat pituitary RNA pool, and the percentage CV was calculated. The reported intraassay CV was derived from the mean percentage CV from 10 assays. To validate this protocol for mouse, serial dilutions of a mouse pituitary RNA pool were compared to a rat pool. The results showed a similar slope for both mouse and rat pituitary RNA for each gonadotropin subunit mRNA. Pituitary samples from intact and castrate mice were also tested. After castration, there was a three- to fivefold increase in gonadotropin subunit mRNA, which was similar to previous observations in the rat *(50)*.

For the data presented in this study, RNA samples were run in a single assay, with representatives of each group included on each filter (to reduce intraassay bias). Increasing amounts of "sense strand" RNA (10, 50, 500, and 1000 pg/dot), specific to the cDNA probe added, were spotted on each hybridization filter. The linear increase in counts seen in the RNA standards for each cDNA probe confirms that alterations in counts (i.e., cDNA binding) observed in experimental groups reflect similar changes in mRNA levels.

Data Analysis

A two-way analysis of variance was employed. Genotype and steroid treatment were the independent factors. Data from male and female mice were analyzed separately. Post-hoc comparisons were made using student Newman-Keuls tests.

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