0013-7227/98/$03.00/0
Endocrinology

Vol. 139, No. 10
Printed in U.S.A.

Effects of Castration and Chronic Steroid Treatments on Hypothalamic Gonadotropin-Releasing Hormone Content and Pituitary Gonadotropins in Male Wild-Type and Estrogen Receptor-α Knockout Mice

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ABSTRACT

Testicular androgens are integral components of the hormonal feedback loops that regulate circulating levels of LH and FSH. The sites of feedback include hypothalamic areas regulating GnRH neurons and pituitary gonadotropes. To better define the roles of androgen receptor (AR), estrogen receptor-α (ERα), and estrogen receptor-β (ERβ) in mediating feedback effects of sex steroids on reproductive neuroendocrine function, we have determined the effects of castration and steroid replacement therapy on hypothalamic GnRH content, pituitary LHβ and FSHβ messenger RNA (mRNA) levels, and serum gonadotropins in male wild-type (WT) and estrogen receptor-α knockout (ERKO) mice. Hypothalami from intact WT and ERKO males contained similar amounts of GnRH, whereas castration significantly reduced GnRH contents in both genotypes. Replacement therapy with estradiol (E_2), testosterone (T), or dihydrotestosterone (DHT) restored hypothalamic GnRH content in castrated (CAST) WT mice; only the androgens were effective in CAST ERKOs.

Analyses of pituitary function revealed that LHβ mRNA and serum LH levels in intact ERKOs were 2-fold higher than those in intact WT males. Castration increased levels of LHβ mRNA (1.5- to 2-fold) and serum LH (4- to 5-fold) in both genotypes. Both E_2 and T treatments significantly suppressed LHβ mRNA and serum LH levels in CAST WT males. However, E_2 was completely ineffective, and T was only partially effective in suppressing these two indexes in the CAST ERKO males. DHT treatments stimulated a 50% increase in LHβ mRNA and serum LH levels in WT males, whereas serum LH was significantly suppressed in DHT-treated ERKO males.

Although the pituitaries from intact ERKO males contained similar amounts of FSHβ mRNA, serum FSH levels were 20% higher than those in the intact WT males. Castration increased FSHβ mRNA levels only in WT males, but significantly increased serum FSH levels in both genotypes. Both E_2 and T treatments significantly suppressed serum FSH in CAST WT males, whereas only E_2 suppressed FSHβ mRNA. DHT treatments of CAST WT mice stimulated a small increase in serum FSH, but failed to alter FSHβ mRNA levels. None of the steroid treatments exerted any significant effect on FSHβ mRNA or serum FSH levels in CAST ERKOs.

These data suggest that hypothalamic GnRH contents can be maintained solely through AR signaling pathways. However, normal regulation of gonadotrope function requires aromatization of T and activation of ERα signaling pathways in the gonadotrope. In addition, serum FSH levels in male ERKOs appear to be regulated largely by nonsteroidal testicular factors such as inhibin. Finally, these data suggest that hypothalamic ERβ may not be involved in mediating the negative feedback effects of T on serum LH and FSH in male mice. (*Endocrinology* **139:** 4092–4101, 1998)

IN MALE vertebrates, the levels of circulating LH and FSH are largely determined by the stimulatory effects of GnRH and activins (1, 2) and the inhibitory effects of steroids and inhibin (1–3). The negative feedback effects of testicular androgens on serum LH and FSH were first demonstrated in studies in which castration resulted in substantial increases in circulating LH and FSH that were prevented if physiological levels of testosterone (T) were administered (4). Subsequent studies have demonstrated that the feedback effects of T are mediated at the level of the hypothalamus and the pituitary. Although there are conflicting data concerning the effects of T on GnRH synthesis and secretion, a number of studies have demonstrated that castration and steroid replacement alter levels of GnRH messenger RNA (mRNA) (5–7), processing of GnRH prohormone (8–11), hypothalamic GnRH contents (12–16), and patterns of pulsatile GnRH release (16–19).

Besides examining hypothalamic sites of action, a number of investigators have also examined feedback directly on the pituitary. T, dihydrotestosterone (DHT), or estradiol (E_2) can suppress GnRH-stimulated LH secretion from pituitary cultures (20, 21), whereas T treatments can increase basal FSH secretion and intrapituitary FSH levels (22). Furthermore, molecular analyses of the promoter regions of the gonadotropin genes have revealed that some of the feedback effects of E_2 or T may be mediated through androgen receptor (AR) or estrogen receptor (ER) interactions with response elements and other transcription factors that regulate expres-

Received March 20, 1998.
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sion of the α-gonadotropin subunit (αGSU), and the FSHβ and LHβ subunits (1, 2, 23).

Although these data support both hypothalamic and pituitary sites of steroid feedback, it is unclear whether the effects of T are primarily mediated directly through the AR or indirectly via aromatization and activation of ER. Support for AR-mediated feedback is found in studies in which treatments with DHT (a nonaromatizable androgen) effectively suppressed serum LH and steady state levels of αGSU and LHβ mRNA in rats (24, 25), androgen blockade with flutamide (an antiandrogen) resulted in elevations of serum LH (26), and AR-mediated suppression of the αGSU gene expression occurred through enhancer elements in the promoter region of that gene (23). Convincing arguments for the role of AR are also found in models of receptor-based androgen resistance, the testicular feminized mouse (Tfm) (27) and human androgen insensitivity syndromes (28), in which serum LH can be significantly elevated. By contrast, other studies have demonstrated robust suppression of serum LH and FSH, and gonadotropin mRNAs by exogenous E_2 treatment (1, 2) and elevations in serum LH after treatments with aromatase inhibitors (29). The roles of estrogens and ERα are further supported clinically by the elevated serum FSH levels in an estrogen-resistant patient (30) and in aromatase-deficient humans (31).

The question of AR- *vs.* ER-mediated negative feedback on serum gonadotropins is further complicated by recent characterizations of a second nuclear ER, termed ERβ (32, 33). Although ERβ mRNA levels are very low in adult mouse pituitaries (34), there are reports of ERβ protein and mRNA in hypothalamic nuclei of both rats and mice (34–36). Thus, androgens could regulate neuronal activity directly through AR signaling pathways or indirectly through aromatization and activation of either ERα or ERβ signaling pathways.

The recent development of an ERα knockout (ERKO) mouse line (37) provides a unique opportunity to explore the roles of AR, ERα, and ERβ in regulating hypothalamic and pituitary function. To better define the roles of these steroid receptors in mediating the negative feedback effects of T on hypothalamic GnRH, pituitary gonadotropin gene expression, and serum levels of gonadotropins, we have conducted castration and steroid replacement experiments using male wild-type (WT) and ERKO mice. The findings from our study indicate that both ERα and AR signaling pathways effectively regulate GnRH content, but that aromatization and activation of ERα enhance the negative feedback effects of T on serum gonadotropins. In addition, these findings suggest that hypothalamic ERβ does not play an obvious role in regulating GnRH content or gonadotrope physiology in adult male mice.

Materials and Methods

Animals

Adult male WT and ERKO mice (C57BL/6J strain; 10–12 weeks of age) were anesthetized and castrated according to standard surgical protocols at the NIEHS. Castrated (CAST) males were implanted (sc) 2 weeks after surgery with SILASTIC brand capsules (Dow Corning, Midland, MI) containing crystalline T (10–12 mg), DHT (10–12 mg), or E_2 (20 μg/capsule dissolved in sesame oil). Intact and CAST control males were implanted with either empty SILASTIC capsules or capsules filled with sesame oil.

Three weeks after implantation, the males were weighed and killed. Blood samples were collected, and pituitaries and hypothalami were frozen on dry ice for subsequent analysis of steady state levels of gonadotropin mRNA and hypothalamic GnRH content. The preoptic-hypothalamic regions were removed as a tissue block, as previously described (38). Seminal fluid was expressed, and the seminal vesicles were weighed to gauge the efficacy of the exogenous androgen treatments. This study was replicated three times, and the samples were assayed as described below. All studies were conducted according to the principles and procedures outlined by the NIH Guidelines for the Care of Experimental Animals and under an approved animal study protocol from the NIEHS animal care and use committee.

Preparation of SILASTIC capsules

Steroid capsules were prepared by cutting SILASTIC tubing (0.62 id × 0.125 od inches) into 15-mm lengths, sealing one end with SILASTIC adhesive, and filling the capsule with either crystalline DHT or T (Steraloids, Wilton, NH) to a length of 1 cm or with 20 μl E_2 (Sigma Chemical Co., St. Louis, MO) suspended in sesame oil (1.0 μg/μl). The tubes were then sealed with SILASTIC adhesive. Immediately before implantation, capsules were rinsed using 70% ethanol and washed with sterile saline.

Gonadotropin, GnRH, and steroid RIAs

Serum LH and FSH were quantified by RIA. The assays were performed using the following materials supplied by the NIDDK: 1) for LH, iodination preparation I-9, reference preparation RP-3, and antiserum S-11; and 2) for FSH, iodination preparation I-8, reference preparation RP-3, and antiserum S-11. These RIAs have been previously described and used in the measuring of mouse gonadotropins (39, 40). Fifty-microliter aliquots of sera were assayed in duplicate for both LH and FSH. Due to limited sample volumes, some samples were assayed for FSH as single tubes. In cases where LH and FSH levels were below the limit of detection, the minimum detectable concentration was assigned to that sample. The lower limits of detection for LH and FSH were 0.12 and 1.6 ng/ml, respectively. The intraassay variations were 5% and 9% for the LH assays and 2% and 4% for the FSH assays. Interassay variations were approximately 10% and 5% for LH and FSH assays, respectively.

In samples in which sufficient serum remained after gonadotropin assays, circulating T, DHT, and E_2 levels were determined using direct serum RIA kits (Diagnostic Systems Laboratories, Webster, TX). Limited sample volumes prevented measurement of E_2, T, and DHT in all treatment groups. Therefore, serum E_2 was measured in E_2-treated CAST males, and serum T levels were measured in both intact and T-treated CAST males. Serum DHT was measured only in the DHT-treated CAST males. Due to limited sample volumes, some of the samples were assayed as single tubes for steroid levels. The sensitivities of the T, DHT, and E_2 assays were 80, 4, and 5 pg/ml, respectively. All samples were assayed within a single assay, and the intraassay variations were approximately 8%, 4%, and 5% for the T, DHT, and E_2 assays, respectively.

Hypothalami were sonicated in 0.1 N acetic acid, boiled for 5 min, and centrifuged at 14,000 rpm in a microfuge at 4 C, and the resulting supernatant was lyophilized overnight. The crude protein pellet was saved for subsequent protein determinations and normalization of GnRH values (41). The lyophilized supernatants were resuspended in assay buffer and subjected to RIA for GnRH as described previously (42). Intra- and interassay variations were approximately 6% and 9%, respectively.

RNA isolation and Northern blot analysis

Total pituitary RNA was isolated from single pituitaries using a modification of the Trizol reagent protocol (Life Technologies, Gaithersburg, MD). Briefly, a single pituitary was homogenized in 200 μl Trizol reagent, incubated at room temperature for 5 min, and extracted with 40 μl chloroform. After thorough mixing, the tubes were centrifuged at 14,000 × *g* for 15 min at 4 C. Glycogen (10 μg/tube) was added to increase the yield of RNA. The aqueous phase was removed, and RNA was precipitated using 1 vol isopropanol for 10 min at room temperature. RNA was centrifuged at 14,000 × *g* for 15 min at 4 C, washed with

70% ethanol, and resuspended in 40 μl ribonuclease-free water, and the concentration was determined by UV spectrophotometry.

Aliquots of total RNA (1.0 μg) were suspended in 20 μl RNA loading buffer (Ambion, Austin, TX), fractionated on formaldehyde gels, and transferred overnight to Hybond nylon membranes (Amersham, Arlington Heights, IL) using 10 × SSC (1.5 M sodium chloride-0.15 M sodium citrate). RNA was cross-linked by UV exposure in a Stratalinker (Stratagene, La Jolla, CA). Blots were prehybridized for a minimum of 6 h in 12 ml Northern Max hybridization solution (Ambion) containing salmon sperm DNA. Antisense riboprobes for LHβ and PL7 were generated using a Maxiscript kit (Ambion), purified on a Nuc-Trap column (Stratagene), counted, and added to the hybridization buffer at 2×10^6 cpm/ml for both LHβ and PL7 probes. After an overnight hybridization at 65 C, blots were washed for 30 min at low stringency (2 × SSC-0.1% SDS, at room temperature), followed by a 30-min wash at high stringency (0.1% SSC-0.1% SDS, at room temperature). Blots were exposed to PhosphorImager screens, and mRNA levels were quantified using a PhosphorImager Storm 860 (Molecular Dynamics, Sunnyvale, CA). Blots were then exposed to Hyperfilm (Amersham) for autoradiography and photography.

Ribonuclease protection assays (RPA)

Antisense riboprobes for the mouse FSHβ and cyclophilin genes were generated using Ambion Maxiscript kits, and RPAs were conducted using a Hybspeed RPA kit (Ambion). Briefly, total RNA (0.5 μg) was ethanol precipitated with 50,000 cpm each of the FSHβ and cyclophilin riboprobes, resuspended in 10 μl Hybspeed hybridization buffer (95 C), and incubated at 65 C for 30 min. After hybridization, samples were treated with a 1:250 dilution of ribonuclease A/T1 mix (Ambion) for 30 min at 37 C. At the end of this period, stop solution was added, and the RNA was precipitated for a minimum of 1 h at −80 C. Precipitates were centrifuged at 14,000 rpm for 15 min at 4 C, the supernatant was carefully removed, and the pellets were resuspended in 8 μl RNA loading buffer (Ambion). The samples were heated at 95–100 C for 3 min, placed on wet ice, and electrophoresed on a denaturing, 6% bis-acrylamide gel (8.3 M urea) at 275 V. Gels were fixed twice for 15 min each time in 10% glacial acetic acid-0.5% glycerol and dried overnight before exposure to PhosphorImager screens and Hyperfilm (Amersham).

Statistical analyses

Data for GnRH content, serum LH and FSH, LHβ and FSHβ mRNA, and steroid levels were analyzed using Levene's test for homogeneity of variance. All of the variables, except the serum DHT levels, exhibited statistically significant heteroscedascticity. Therefore, although raw data are graphically presented in the figures, all data (except for serum DHT) were log transformed for the statistical analyses. Data were analyzed using two-way ANOVA, and in most cases, significant interactions between genotype and treatment were noted (see figure legends). *Post-hoc* analyses were conducted using a Student Newman-Keuls test. In all cases, statistical significance was accepted at $P < 0.05$.

Results

Seminal vesicle weights (SVWs) and circulating levels of steroids

To determine whether physiologically relevant levels of steroids were released from the steroid implants, fluid was expressed from seminal vesicles, and wet weights were determined; serum concentrations of steroids were measured by RIA. Castration significantly reduced SVWs and both T and DHT treatments restored SVW to approximately 80% of the values for intact WT and ERKO mice (Fig. 1). No effect was observed with E_2 treatment. Based on SVWs, there were no obvious genotypic differences in peripheral responses to the androgens.

Serum E_2 levels in E_2-treated CAST WT and ERKO males were 12.7 ± 2.3 pg/ml (n = 7) and 13.0 ± 2.3 pg/ml (n = 11), respectively. These values are comparable to levels previously reported for intact WT and ERKO males (43). Serum T levels in this study were approximately 2-fold higher in intact ERKO males (8.5 ± 2.2 ng/ml; n = 12; $P < 0.05$) than in intact WT males (3.9 ± 2.4 ng/ml; n = 12). This 2-fold elevation is consistent with a previous report of elevated serum T in ERKO males (44). Although T-treated CAST ERKO males had 2-fold lower serum T levels (3.4 ± 0.5 ng/ml; n = 12; $P > 0.05$) than T-treated CAST WT males (7.1 ± 2.1 ng/ml; n = 12) or intact ERKO males (see above), these levels were similar to those of intact WT males (see above). Serum levels of DHT were 3.6 ± 0.4 (n = 14) and 3.2 ± 0.3 ng/ml (n = 14) in DHT-treated WT and ERKO males.

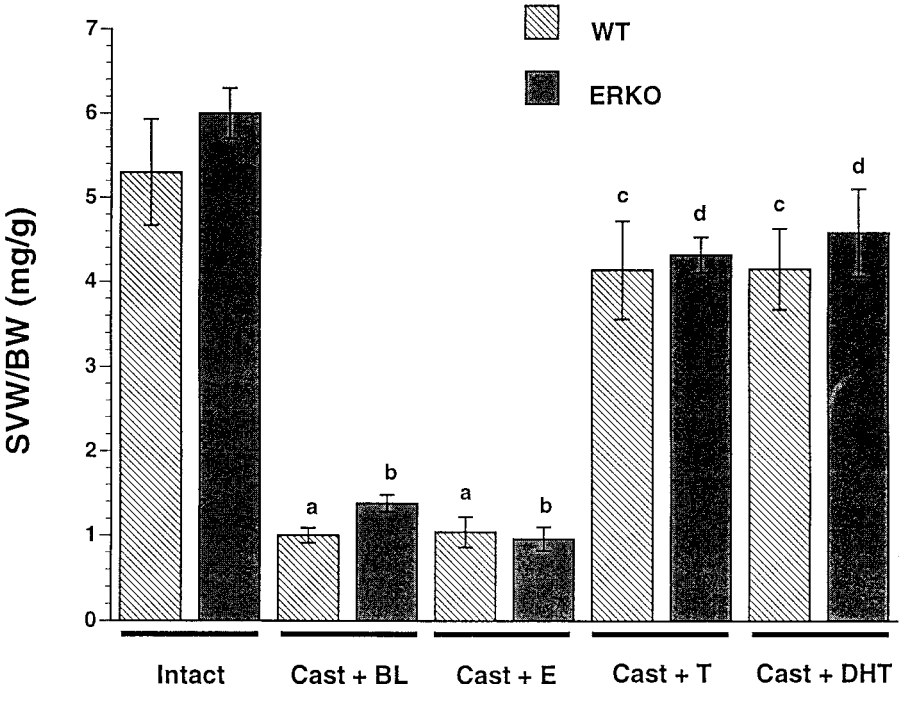

FIG. 1. SVWs in intact, CAST, and steroid-treated WT and ERKO males. SVWs were normalized to body weights (BW). The data are from one of the replicate experiments and are depicted as the mean and SEs for each group. The CAST groups consisted of mice given a blank SILASTIC capsule (BL) or a capsule containing 17β-estradiol (E), T, or DHT. Sample size ranged from five to seven mice per group. Two-way ANOVA demonstrated significant treatment effects. By Student-Newman-Keuls test: a, $P < 0.05$ *vs.* intact WT; b, $P < 0.05$ *vs.* intact ERKO; c, $P < 0.05$ *vs.* CAST WT; d, $P < 0.05$ *vs.* CAST ERKO.

Coupled with the data on SVW, these data suggest that the levels of E_2 and T produced by the SILASTIC capsules were in physiologically relevant ranges.

Hypothalamic GnRH content

As sex steroids are known to exert feedback effects on LHRH neurons (5, 7, 9, 11, 45–47), hypothalamic GnRH contents were examined. There were no significant differences between mean GnRH contents of intact WT and ERKO males, and castration significantly reduced these concentrations by 42% and 33%, respectively (Fig. 2). Treatment with E_2 restored GnRH contents in CAST WT males to levels comparable to those found in intact WT males, but it failed to have any effect in CAST ERKO males. However, T treatments restored GnRH content to precastration levels in both WT and ERKO castrates. Interestingly, DHT treatment completely reversed the castration effect on GnRH content in ERKO males, but it only produced a partial reversal (51%) of the castration effect in WT males.

LHβ mRNA expression and serum LH values

Due to evidence that sex steroids exert strong feedback effects on LHβ gene expression and serum levels of LH (1, 2), we measured steady state levels of LHβ mRNA and circulating levels of LH. Northern blot analyses revealed that pituitaries from intact, adult male ERKOs contain approximately 2-fold higher levels of LHβ mRNA ($P > 0.05$) compared with those from intact WT males (Fig. 3, A and B). Long term castration caused 2.0-fold ($P > 0.05$) and 1.4-fold ($P > 0.05$) increases in steady state levels of LHβ mRNA in WT and ERKO males, respectively. Treatments of CAST WT males with E_2 and T suppressed levels of LHβ mRNA to 29% ($P < 0.05$) and 23% ($P < 0.05$), respectively, of the levels found in CAST WT controls (Fig. 3, A and B), whereas DHT treatments resulted in a 47% increase in LHβ mRNA ($P > 0.05$). Unlike steroid treatments of CAST WT males, neither E_2, T, nor DHT treatment of CAST ERKOs resulted in significant changes in steady state levels of LHβ mRNA (Fig. 3, A and B).

Although an earlier study did not find significant elevations of serum LH levels in intact ERKO males (44), we sampled a larger number of males and found that serum LH values were 2-fold higher ($P < 0.05$) in intact ERKO males (Fig. 4). This increased level of serum LH agrees roughly with the 2-fold higher levels of LHβ mRNA found in intact ERKO pituitaries. Although castration did not significantly alter LHβ mRNA levels in either WT or ERKO males, castration resulted in 5-fold ($P < 0.05$) and 4-fold ($P < 0.05$) elevations in serum LH in WT and ERKO males, respectively. Treatments of WT castrates with E_2 and T suppressed serum LH to 14% and 6% ($P < 0.05$), respectively, of the values in CAST WT males (Fig. 4). However, E_2 treatments of CAST ERKO males failed to suppress serum LH. On the other hand, T treatments of CAST ERKO animals suppressed serum LH by 32% ($P > 0.05$) relative to levels in CAST ERKO controls. Interestingly, DHT treatment resulted in a 52% increase ($P > 0.05$) in serum LH values in CAST WT while producing a 58% decrease ($P < 0.05$) in ERKO males.

Expression of FSHβ mRNA and serum FSH

Steroidal and nonsteroidal testicular factors have been demonstrated to exert feedback effects on FSH synthesis and secretion (1, 2). For this reason, we determined the effects of castration and steroid replacement on steady state levels of FSHβ mRNA and serum FSH levels in WT and ERKO males. RPAs revealed no significant genotypic differences in steady state levels of FSHβ mRNA in pituitaries from intact WT and ERKO male mice (Fig. 5). Castration resulted in a 1.7-fold increase ($P < 0.05$) in FSHβ mRNA levels in WT males (Fig. 5), but it failed to have any appreciable effect in ERKO males

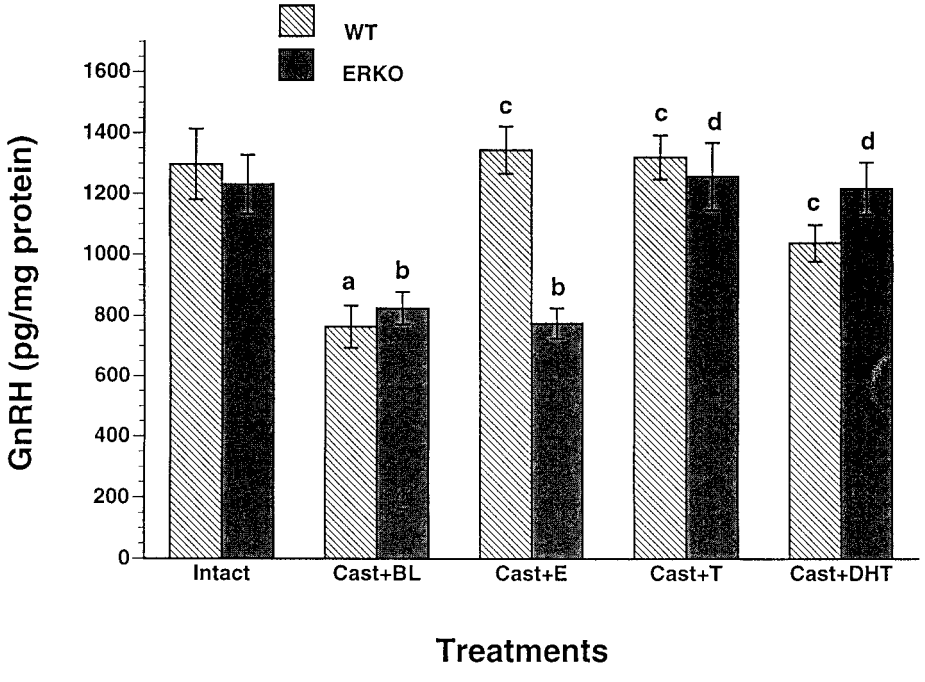

FIG. 2. Hypothalamic GnRH contents in intact, CAST, and steroid-treated WT and ERKO males. See Fig. 1 for group details. Depicted are the mean and SEs for each group. Sample size ranged from 12–20 mice/group. Two-way ANOVA demonstrated no significant genotype effect, but there were significant treatment effects and interactive effects. By Student-Newman-Keuls test: a, $P < 0.05$ *vs.* intact WT; b, $P < 0.05$ *vs.* intact ERKO; c, $P < 0.05$ *vs.* CAST WT; d, $P < 0.05$ *vs.* CAST ERKO.

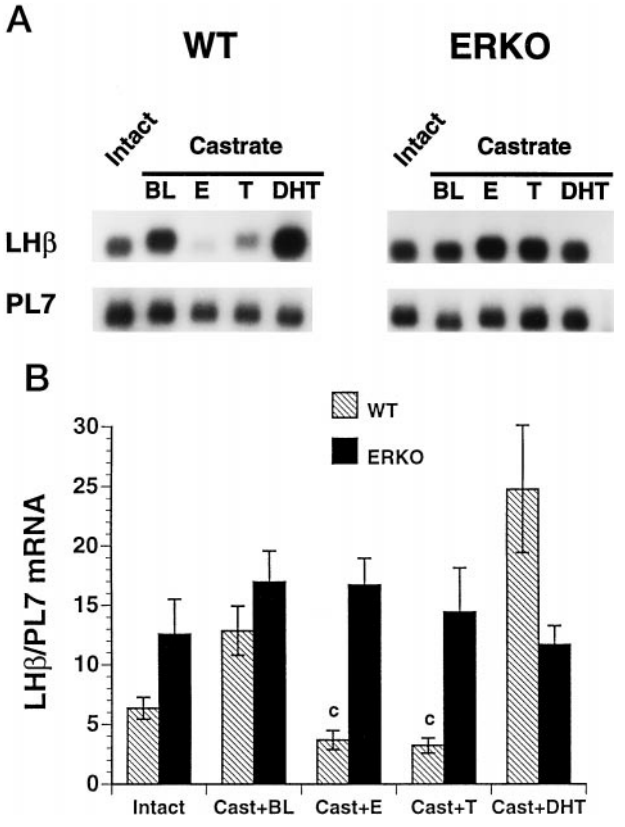

FIG. 3. Northern blot analysis of LHβ and PL7 mRNA levels in the pituitaries of intact, CAST, and steroid-treated WT and ERKO males. See Fig. 1 for group details. A, Representative blots of LHβ and PL7 are displayed. B, Blots were scanned with a PhosphorImager, and levels of LHβ mRNA were normalized to PL7 expression. The sample size was seven mice per group. Two-way ANOVA demonstrated significant genotype, treatment, and interactive effects ($P < 0.05$). By Student-Newman-Keuls test: c, $P < 0.05$ *vs.* CAST WT.

(Fig. 5). Compared with CAST WT controls, E_2 treatments of CAST WT males decreased steady state levels of FSHβ mRNA by approximately 50% ($P < 0.05$), whereas T and DHT treatments had no effect. In contrast to WT males, neither castration nor steroid replacement therapy significantly altered FSHβ mRNA levels in ERKO males.

Mean serum FSH values were 20% higher ($P > 0.05$) in intact ERKO males than in intact WT males (Fig. 6). Whereas castration elevated FSHβ mRNA only in WT males, it resulted in significant increases (~68%) in serum FSH in both WT and ERKO males. E_2 and T treatments of CAST WT males suppressed serum FSH by 36% ($P < 0.05$) and 47% ($P < 0.05$), respectively, whereas DHT resulted in a small increase in serum FSH ($P > 0.05$). Despite the fact that castration significantly increased serum FSH in ERKO males, none of the steroid treatments significantly altered serum FSH in ERKO males (Fig. 6). Thus, in WT males, the E_2-induced suppression of serum FSH is paralleled by a suppression of FSHβ mRNA levels, whereas the T-induced suppression of serum FSH is not accompanied by a similar suppression of FSHβ mRNA levels. Furthermore, in ERKO males, the postcastration rise in serum FSH levels without accompanying FSHβ mRNA increases is probably due to the removal of testicular inhibins.

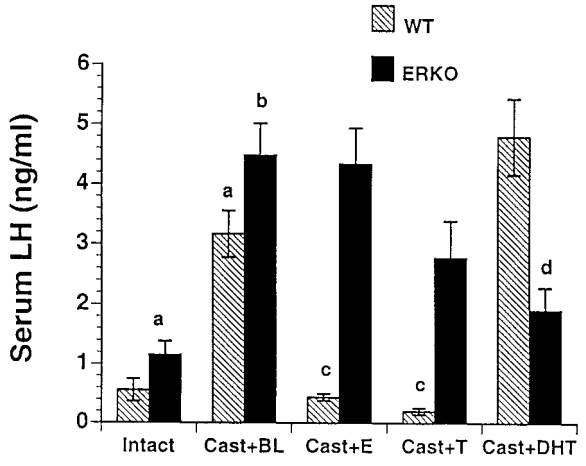

FIG. 4. Serum LH concentrations in intact, CAST, and steroid-treated WT and ERKO males. See Fig. 1 for group details. Sample size ranged from 12–22 mice/group. Two-way ANOVA demonstrated significant genotype, treatment, and interactive effects ($P < 0.05$). By Student-Newman-Keuls test: a, $P < 0.05$ *vs.* intact WT; b, $P < 0.05$ *vs.* intact ERKO; c, $P < 0.05$ *vs.* CAST WT; d, $P < 0.05$ *vs.* CAST ERKO.

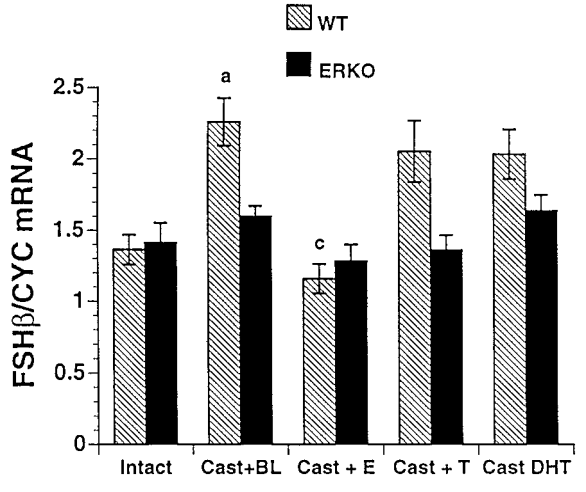

FIG. 5. Steady state levels of expression of FSHβ mRNA in pituitaries from intact, CAST, and steroid-treated WT and ERKO males. See Fig. 1 for group details. Gels from RPAs were dried and quantified using a PhosphorImager. Levels of FSHβ mRNA were normalized to cyclophilin expression. Sample size ranged from 6–19 mice/group. Two-way ANOVA demonstrated significant genotype, treatment, and interactive effects ($P < 0.05$). By Student-Newman-Keuls test: a, $P < 0.05$ *vs.* intact WT; c, $P < 0.05$ *vs.* CAST WT.

Discussion

Sex steroids are critical components of the hormonal feedback loops that regulate serum LH and FSH. The sites for these feedback effects include neurons and cells in the hypothalamus that are in close proximity to the GnRH neurons and the gonadotropes in the pituitary. The ability of these target cells to respond directly to androgens is due to the expression of nuclear AR, whereas the presence of aromatase and nuclear ER allows for the conversion of androgens into estrogens and subsequent activation of ER signaling pathways. Indeed, AR, aromatase and ERα are coexpressed in many hypothalamic nuclei and in gonadotropes (48, 49).

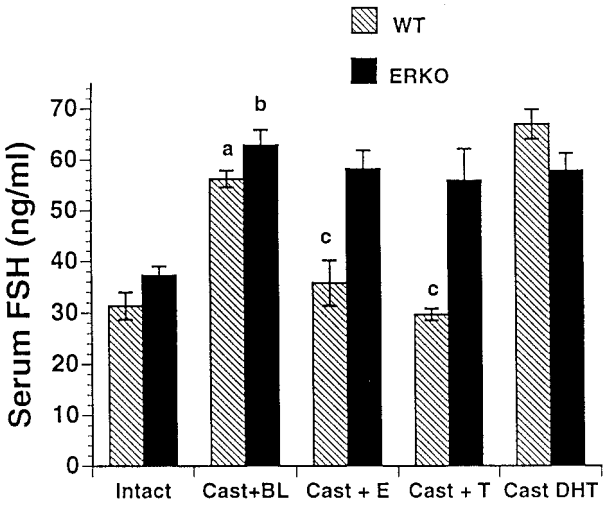

FIG. 6. Serum FSH concentrations in intact, CAST, and steroid-treated WT and ERKO males. See Fig. 1 for group details. Sample size ranged from 6–19 mice/group. Two-way ANOVA demonstrated significant genotype, treatment, and interactive effects ($P < 0.05$). By Student-Newman-Keuls test: a, $P < 0.05$ *vs.* intact WT; b, $P < 0.05$ *vs.* intact ERKO; c, $P < 0.05$ *vs.* CAST WT.

Recent data also indicate that the hypothalamus in rats and mice contains both ERβ mRNA and protein (34–36). To explore the roles of AR, ERα, and ERβ in mediating the negative feedback effects of sex steroids on the hypothalamus and gonadotropes, we examined the effects of castration and steroid replacement on GnRH content, gonadotropin gene expression, and serum gonadotropins in WT and ERKO mice.

Hypothalamic GnRH content

Many studies have documented that GnRH plays a major role in determining both the synthesis and release of LH from gonadotropes. However, the exact roles of T and E_2 in regulating GnRH synthesis and secretion remain somewhat controversial. Indeed, studies have demonstrated that castration can increase (5–7), decrease (50), or have no effect (51) on GnRH mRNA levels, whereas exogenous androgens can either decrease (5, 7) or increase (50) levels of GnRH mRNA. Some of these differences may be explained by differences in experimental designs. In several species, castration leads to a rapid increase in the frequency of GnRH pulses (16–19), which can be suppressed by T treatment (17, 19). Although GnRH pulse frequencies increase rapidly and persist for weeks after castration, a number of studies have also demonstrated that long term castration results in diminished GnRH content and that either E_2 or androgen replacement can restore GnRH contents to intact levels (11–13). Indeed, one study has demonstrated that in long term castrate guinea pigs, hypothalamic GnRH contents are diminished, whereas GnRH pulse frequencies are increased (16). As GnRH content reflects the net effects of changes in transcription, translation, pro-GnRH processing, GnRH secretion, and degradation, we measured GnRH content as an initial step in addressing the roles of AR, ERα, and ERβ in regulating GnRH neuronal function.

In these studies, we found that GnRH contents were similar in intact WT and ERKO males despite the fact that castrated ERKO males were completely insensitive to exogenous E_2. As ERβ mRNA levels appear unaltered in ERKO mice (34–36), this suggests that hypothalamic ERβ may not play an obvious role in mediating T or E_2 regulation of GnRH content in male mice, and that in the absence of functional ERα, physiological levels of T can effectively regulate GnRH content directly through AR signaling pathways. However, given the *in vitro* evidence that ERβ and ERα form heterodimers (52), it is possible that disruption of ERα has ablated any role of ERβ in regulating GnRH content. This possibility will need to be addressed by determining the extent to which ERα and ERβ are colocalized in neurons. Future development of ERβ-specific agonists and antagonists and ERβ knockout models will also allow further delineation of any roles that ERβ may play in regulating the GnRH content in male mice.

The fact that both T and DHT treatments of castrated ERKO mice restored the hypothalamic GnRH content to levels observed in intact ERKO animals confirms that AR signaling pathways can effectively regulate the neuronal GnRH content. Although DHT treatments of WT males were less effective than that of CAST ERKO males, both DHT and T treatments of CAST WT males also resulted in significant elevations of GnRH content. The ability of both E_2 and DHT to regulate GnRH content in WT males suggests that both AR and ERα signaling pathways can regulate hypothalamic GnRH contents in male WT mice. However, the increased effectiveness of DHT in ERKO male mice suggests that ERα disruption has increased the sensitivities of hypothalamic target cells to androgens. This raises the possibility that the ERα disruption may have resulted in developmental perturbations that lead to increased sensitivity to DHT.

These experiments measured only GnRH content, which is a reflection of the net rates of pro-GnRH synthesis, processing, and secretion. Thus, it is possible that AR and ERα receptor systems may regulate different steps in the synthesis and secretion of GnRH that yield similar results in terms of GnRH contents. Indeed, it will be interesting to determine the effects of these treatments on GnRH mRNA levels and GnRH secretion patterns in both WT and ERKO males. Nonetheless, these data are consistent with studies suggesting that both AR and ERα signaling pathways can regulate hypothalamic GnRH content (5, 11–13).

LHβ mRNA expression and serum LH

In addition to regulating GnRH synthesis and secretion, various investigators have shown that E_2 and androgens can suppress LHβ mRNA and serum LH levels (1, 2, 20, 21, 23). Despite complete E_2 insensitivity, we found that both LHβ mRNA and serum LH levels in intact male ERKOs were only 2-fold higher than those in intact WT males. As GnRH contents were comparable, these data suggest that the slightly higher levels of LHβ mRNA and serum LH in intact ERKO males stem from estrogen insensitivity at the level of the gonadotrope. However, this hypothesis must be examined by testing for genotypic differences in GnRH release patterns and *in vitro* challenges of primary pituitary cell cultures with

steroids and GnRH. Nonetheless, it is apparent that ERα disruption results in relatively small elevations in LHβ mRNA and serum LH levels in male ERKO mice compared with the 8- to 10-fold increase in LHβ mRNA and serum LH levels in ERKO females (53, 54). This suggests that ERα plays a more dominant role in regulating LHβ mRNA and serum LH levels in females, whereas AR signaling pathways are relatively effective in regulating LH synthesis and secretion in intact male mice. This hypothesis is supported by the fact that intact Tfm mice have significantly elevated serum LH values that are not further elevated by castration (27).

Long-term castration of both WT and ERKO males induced small (1.5- to 2-fold) elevations in LHβ mRNA levels compared with the effects of castration in male rats (1, 2). The discrepancy between the data from this study and those from rat studies may reflect species differences, as an earlier mouse study reported similar elevations of LHβ mRNA after castration (27). Alternatively, as reported for male rats, it is possible that LHβ mRNA is rapidly induced after castration and subsequently decreases with increasing length of castration. Interestingly, the large increases in serum LH levels (4- to 5-fold) in castrated male WT and ERKO mice were of much greater magnitude than the increases in LHβ mRNA levels. This suggests that alterations in pulsatile GnRH secretion and/or gonadotrope sensitivity to GnRH may be primarily responsible for the elevated serum LH observed in long term castrated WT and ERKO males. This interpretation is consistent with experiments demonstrating that castration leads to increased GnRH pulse frequency and elevated serum LH in several species (16–19).

Both E_2 and T significantly suppressed LHβ mRNA and serum LH levels in WT castrates while exerting no significant effects in castrated ERKOs. Thus, it appears that aromatization and activation of ERα enhance the feedback effects of T on LH synthesis and secretion in male mice. It is possible that the lower serum T levels found in T-treated ERKO mice may account for this genotypic difference in the effects of T. However, the range of serum T levels found in T-treated CAST ERKOs overlapped the range of serum T values in intact ERKOs. Furthermore, despite the fact that intact ERKOs have 2-fold higher serum T levels than intact WT males, serum LH values remain 2-fold higher in intact ERKO males. Thus, the genotypic difference in the ability of exogenous T to suppress serum LH may be primarily due to the ERα disruption. In theory, ERα disruption could compromise T-induced suppression of GnRH pulse frequency or alter gonadotrope sensitivity to GnRH and rates of transcription of the gonadotropin subunits. We are presently addressing these possibilities with experiments that examine GnRH pulse frequencies and responses of pituitary cell cultures to steroid and GnRH challenges. Studies have suggested that direct effects of E_2 and T on gonadotropes may include the regulation of GnRH receptor numbers (55–57), intracellular responses to GnRH (56, 57), or transcriptional activity of gonadotropin subunit genes (23, 58). It will be interesting to determine the extent to which ERα *vs.* AR signaling pathways regulate GnRH receptor levels, basal levels of transcription of the αGSU or LHβ genes, and gonadotrope responsivity to GnRH.

Despite data indicating that aromatization and activation of ERα greatly enhance the feedback effects of T on LHβ mRNA and serum LH levels, it is unclear why T treatments of CAST ERKO males did not suppress serum LH closer to the levels found in intact ERKO males. Some studies have demonstrated that the duration of castration and steroid therapy or steroid dosages may influence the magnitudes of change in LHβ mRNA and serum LH responses (1). Thus, it is possible that different lengths of castration and T treatments or different dosages of T might result in a more robust suppression of serum LH in ERKO mice. An intriguing possibility is that AR signaling pathways may suffice to maintain negative feedback control over serum LH levels, but that ERα signaling pathways are more potent and, therefore, are better able to reinstate negative feedback control over highly elevated serum LH levels found in CAST males. Finally, it is also possible that T was not as effective in male ERKOs because, in the absence of functional ERα, nonsteroidal testicular factors may play an enhanced role in mediating LH synthesis and secretion.

Based on experiments demonstrating that exogenous DHT suppressed GnRH mRNA levels (7) and serum LH in rats (24), and that serum LH is elevated in Tfm mice (27), we had anticipated that DHT would effectively suppress serum LH in both WT and ERKO males. Surprisingly, we found that DHT treatments consistently resulted in small increases in serum LH and LHβ mRNA levels in CAST WT males while significantly suppressing serum LH in CAST ERKO males. Furthermore, DHT was also less effective in raising GnRH content in CAST WT males. It is possible that DHT failed to suppress serum LH levels in CAST WT because of differences in experimental design (chronic castration and DHT treatments) or because of a pharmacological DHT effect. Nonetheless, these possibilities do not address the observation that identical DHT treatments exerted opposite effects on serum LH levels in male WT and ERKO mice. Perhaps the most parsimonious explanation of this phenomenon would be genotypic differences in metabolism and clearance that resulted in different levels of circulating DHT. However, RIA confirmed that serum DHT levels were similar in DHT-treated WT and ERKO males. Thus, it appears that the absence of functional ERα has altered hypothalamic and/or pituitary sensitivities to DHT. Such altered sensitivities may be due to developmental changes in patterns of AR expression, expression of steroid-metabolizing enzymes, or developmental selection for cells that are more sensitive to androgens. It is also possible that ERα disruption has simply increased the availability of transcription factors common to both ERα and AR signaling pathways. Recent studies indicate no obvious genotypic difference in the levels of hypothalamic AR found by immunohistochemistry (59) or in the levels of AR mRNA in the pituitaries of WT and ERKO mice (our unpublished data). We are presently determining whether ERα disruption has altered patterns of expression of steroid-metabolizing enzymes in androgen target cells or altered availability of transcription cofactors common to both ERα and AR signaling pathways.

Although the ERKO mouse has provided some interesting insights into the nuclear receptor systems mediating the feedback effects of T on serum LH, it is important to recog-

nize the possibility that ablation of ERα signaling pathways has led to the development or enhancement of alternative feedback mechanisms. Indeed, the genotypic differences in the effects of DHT suggest that this may have occurred and that, therefore, ERα signaling pathways may play a more important role in WT mice. However, studies of Tfm mice indicate that serum LH levels are much higher than those in ERKO males and that, unlike ERKO males, castration of Tfm mice does not lead to a further increase in serum LH levels (27). Thus, comparisons of these two models of steroid insensitivity support the general conclusion that AR signaling pathways are more dominant than ERα signaling pathways in regulating serum LH levels in male mice.

FSHβ mRNA expression and serum FSH

The synthesis and secretion of FSH are stimulated by GnRH and activins and suppressed by inhibins and estrogens. However, the feedback effects of T are more complex. For instance, some studies demonstrate that *in vivo* T treatments can suppress serum FSH (1), whereas other studies illustrate that T can stabilize or stimulate levels of FSHβ mRNA, FSH secretion, and serum FSH when endogenous GnRH stimulation is eliminated through the use of GnRH antagonists *in vivo* (60) or primary pituitary cultures (1). Given the absence of functional ERα and elevated serum T levels in ERKO males, it was anticipated that FSHβ mRNA levels might be elevated in ERKO males. However, FSHβ mRNA levels were comparable in intact WT and ERKO mice. This suggests that estrogen insensitivity at the level of the pituitary does not have a significant impact on basal levels of FSHβ mRNA in pituitaries of intact ERKO males. In contrast, ERα disruption in female mice leads to a 7- to 8-fold increase in FSHβ mRNA levels (53). This suggests that a sex difference exists in the importance of ERα in regulating FSHβ mRNA synthesis.

Consistent with earlier rodent studies (1, 2), we found that castration increased FSHβ mRNA levels in WT males and that E_2 treatments suppressed this increase in WT males. The absence of an androgen effect on FSHβ mRNA levels agrees with several studies suggesting that androgens may stabilize FSHβ mRNA levels (1, 2). In contrast to WT males, FSHβ mRNA levels in ERKO males were not significantly altered by any of the treatments, and therefore, it appears that FSHβ mRNA levels are relatively static in long term castrated and steroid-replaced ERKO males. However, it is unclear why the large androgen-induced changes in GnRH content did not induce changes in steady state levels of FSHβ mRNA. These data suggest that either the changes in hypothalamic GnRH content in ERKO mice are not accompanied by changes in GnRH secretion patterns or, more likely, that ERα-mediated effects on the gonadotrope are required for GnRH regulation of FSHβ mRNA levels.

In agreement with the normal levels of FSHβ mRNA in intact ERKO pituitaries, we found that serum FSH levels were only slightly elevated in ERKO males. In addition, castration significantly increased serum FSH levels in both WT and ERKO males, whereas FSHβ mRNA levels were increased only in WT males. This indicates that the postcastration rise in serum FSH in ERKO males is independent of changes in steady state levels of FSHβ mRNA. Furthermore, as none of the steroid treatments exerted any significant effect on serum FSH levels in ERKO males, we believe that the postcastration rise in serum FSH levels in ERKO males is due to removal of nonsteroidal testicular factors such as inhibin.

Whereas inhibins may play a dominant role in regulating serum FSH levels in ERKO males, it is clear that both E_2 and T can effectively suppress serum FSH in WT males. Interestingly, only the E_2 treatments of CAST WT males were effective at suppressing postcastration increases in FSHβ mRNA, whereas both E_2 and T suppressed the postcastration rise in serum FSH. This indicates that mechanisms underlying T suppression of serum FSH in WT males are separate and distinct from the effects of T on steady state levels of FSHβ mRNA. It is possible that T treatments stabilized or increased FSHβ mRNA levels as reported in rats (1, 60), but suppressed serum FSH in WT males through a reduction in GnRH pulse frequency. However, T treatments elevated GnRH content but failed to suppress serum FSH in male ERKOs. Thus, in WT mice, it is likely that T is aromatized and acts through gonadotrope ERα to modulate the translation and/or secretion of FSH independently of changes in FSHβ mRNA levels. The fact that T treatments failed to suppress serum FSH in ERKO males lends further support to this hypothesis.

In summary, we have employed the ERKO mouse, a genetic model of estrogen insensitivity, to explore the roles of AR, ERα, and ERβ in regulating gonadotropins. The data presented here indicate that ERα is the predominant signaling pathway involved in mediating E_2 suppression of GnRH content, serum gonadotropins, and gonadotropin subunit mRNA expression in male mice. Thus, it does not appear that hypothalamic ERβ plays an obvious role in mediating negative feedback regulation of gonadotropins in male mice. Comparisons of serum LH data in this paper with serum LH values in Tfm mice also indicate that AR signaling pathways are the dominant signaling pathways by which T suppresses serum LH in male mice. Indeed, in the absence of functional ERα, androgens can effectively act through AR signaling pathways to regulate GnRH content, and this may be the predominant pathway by which androgens regulate serum LH in ERKO males. Nonetheless, it does appear that aromatization and activation of gonadotrope ERα are required to achieve the levels of LHβ mRNA and serum LH found in WT males. Interestingly, the ability of both E and T to suppress serum FSH in WT mice is clearly dependent on the presence of ERα. It also appears that T suppresses serum FSH levels through ERα-mediated posttranscriptional effects that are independent of steady state levels of FSHβ mRNA. Finally, in the absence of functional ERα, nonsteroidal testicular factors (*i.e.* inhibins) may effectively regulate serum FSH levels in male mice.

Acknowledgments

We thank Marianna Molina, Todd Washburn, James Clark, Page Myers, and other members of the NIEHS Comparative Medicine Branch staff for their surgical expertise and animal care. Constructs for generating the LHβ and FSHβ riboprobes were generously provided by Drs. John Nilsson and Malcolm Low, respectively. We also thank Drs. W.

Bocchinfuso, E. M. Eddy, and J. Webster for their careful reading and critiques of this manuscript. In addition, we thank Drs. Beth Gladen and Joseph Haseman for statistical advice.

References

1. **Gharib S, Wierman M, Shupnik M, Chin W** 1990 Molecular biology of the pituitary gonadotropins. Endocr Rev 11:177–199
2. **Haisenleder DJ, Dalkin AC, Marshall JC** 1994 Regulation of gonadotropin gene expression. In: Knobil E, Neill JD (eds) The Physiology of Reproduction. Raven Press, New York, vol 1:1793–1814
3. **Vale W, Bilezikjian LM, Rivier C** 1994 Reproductive and other roles of inhibins and activins. In: Knobil, Neill (eds) The Physiology of Reproduction. Raven Press, New York, vol 1:1861–1878
4. **Ramirez V, McCann S** 1965 Inhibitory effect of testosterone on luteinizing hormone secretion in immature and adult rats. Endocrinology 76:412
5. **Toranzo D, Dupont E, Simard J, Labrie C, Couet J, Labrie F, Pelletier G** 1989 Regulation of pro-gonadotropin-releasing hormone gene expression by sex steroids in the brain of male and female rats. Mol Endocrinol 3:1748–1756
6. **Selmanoff M, Shu C, Petersen S, Barraclough C, Zoeller R** 1991 Single cell levels of hypothalamic messenger ribonucleic acid encoding luteinizing hormone-releasing hormone in intact, castrated, and hyperprolactinemic male rats. Endocrinology 128:459–466
7. **Li S, Pelletier G** 1992 Role of dopamine in the regulation of gonadotropin-releasing hormone in the male rat brain as studied by *in situ* hybridization. Endocrinology 131:395–399
8. **Culler M, Valenca M, Merchenthaler I, Flerko B, Negro-Vilar A** 1988 Orchidectomy induces temporal and regional changes in the processing of the luteinizing hormone-releasing hormone prohormone in the rat brain. Endocrinology 122:1968–1976
9. **Wetsel W, Negro-Vilar A** 1989 Combined antibody-high performance liquid chromatography approach to assess prohormone processing. Methods Enzymol 168:517–545
10. **Wetsel W, Negor-Vilar A** 1989 Testosterone selectively influences protein kinase C-coupled secretion of proLHRH-derived peptides. Endocrinology 125:538–547
11. **Roselli C, Kelly M, Ronnekleiv O** 1990 Testosterone regulates progonadotropin-releasing hormone levels in the preoptic area and basal hypothalamus of the male rat. Endocrinology 126:1080–1086
12. **Gross D** 1980 Effects of castration and steroid replacement on immunoreactive gonadotropin-releasing hormone in the hypothalamus and preoptic area. Endocrinology 106:1442–1450
13. **Kalra P, Kalra S** 1980 Modulation of hypothalamic luteinizing hormone-releasing hormone levels by intracranial and subcutaneous implants of gonadal steroids in castrated rats: effects of androgen and estrogen antagonists. Endocrinology 106:390–397
14. **Shivers B, Harlan R, Morrell J, Pfaff D** 1983 Immunocytochemical localization of luteinizing hormone-releasing hormone in male and female rat brains. Neuroendocrinology 36:1–12
15. **Zanisi M, Celotti F, Motta M** 1986 Testosterone metabolites do not participate in the control of hypothalamic LH-releasing hormone. J Endocrinol 109:291–296
16. **Giri M, Kaufman J** 1994 Effects of long term orchidectomy on *in vitro* pulsatile gonadotropin-releasing hormone release from the medial basal hypothalamus of the adult guinea pig. Endocrinology 134:1621–1626
17. **Steiner R, Bremner W, Clifton D** 1982 Regulation of luteinizing hormone pulse frequency and amplitude by testosterone in the adult male rat. Endocrinology 111:2055–2061
18. **Levine J, Duffy M** 1988 Simultaneous measurement of luteinizing hormone (LH)-releasing hormone, LH and follicle-stimulating hormone release in intact and short-term castrate rats. Endocrinology 122:2211–2221
19. **Jackson G, Kuehl D, Rhim T** 1991 Testosterone inhibits gonadotropin-releasing hormone pulse frequency in the male sheep. Biol Reprod 45:188–194
20. **Drouin J, Labrie F** 1976 Selective effects of androgens on LH and FSH release in anterior pituitary cells in culture. Endocrinology 98:1528–1534
21. **Frawley S, Neill J** 1984 Biphasic effects of estrogen on gonadotropin-releasing hormone-induced luteinizing hormone release in monolayer cultures of rat and monkey pituitary cells. Endocrinology 114:659–663
22. **Kennedy J, Chappel S** 1985 Direct pituitary effects of testosterone and luteinizing hormone-releasing hormone upon follicle-stimulating hormone: analysis by radioimmuno- and radioreceptor assay. Endocrinology 116:741–748
23. **Heckert L, Wilson E, Nilson J** 1997 Transcriptional repression of the α-subunit gene by androgen receptor occurs independently of DNA binding but requires the DNA-binding and ligand-binding domains of the receptor. Mol Endocrinol 11:1497–1506
24. **Gill M, Karanth S, Dutt A, Juneja H** 1985 Effect of castration and steroid treatment on the release of gonadotropins by the rat pituitary-hypothalamus complex *in vitro*. Horm Metab Res 17:141–146
25. **Wierman M, Gharib S, LaRovere J, Badger T, Chin W** 1988 Selective failure of androgens to regulate follicle stimulating hormone β messenger ribonucleic acid levels in the male rat. Mol Endocrinol 2:492–498
26. **Urban R, Davis M, Rogol A, Johnson M, Veldhuis J** 1988 Acute androgen receptor blockade increases luteinizing hormone secretory activity in men. J Clin Endocrinol Metab 67:1149–1155
27. **Scott IS, Bennett MK, Porter-Goff AE, Harrison CJ, Cox BS, Grocock CA, O'Shaughnessy PJ, Clayton RN, Craven R, Furr BJA, Charlton HM** 1992 Effects of the gonadotrophin-releasing hormone agonist "Zoladex" upon pituitary and gonadal function in hypogonadal (hpg) male mice: a comparison with normal male and testicular feminized (tfm) mice. J Mol Endocrinol 8:249–258
28. **Brown T** 1995 Human androgen insensitivity syndrome. J Androl 16:299–303
29. **Juniewicz P, Oesterling J, Walters J, Steele R, Niswender G, Coffey D, Ewing L** 1988 Aromatase inhibition in the dog: I. effect on serum LH, serum testosterone concentrations, testicular secretions and spermatogenesis. J Urol 139:827–831
30. **Smith EP, Boyd J, Frank G, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS** 1994 Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. N Engl J Med 331:1056–1061
31. **Mullis P, Yoshimura N, Kuhlman B, Lippuner K, Jaeger P, Harada H** 1997 Aromatase deficiency in a female who is compound heterozygote for two new point mutations in the P450arom gene: impact of estrogens on hypergonadotropic hypogonadism, mulitcystic ovaries, and bone densitometry. J Clin Endocrinol Metab 82:1739–1745
32. **Kuiper GGJM, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson J-A** 1996 Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 93:5925–5930
33. **Tremblay GB, Tremblay A, Copeland NG, Gilbert DJ, Jenkins NA, Labrie F, Giguere V** 1997 Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor β. Mol Endocrinol 11:353–365
34. **Couse JF, Lindzey J, Grandien K, Kuiper GGJM, Gustafsson J-A, Korach KS** 1997 Tissue distribution and quantitative analysis of estrogen receptor-α (ERα) and estrogen receptor-β (ERβ) mRNA in the wild-type and ERα-knockout mouse. Endocrinology 138:4613–4621
35. **Shughrue PJ, Komm B, Merchenthaler I** 1996 The distribution of estrogen receptor-β mRNA in the rat hypothalamus. Steroids 61:678–681
36. **Li X, Schwartz P, Rissman E** 1997 Distribution of estrogen receptor-β-like immunoreactivity in rat forebrain. Neuroendocrinology 66:63–67
37. **Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O** 1993 Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Natl Acad Sci USA 90:11162–11166
38. **Wetsel W, Liposits Z, Seidah N, Collins S** 1995 Expression of candidate pro-LHRH processing enzymes n rat hypothalamus and an immortalized hypothalamic neuronal cell line. Neuroendocrinology 62:166–177
39. **Darney K, Goldman J, Vandenburgh J** 1992 Neuroendocrine responses to social regulation of puberty in the female mouse. Neuroendocrinology 55:434–443
40. **Beamer W, Murr S, Geschwind I** 1972 Radioimmunoassay of mouse luteinizing and follicle stimulating hormone. Endocrinology 90:823–827
41. **Lowry O, Rosebrough N, Farr A, Randall R** 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
42. **Wetsel W, Hill D, Ojeda S** 1996 Basic fibroblast growth factor regulates the conversion of pro-luteinizing hormone-releasing hormone (pro-LHRH) to LHRH in immortalized hypothalamic neurons. Endocrinology 137:2606–2616
43. **Lindzey J, Korach K** 1997 Developmental and physiological effects of estrogen receptor gene disruption in mice. Trends Endocrinol Metab 8:137–145
44. **Eddy EM, Washburn TF, Bunch DO, Goulding EH, Gladen BC, Lubahn DB, Korach KS** 1996 Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility. Endocrinology 137:4796–4805
45. **Zoeller R, Seeburg P, Young W** 1988 *In situ* hybridization histochemistry for messenger ribonucleic acid (mRNA) encoding gonadotropin-releasing hormone (GnRH): effects of estrogen on cellular levels of GnRH mRNA in female rat brain. Endocrinology 122:2570–2577
46. **Rothfeld J, Heftmancik J, Conn PM, Pfaff D** 1989 *In situ* hybridization of LHRH mRNA following estrogen treatment. Brain Res Mol Brain Res 6:121–125
47. **Petersen S, McCrone S, Shores S** 1993 Localized changes in LHRH mRNA levels and cellular correlates of the positive feedback effects of estrogen on LHRH neurons. Am Zool 33:255–265
48. **Handa R, Resko J** 1988 Effects of gonadectomy and hormone replacement on steroid hormone receptors and 5α-reductase activity in pituitaries of male rhesus macaques. J Clin Endocrinol Metab 66:1251–1258
49. **Meisel R, Sachs B** 1994 The physiology of male sexual behavior. In: Knobil E, Neill JD (eds) The Physiology of Reproduction. Raven Press, New York, vol 2:3–106
50. **Park Y, Park S, Cho W, Kim K** 1988 Testosterone stimulates LHRH-like mRNA level in the rat hypothalamus. Brain Res 451:255–260
51. **Wiemann J, Clifton D, Steiner R** 1990 Gonadotropin-releasing hormone messenger ribonucleic acid levels are unaltered with changes in the gonadal hormone milieu of the adult male rat. Endocrinology 127:523–532

52. **Cowley SM, Hoare S, Mosselman S, Parker MG** 1997 Estrogen receptors α and β form heterodimers on DNA. J Biol Chem 272:19858–19862
53. **Scully KM, Gleiberman AS, Lindzey J, Lubahn DB, Korach KS, Rosenfeld MG** 1997 Role of estrogen receptor α in the anterior pituitary gland. Mol Endocrinol 11:674–681
54. **Rissman E, Wersinger S, Taylor J, Lubahn D** 1997 Estrogen receptor function as revealed by knockout studies: neuroendocrine and behavioral aspects. Horm Behav 31:232–243
55. **Naik S, Young L, Charlton H, Clayton R** 1984 Pituitary gonadotropin-releasing hormone receptor regulation in mice. I. Males. Endocrinology 115:106–113
56. **McArdle C, Schomerus E, Groner I, Poch A** 1992 Estradiol regulates gonadotropin-releasing hormone receptor number, growth and inositol phosphate production in αT3–1 cells. Mol Cell Endocrinol 87:95–103
57. **Tobin V, Millar R, Canny B** 1997 Testosterone acts directly at the pituitary to regulate gonadotropin-releasing hormone-induced calcium signals in male rat gonadotropes. Endocrinology 138:3314–3319
58. **Keri R, Andersen B, Kennedy G, Hamernik D, Clay C, Brace A, Nett T, Notides A, Nilson J** 1991 Estradiol inhibits transcription of the human glycoprotein hormone α-subunit gene despite the absence of high affinity binding site for estrogen receptor. Mol Endocrinol 5:725–733
59. **Ogawa S, Lubahn DB, Korach KS, Pfaff DW** 1997 Behavioral effects of estrogen receptor gene disruption in male mice. Proc Natl Acad Sci USA 94:1476–1481
60. **Wierman M, Wang C** 1990 Androgen selectively stimulates follicle-stimulating hormone-β mRNA levels after gonadotropin-releasing hormone antagonist administration. Biol Reprod 42:563–571