The bi-modal effects of estradiol on gonadotropin synthesis and secretion in female mice are dependent on estrogen receptor- α

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Abstract

Depending on the estrous/menstrual cycle stage in females, ovarian-derived estradiol (E_2) exerts either a negative or a positive effect on the hypothalamic–pituitary axis to regulate the synthesis and secretion of pituitary gonadotropins, LH, and FSH. To study the role of estrogen receptor- α (ER α) mediating these effects, we assessed the relevant parameters in adult wild-type (WT) and ER α -null (α ERKO) female mice *in vivo* and in primary pituitary cell cultures. The α ERKO mice exhibited significantly higher plasma and pituitary LH levels relative to WT females despite possessing markedly high levels of circulating E_2 . In contrast, hypothalamic GnRH content and circulating FSH levels were comparable between genotypes. Ovariectomy led to increased plasma LH in WT females but no further increase in α ERKO females, while plasma FSH levels increased in both genotypes. E_2 treatment suppressed the high plasma LH and pituitary *Lhb* mRNA expression in ovariectomized WT females but had no effect in α ERKO. In contrast, E_2 treatments only partially suppressed

plasma FSH in ovariectomized WT females, but this too was lacking in α ERKO females. Therefore, negative feedback on FSH is partially E_2 /ER α mediated but more dependent on ovarian-derived inhibin, which was increased threefold above normal in α ERKO females. Together, these data indicate that E_2 -mediated negative feedback is dependent on functional ER α and acts to primarily regulate LH synthesis and secretion. Studies in primary cultures of pituitary cells from WT females revealed that E_2 did not suppress basal or GnRH-induced LH secretion but instead enhanced the latter response, indicating that the positive influence of E_2 on gonadotropin secretion may occur at the level of the pituitary. Once again this effect was lacking in α ERKO gonadotropes in culture. These data indicate that the aspects of negative and positive effects of E_2 on gonadotropin secretion are ER α dependent and occur at the level of the hypothalamus and pituitary respectively.

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Introduction

Gonadotropin-releasing hormone (GnRH) is secreted in a pulsatile pattern from the hypothalamus into the hypothalamic–hypophyseal portal veins that drain into the anterior pituitary, and is the primary stimulus for the synthesis and secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in the latter organ (Gharib *et al.* 1990, Haisenleder *et al.* 1994, Vale *et al.* 1994, Shupnik 1996). However, the endocrine role of gonad-derived steroids and peptides (e.g. estradiol (E_2) and inhibins) on the hypothalamic–pituitary (HP) axis to modulate gonadotropin synthesis and secretion is also well described and critical to reproductive function (Gharib *et al.* 1990, Haisenleder *et al.* 1994, Vale *et al.* 1994, Shupnik 1996). In most mammals, the estrous cycle is dictated by the bi-modal actions of ovarian-derived estrogens on the HP axis such that moderate levels of E_2 during early folliculogenesis are suppressive to LH secretion, while an acute rise in E_2 levels at proestrus acts to prime the hypothalamus and/or pituitary to

produce the hallmark pre-ovulatory gonadotropin surge that induces ovulation (Freeman 1994). The mechanisms and precise sites of action by which estrogens exert both negative and positive effects on the HP axis are still under investigation. Feedback at the level of the hypothalamus is illustrated by reports that ovariectomy results in upregulation of GnRH and that exogenous E_2 treatment of ovariectomized female rodents restores to normal the hypothalamic levels of GnRH mRNA (Zoeller & Young 1988, Zoeller *et al.* 1988), GnRH content (Wise *et al.* 1981a), and GnRH secretion (Wise & Ratner 1980, Wise *et al.* 1981b). However, *in vitro* studies demonstrate that some effects of E_2 on the pituitary are clearly independent of hypothalamic influence, including a suppression of *Fshb* (FSH- β mRNA) expression (Miller & Miller 1996; sheep) and an increase of *Lhb* (LH- β mRNA) expression (Shupnik *et al.* 1989a; rat). Furthermore, such effects of E_2 on gonadotropin gene expression appear to be estrogen receptor (ER) mediated, either via estrogen-response elements within the promoter regions of the gonadotropin subunit genes (Shupnik *et al.* 1989b)

or via interactions with other key transcription factors (Miller & Miller 1996). E₂ may also directly modulate the pituitary response to GnRH via regulation of GnRH-receptor (GnRH-R) levels (Naik *et al.* 1984, Turgeon *et al.* 1996).

Historically, a single form of nuclear ER, now known as ER α , was thought to mediate the effects of E₂ on gonadotropin secretion. However, the discovery of a second form of nuclear ER, the ER β (Kuiper *et al.* 1996, Mosselman *et al.* 1996, Tremblay *et al.* 1997), presents the possibility that two independent receptor forms may mediate the differential effects of E₂ feedback on the HP axis. The comparable binding affinities of ER α and ER β for various estrogenic ligands (Kuiper *et al.* 1997) have hampered the development of selective agonists or antagonists that could be employed to differentiate the actions of each receptor form. Therefore, the ER α and ER β -null (α ERKO and β ERKO respectively) mice provide unique models to discern the contribution of each receptor form in mediating the feedback effects of E₂ in the HP axis. ER α -null female mice have a hypoplastic reproductive tract. They are anovulatory, invariably possess ovaries that exhibit multiple atretic and enlarged cystic follicles and hypertrophied theca (Couse & Korach 1999). Plasma levels of gonadal steroids are significantly elevated in α ERKO females compared with wild-type (WT) females (Couse *et al.* 2003), as are plasma LH concentrations (Couse *et al.* 2003), presumably due to the lack of E₂-mediated negative feedback in the HP axis of α ERKO females. However, plasma FSH levels are similar in α ERKO and WT adult female mice (Couse *et al.* 2003).

Both ER α and ER β transcripts and immunoreactivity have been localized to hypothalamic nuclei thought to be involved in regulating GnRH secretion in rats and mice (Li *et al.* 1997, Shughrue *et al.* 1997a, Laflamme *et al.* 1998). Furthermore, high-affinity E₂ binding and transcripts encoding ER β (Skynner *et al.* 1999, Hrabovszky *et al.* 2000), estrogen-related receptor- α (ERR α ; Herbison & Pape 2001) but not ER α (Herbison & Pape 2001) are reportedly present in GnRH-secreting neurons of the mouse hypothalamus; thereby, challenging the long-held hypothesis that estrogen actions in these cells are indirect. In the pituitary, both ER α and ER β are expressed in the adult rat (Wilson *et al.* 1998), whereas adult mice may possess ER α only (Couse *et al.* 1997, Couse & Korach 1999).

Thus, in the present study, we characterized the role of ER α in regulating gonadotropin synthesis and secretion by evaluating and comparing the following parameters in wild-type and α ERKO female mice: (1) hypothalamic GnRH content, (2) circulating and pituitary gonadotropin levels, (3) effects of E₂ replacement on circulating gonadotropin levels and pituitary *Lhb* expression in ovariectomized wild-type and α ERKO females, and (4) secretory response of gonadotropes to E₂ and GnRH challenges *in vitro*. Our results indicate that female α ERKO mice exhibit normal hypothalamic GnRH content, increased plasma and pituitary LH levels despite excessively high levels of circulating E₂, but relatively normal plasma FSH levels and low pituitary FSH

levels that may be attributed to increased ovarian production of inhibin. Furthermore, ovariectomized α ERKO females were refractory to the negative feedback effects of exogenous E₂ that effectively suppressed LH gonadotropin synthesis and secretion in WT females. Likewise, α ERKO pituitary cells in culture were refractory to the positive effects of E₂ that effectively increased GnRH-stimulated LH release from WT pituitary cells.

Materials and Methods

Animals

All procedures involving animals were pre-approved by the National Institute of Environmental Health Sciences (NIEHS) Animal Care and Use Committee. Animals were maintained in plastic cages in a temperature-controlled room (21–22 °C) under a 12 h light:12 h darkness schedule and provided with NIH 31 mouse chow and fresh water available *ad libitum*. WT (*Esr1*^{+/+}) and *Esr1*^{-/-} (α ERKO) mice of the C57BL/6 strain were obtained from our colony at Taconic Farms, Germantown, NY, USA and used at 10–16 weeks of age. All animals were genotyped by PCR on DNA extracted from tail biopsies using the Wizard SV 96 Genomic DNA extraction kit (Promega) as previously described (Couse *et al.* 2003). Mice were ovariectomized using isoflurane anesthesia according to NIEHS approved surgical procedures and allowed to rest 2 weeks prior to experimental use.

Experimental design

Experiment 1 Intact, adult WT and α ERKO female mice were killed during 0900–1100 h to determine the basal levels of (1) hypothalamic GnRH content, (2) pituitary gonadotropin content, (3) plasma gonadotropin levels, and (4) serum and plasma inhibin-A levels. Due to sample limitations, not all endpoints were measured in the same group of animals. Animals were killed by CO₂ asphyxiation, whole blood was immediately collected from the inferior vena cava; hypothalami and pituitaries were then immediately removed and snap-frozen, and then stored at –70 °C until analysis. Whole blood or whole blood mixed with heparin (60 mg/ml) was centrifuged at 8000 *g* at 4 °C to collect serum or plasma respectively and stored at –70 °C until further analysis.

Experiment 2 To examine the effect of ovariectomy and E₂ replacement on plasma gonadotropin levels and pituitary gonadotropin gene expression, ovariectomized adult female WT and α ERKO mice were injected subcutaneously with 100 μ l vehicle (sesame oil) or 17 β -E₂ (Research Plus, Inc., Manasquan, NJ, USA) at 50 μ g/kg body weight for three consecutive days between 0900 and 1100 h. E₂ treatments of 15–50 μ g/kg per day for three consecutive days consistently induce a maximum uterotrophic response in ovariectomized

WT mice (Lubahn *et al.* 1993, Hewitt *et al.* 2003). We used the higher dose in the present studies to more closely mimic the elevated E₂ levels that are endogenous to intact α ERKO female mice (Couse *et al.* 2003). Blood and tissues were collected 24 h after the final treatment.

Experiment 3 To examine the effects of E₂ on basal and GnRH-induced LH secretion in the absence of hypothalamic influence, dispersed pituitary cell cultures were prepared from adult WT and α ERKO females according to the procedure of Huang *et al.* (2001) with the following modifications. Pituitaries were harvested and pooled according to genotype ($n > 9$ per genotype per experiment), then minced in 1× Hank's balanced salt solution (HBSS; Invitrogen) supplemented with 25 mM HEPES (Sigma) and 0.15 mM calcium chloride. The resulting tissue fragments were then digested in HBSS with HEPES containing Cls-2 collagenase (640 U/ml; 200 μ l/5 pituitaries; Worthington, Lakewood, NJ, USA) at 36.5 °C for 2–3 h with periodic vortexing; followed by incubation in calcium–magnesium free HBSS with HEPES containing 0.25% pancreatin (Invitrogen) for 15–20 min at 36.5 °C. The dispersed cells were then vigorously vortexed, pelleted, and washed three times in culture media (DMEM without phenol red; 10% stripped fetal calf serum; with PenStrep; Invitrogen) and filtered through 50 μ m Nitex (Sefar Filtration, Depew, NY, USA) to remove aggregates and debris. Cells were counted and plated (1.5×10^5 cells/well; four to five wells per treatment) in 96-well plates coated with Matrigel (BD Biosciences, San Jose, CA, USA) diluted 1:3 with DMEM. Cells were incubated in a humidified chamber of 95% O₂:5% CO₂ at 37 °C. After 24 h, the media were changed to culture media containing either vehicle (ethanol) or E₂ (10 and 100 pg/ml). After 48 h, the spent medium was carefully removed by aspiration and replaced with experimental medium (DMEM without phenol red; no serum; 0.1% BSA) containing vehicle or E₂ (10 and 100 pg/ml), and/or GnRH (10 nM; Sigma). The cells were allowed to incubate for an additional 2 h, after which the media were rapidly collected and stored at –70 °C until analysis. The cells were then processed later for RNA extraction. This experiment was repeated three times with similar results.

Hormone measurements

To measure hypothalamic GnRH content, hypothalami were collected from individual animals and frozen immediately upon animal death. An aqueous supernatant was prepared from each hypothalamus by sonicating in 0.1 N (0.1 M) acetic acid (500 μ l), boiling for 5 min, and pelleting the cellular debris by centrifugation at 14 000 *g* at 4 °C. The resulting supernatant containing GnRH was then lyophilized overnight, resuspended in a fixed volume of RIA buffer, and then duplicate aliquots per preparation were subjected to RIA for mature GnRH as previously described (Wetsel *et al.* 1996). The cellular debris pellets were stored at –70 °C and later used to determine the total protein content using the BCA assay (Bio-Rad) according

to the manufacturer's protocol. Intra- and inter-assay coefficients of variation were 6 and 9% respectively.

Plasma and pituitary gonadotropin levels were assessed using rat LH and FSH RIAs that were previously reported to accurately measure mouse gonadotropins (Beamer *et al.* 1972, Darney *et al.* 1992). For plasma samples, 50 μ l aliquots were assayed for LH and FSH in duplicate when sample volume allowed. To assess pituitary gonadotropin content, individual pituitaries were sonicated in 500 μ l RIA buffer and duplicate 5 μ l aliquots of a tenfold dilution of the homogenate were assayed. Individual LH and FSH levels below the limit of detection were assigned a value equal to the lower limit for each respective assay (FSH 1.6 ng/ml; LH 0.12 ng/ml).

LH secretion by cultured pituitary cells was assessed using rat LH kits previously reported to accurately measure mouse LH (Couse *et al.* 2003; GE Healthcare Life Sciences, Piscataway, NJ, USA). Aliquots (75 μ l) were assayed singly. The limit of detection was 0.08 ng/ml and the intra-assay coefficient of variation was 6%.

Inhibin-A levels were assessed in 50 μ l aliquots of serum or plasma using the active inhibin-A ELISA (Diagnostic Systems Laboratories, Webster, TX, USA) according to the manufacturer's protocol. Serum samples ($n = 12$ per genotype) were assayed in duplicate and plasma samples ($n = 9–11$ per genotype) were assayed as singlets. Results did not differ between serum and plasma samples and were combined before final statistical analysis. The range of detection was 10–1000 pg/ml. The inter- and intra-assay coefficients of variation were 7 and 10% respectively.

RNA isolation and analysis

Total RNA was isolated from individual snap-frozen pituitaries or pituitary cell cultures using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Glycogen (10 μ g/tube) was added prior to the final alcohol precipitation to maximize RNA yield. The concentration of all final preparations was calculated via an A₂₆₀ reading using a Molecular Devices Spectramax (Sunnyvale, CA, USA) spectrophotometer followed by electrophoresis of a 1 μ g aliquot to ensure integrity prior to further analysis.

Pituitary levels of *Lhb* mRNA were assessed by northern blot analysis on 1 μ g aliquots of total RNA from individual pituitaries, then normalized by subsequent probing for *Rpl7* mRNA as previously described (Lindzey *et al.* 1998). Pituitary levels of *Fshb* mRNA were assessed by ribonuclease protection assays (RPAs) on 1 μ g aliquots of total RNA from individual pituitaries, and included a probe for *Ppia* (cyclophilin) to normalize among samples, as previously described (Couse *et al.* 2003).

Gene expression in total RNA from cultured pituitary cells was assessed by quantitative real-time reverse-transcriptase PCR (qRT-PCR). For each sample (each well), cDNA was generated from 1 μ g RNA in a 25 μ l reaction mixture using random hexamers and the superscript cDNA synthesis system (Invitrogen) according to the manufacturer's protocol. Applied

Biosystems Primer Express (Foster City, CA, USA) software was used to select primers specific for the amplification of murine gonadotropin-releasing hormone receptor (*Gnrhr*), common α-glycoprotein (*Cga*), *Lhb*, and *Fshb* cDNAs (Table 1). All primer sets were designed to lie in separate exons to avoid erroneous amplification of contaminating genomic DNA and confirmed to amplify a single product of the expected size via dissociation analysis and gel electrophoresis. Each sample was assayed in duplicate using the equivalent of 0.1 μl cDNA (prepared as described previously), 20 pmol of each primer and 1 × SYBR Green Master Mix (Applied Biosystems) in a total reaction volume of 50 μl. For normalization purposes, an identical set of reactions was prepared using primers specific for *Rpl7* as previously described (Hewitt *et al.* 2003; Table 1). Amplification was carried out in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) as follows: 50 °C/2 min, 95 °C/10 min (1 ×); 95 °C/15 s, 60 °C/30 s (40 ×). Quantitative differences in the cDNA target between samples were determined using the mathematical model of Pfaffl (2001), in which an expression ratio was determined for each sample by calculating $(E_{\text{target}})^{\Delta Ct(\text{target})}/(E_{Rpl7})^{\Delta Ct(Rpl7)}$, where E is the efficiency of a primer set and $\Delta Ct = Ct(\text{calibrator sample}) - Ct(\text{experimental sample})$ and calibrator sample = 'control' = WT noGnRH noE₂. The amplification efficiency of each primer set was calculated from the slope of a standard amplification curve of log μl cDNA vs Ct value over at least four orders of magnitude ($E = 10^{-(1/\text{slope})}$).

Statistical analysis

All data were analyzed using Levene's test for homogeneity of variance. If significant heteroscedascity was observed, data were log transformed prior to statistical analyses. Data were initially analyzed using a two-way ANOVA and the Bonferroni–Dunn *post hoc* test. If significant genotype and treatment interactive effects were noted, data for each genotype were analyzed separately using a one-way ANOVA and the Bonferroni–Dunn *post hoc* test or by *t*-tests. In addition, some comparisons between intact WT and αERKO females were made using one-tail, unpaired *t*-tests. In all cases, statistical significance was accepted at $P < 0.05$.

Results

Experiment I: evaluation of reproductive hormone levels in intact WT and αERKO females

Despite possessing comparable levels of hypothalamic GnRH content (Fig. 1A), αERKO females exhibited conspicuous dysregulation of gonadotropin synthesis and secretion in the pituitary compared with WT females (Fig. 1B and C). Pituitary LH content and plasma LH levels were both increased 2.7– ($P < 0.05$) and 8-fold ($P < 0.05$) respectively, in αERKO females relative to wild type (Fig. 1C). In contrast, plasma FSH levels did not differ between genotypes, but the average pituitary FSH content in αERKO females was 50% below that of WT females ($P < 0.05$). Inhibin-A levels in αERKO females were dramatically increased (3.7-fold; $P < 0.05$) compared with WT females (Fig. 1D).

Experiment II: effect of ovariectomy and E₂ treatment on the αERKO HP axis in vivo

As expected, ovariectomy led to plasma FSH and LH levels that were increased almost 4- and 12-fold respectively in WT females (Figs 1 and 2). Ovariectomized αERKO females exhibited similar increases in plasma FSH but showed no further rise in plasma LH levels compared with intact αERKO females (Figs 1 and 2). In ovariectomized WT females, E₂ treatments suppressed plasma LH levels to pre-surgery levels but decreased the heightened plasma FSH levels by only 30% (compare Figs 1 and 2). In contrast, E₂ treatments of ovariectomized αERKO females failed to suppress plasma LH and FSH levels.

The effects of ovariectomy and E₂ replacement on plasma gonadotropin levels in each respective genotype were mirrored by changes in *Lhb* expression in the pituitary. As previously reported, intact αERKO females possess significantly increased levels of *Lhb* expression in the pituitary (Couse *et al.* 2003). As shown in Fig. 2, ovariectomy abolished the genotypic difference in *Lhb* expression. However, while WT females exhibited a 33% ($P < 0.05$) reduction in *Lhb* mRNAs following exogenous E₂ treatment, αERKO females exhibited no such change.

Table 1 Primers used for RT-PCR

	Accession no.	Amplified sequences (bp)	Forward (5'–3')	Reverse (5'–3')
Gene				
<i>Cga</i>	NM 009889	182–281	GAC TTTATTATT CAGGGTTGCCCA	AGAAGCAACAGCCCATACACTG
<i>Fshb</i>	NM 008045	28–153	GACTGCACAGGACGTAGCTGTTTA	CTGAGATGGTGATGTTGGTCAATT
<i>Gnrhr</i>	NM 010323	767–866	TTCATCAAGACCCACGCAAA	GAGGTAGCGAATGCGACTGTC
<i>Lhb</i>	NM 008497	100–204	TGTCAACGCAACTCTGGCC	GGCAGTACTCGGACCATGCT
<i>Rpl7</i>	NM 011291	416–436	AGCTGGCCTTGTGCATCAGAA	GACGAAGGAGCTGCAGAACCT

Experiment III: effect of E₂ treatment on WT and α ERKO pituitary cells in vitro

To examine any direct effects that E₂ may have on gonadotrope function, basal and GnRH-induced LH secretions were evaluated in primary pituitary cell cultures from WT and α ERKO females. Cells were first exposed to either vehicle or E₂ (10 or 100 pg/ml) for 48 h and then subjected to an acute (2 h) challenge with GnRH in the continued presence or absence of E₂. Interestingly, α ERKO pituitary cells exhibited increased basal *Lhb* expression and LH secretion relative to WT cells following 48 h in culture, regardless of the presence or dose of E₂ (Fig. 3). Upon GnRH stimulation, non-estrogen exposed WT pituitary cells exhibited a 13-fold increase in LH secretion ($P < 0.05$), and this was further enhanced to > 20 -fold in WT cells when pretreated with 10 or 100 pg E₂/ml ($P < 0.05$ versus no E₂; Fig. 3). α ERKO pituitary cells also exhibited increased LH secretion (two- to fourfold, depending on E₂ dose) when challenged with GnRH; however, prior E₂ exposure had no enhancing effect (Fig. 3). The GnRH stimulated LH-secretory response from α ERKO pituitary cells, when expressed as fold increase over basal LH secretion, was blunted in comparison with the WT response. However, the absolute GnRH-stimulated LH secretory response reached similar levels in α ERKO (21.3 ± 0.8 ng/ml) and WT pituitary cells (20.4 ± 0.2 ng/ml). In contrast to the effect of GnRH on LH secretion, no parallel increase in *Lhb* expression was observed in either genotype (Fig. 3). Similar assays for *Cga*, *Fshb*, and *Gnrhr* expression indicated no significant genotypic or treatment effects (data not shown).

Discussion

The endocrine actions of ovarian-derived E₂ on the HP axis are vital to gonadal function and fertility in female mammals. The presence of both known ER forms in the hypothalamus and pituitary, however, confounds our abilities to discern the precise site of such actions and the contribution of each receptor form in mediating the estrogenic effects. We contend that this obstacle may be largely overcome by the study of α ERKO mice. Since these animals lack functional ER α but maintain normal ER β expression (Couse *et al.* 1997), they are especially suited to reveal phenotypes that may distinguish the actions of the two ERs. Herein, we have employed α ERKO female mice to demonstrate that ER α functions are fundamental to the negative-feedback actions of E₂ in the HP axis, congruent with earlier reports (Wersinger *et al.* 1999, Couse & Korach 1999, Couse *et al.* 1999, 2003). Furthermore, our studies indicate that ER α -mediated actions are critical to the negative modulation of LH secretion, while FSH secretion is only partially mitigated by ER α -E₂ actions and are more effectively regulated by ovarian-derived inhibin. We have also found evidence that ER α -mediated actions are important for the positive modulation of LH secretion at the pituitary, where E₂ effectively increased GnRH-stimulated LH release.

The elevated pituitary and plasma LH levels consistently found in α ERKO females are demonstrative of the critical role for ER α in negatively regulating LH synthesis and secretion. WT females exhibited comparable increases in plasma LH only after being rid of circulating sex steroids via

Figure 1 Reproductive hormone levels in the plasma of intact wild-type (WT) and α ERKO females. Shown are the levels (mean $\pm$ S.E.M.) of (A) hypothalamic gonadotropin-releasing hormone (GnRH) content, (B) pituitary (top) and plasma (bottom) follicle-stimulating hormone (FSH) levels, (C) pituitary (top) and plasma (bottom) luteinizing hormone (LH) levels, and (D) circulating inhibin-A levels. Statistically significant differences between genotypes were determined by Student's *t*-test ($P < 0.05$) and are indicated by an asterisk. Sample sizes equalled 7–10 per genotype for the data shown in (A)–(C), and ≥ 21 per genotype for the data shown in (D).

Figure 2 Pituitary gonadotropin gene expression and plasma gonadotropin levels in ovariectomized and estradiol-treated (E_2) female mice. Shown are the levels (mean $\pm$ S.E.M.) of plasma follicle-stimulating hormone (FSH) (left) and plasma luteinizing hormone (LH) and pituitary *Lhb* mRNA (right) in ovariectomized wild-type (WT) and α ERKO females following treatment with either vehicle (sesame oil) or E_2 (50 μ g/kg body weight, once per day for 3 days). *Lhb* mRNA levels were determined by northern blot and normalized to levels of *Rpl7* mRNA per sample; sample sizes were seven to eight animals per group. Statistical differences between genotypes and treatments, as indicated by *, were determined by first employing a two-way ANOVA ($P < 0.05$), followed by the Bonferroni–Dunn *post hoc* test ($P < 0.05$ vs vehicle-treated WT) where appropriate.

ovariectomy. Similarly, female mice devoid of endogenous E_2 due to targeted disruption of the *Cyp19* (P450_{aromatase}) gene also exhibit increased plasma LH, and this is abated upon exogenous E_2 treatments (Britt *et al.* 2004). α ERKO females consistently possess increased LH levels despite possessing excessively high levels of circulating E_2 (Couse *et al.* 2003) and normal ER β expression (Couse *et al.* 1997). Furthermore, ER β -null (β ERKO) females exhibit normal plasma LH levels (Couse *et al.* 2003). These data collectively indicate that E_2 -mediated negative feedback on the HP axis is an ER α -specific action and that any role of ER β is minimal.

In contrast to LH, FSH synthesis and secretion were not increased in intact α ERKO females. As ovariectomy resulted in large increases in circulating FSH in both genotypes, an ovarian factor(s) is obviously required to maintain normal FSH

synthesis and secretion, and this factor is also present in the α ERKO. E_2 and inhibins are the two primary ovarian-derived hormones that feedback upon the female HP axis to negatively modulate gonadotropin secretion. We have previously reported that α ERKO females possess plasma E_2 levels that are elevated almost eightfold above those of WT females (320 ± 17 vs 40 ± 3 pg/ml; Couse *et al.* 2003). Interestingly, E_2 treatments only partially suppressed (30%) FSH levels in ovariectomized WT females. Thus, unlike the regulation of LH secretion, E_2 may only play a minor role within the HP axis in negatively regulating FSH secretion, and this effect is mediated by ER α and not ER β , as E_2 treatments of α ERKO females completely failed to suppress FSH levels. Congruent with the above findings, the regulation of FSH synthesis and secretion is known to be more dependent on the inhibin/activin family of peptide hormones (Woodruff & Mather 1995, Gregory & Kaiser 2004). Therefore, the reason that FSH levels are not elevated in α ERKO females is probably due to their plasma inhibin-A levels, which were threefold above normal. Circulating inhibin-A is primarily derived from the granulosa cells of large, pre-ovulatory follicles in the ovary (Rajkovic *et al.* 2006). Therefore, increased circulating inhibin-A in α ERKO females is not unexpected given that these animals are anovulatory and exhibit ovaries that consistently possess multiple differentiated, albeit unhealthy, follicles (Couse & Korach 1999). Additional markers also considered to be indicative of pre-ovulatory follicles, such as E_2 synthesis (Couse *et al.* 2003) and LH receptor (Couse & Korach 1999), are also increased in α ERKO ovaries. Interestingly, female CYP19-null mice share several aspects of the α ERKO ovarian phenotype but exhibit a greater than eightfold decrease in inhibin-A levels compared with WT females (Britt *et al.* 2005) and, accordingly, possess severely elevated levels of plasma FSH (Fisher *et al.* 1998). Therefore, the once perplexing difference in plasma FSH levels between α ERKO and CYP19-null mice is due to a drastic disparity in circulating inhibin-A levels. These data suggest that ligand-dependent actions of ER β in granulosa cells may facilitate inhibin synthesis in the ovary by promoting granulosa cell growth and function.

The precise site of ER α -mediated negative feedback on gonadotropin synthesis and secretion may be inferred by our findings that basal LH secretion by cultured pituitary cells from either genotype was not altered by E_2 . This absence of direct E_2 effects on gonadotrope behavior strongly suggests that E_2 /ER α -mediated negative feedback occurs at the level of the hypothalamus. Other studies also indicate that E_2 negatively modulates gonadotropin secretion by decreasing the frequency of hypothalamic GnRH pulses (Sarkar & Fink 1980, Weick & Noh 1984). The loss of ER α -mediated E_2 actions leading to increased frequency of GnRH secretion may also lead to increased GnRH synthesis, resulting in unaltered net hypothalamic GnRH content, congruent with our observation that α ERKO females exhibited hypothalamic GnRH content levels that were not different from wild type.

Despite the above findings, ER α -null gonadotropes placed in culture and therefore removed from any hypothalamic influence

continue to exhibit increased basal LH secretion and *Lhb* expression relative to WT gonadotropes. A difference in the gonadotrope population between WT and α ERKO pituitaries is an unlikely explanation for the increased *in vitro* basal LH secretion in the latter. In fact, female α ERKO pituitaries do not exhibit an increased number of gonadotropes (Scully *et al.* 1997) and are slightly decreased in weight (data not shown). Furthermore, our finding that other relevant gonadotrope mRNAs, such as *Cga*, *Fshb*, or *Gnrhr*, were not similarly increased in cultured α ERKO pituitary cells supports the existence of a comparable gonadotrope population in α ERKO pituitaries. Expression of other regulators known to be involved in gonadotrope function and regulation, such as *Nr5a1* (SF-1), *Egr-1*, or *Nr0b1* (DAX-1; Achermann & Jameson 1999) may also be altered and may contribute to the abnormally high LH synthesis and secretion in the α ERKO. The preservation of abnormally high LH synthesis and secretion by ER α -null pituitary cells, even when removed from hypothalamic influence, may be due to lingering effects of chronic GnRH hyperstimulation of the α ERKO pituitary prior to tissue collection. Indeed, *Lhb* mRNA levels are reported to remain stable for several days following GnRH blockade in male rats (Paul *et al.* 1990). Alternatively, abnormal LH secretion and *Lhb* expression in α ERKO gonadotropes could be attributed to aberrant development of the anterior pituitary due to the absence of ER α .

In contrast to the negative effects of E₂/ER α actions on LH secretion that occurs primarily via the hypothalamus, our *in vitro* studies indicated that E₂ had a positive effect at the level of the pituitary. E₂ enhanced GnRH-induced LH secretion in cultured pituitary cells from WT mice. E₂ priming before GnRH stimulation did not lead to similar increases in *Cga*, *Fshb*

(data not shown), or *Lhb* expression in cells from either genotype, indicating that this is primarily a secretory response. *Gnrhr* expression in these cultures was also unaltered (data not shown), but our analyses are limited to the transcriptional levels and may not reflect true GnRH-R protein levels or even the level of active receptor on the gonadotrope cell surface.

However, E₂ treatment of α ERKO-derived pituitary cells failed to enhance LH-secretory responses to GnRH, indicating that this positive effect of E₂ on LH secretion is also dependent on the presence of functional ER α . The fact that in our cultures, the absolute amount (ng/ml) of LH released in response to a GnRH stimulus was not greater in α ERKO than in WT pituitary cells suggests that the elevated levels of plasma LH consistently present in α ERKO females stem from high hypothalamic GnRH secretion and not from increased pituitary responsiveness.

An attractive experimental use of α ERKO mice is to explore E₂-mediated actions within the HP axis that may be independent of ER α . For example, we have previously reported that *Esr2* (ER β) expression in the hypothalamus and pituitary of α ERKO females is not different from wild type (Couse *et al.* 1997, Couse & Korach 1999), and therefore any role of ER β in mediating E₂ actions is presumably intact. The possible involvement of ER β allows for the intriguing prospect that the bi-modal feedback effects of E₂ on gonadotropin regulation are via a dual-receptor system. However, the present study produced no evidence that ER β is involved in mediating the positive feedback actions of E₂ at the pituitary. Indeed, female β ERKO mice are able to spontaneously ovulate and therefore presumably capable of producing a gonadotropin surge at proestrus (Couse & Korach 1999), but they also exhibit reduced fecundity that

Figure 3 Evaluation of *Lhb* expression and LH secretion by wild-type and α ERKO pituitary cells dispersed in culture. Dispersed pituitary cells were cultured at 1.5×10^5 cells per sample and exposed to vehicle (open) or estradiol E₂ at 10 pg/ml (cross-hatched) or 100 pg/ml (filled bar) for 48 h. The cells were then challenged with vehicle or GnRH (10 nM), while maintaining the prior E₂ treatments. The medium and cells were removed after 2 h and assayed for LH concentration and *Lhb* mRNA levels respectively, as described in Materials and Methods ('control' = WT noGnRH noE₂). Shown are means $\pm$ S.E.M. from a representative experiment (n = four to five wells per treatment). Statistical differences between genotypes and treatments were determined by first employing a two-way ANOVA that detected significant interactive effects. Data for each genotype were subsequently analyzed using a one-way ANOVA ($P < 0.05$), followed by the Bonferroni *post hoc* test when appropriate. The indicated statistical differences are as follows: (a) $P < 0.05$ vs wild-type groups without GnRH; (b) $P < 0.05$ vs wild type + 0.00 pg/ml E₂ + GnRH; (c) $P < 0.05$ vs α ERKO groups without GnRH; and (d) $P < 0.05$ vs wild type + 0.00 pg/ml E₂.

may be attributed to infrequent and/or blunted LH surges. ER β has been postulated to facilitate the LH surge by mediating an estrogen-induced increase in progesterone receptor (PR) expression in the hypothalamus, which is required for the LH surge (Chappell *et al.* 1997, Chappell & Levine 2000). This is supported by the preservation of E₂-induced PR expression in the hypothalami of α ERKO females (Shughrue *et al.* 1997b, Moffatt *et al.* 1998). In addition, ER α and ER β transcripts are detected in an immortalized GnRH neuronal cell line (Butler *et al.* 1999, Roy *et al.* 1999), while ER β but not ER α transcripts are reportedly present in GnRH-secreting neurons within the medial preoptic area of female rats (Hrabovszky *et al.* 2000).

In summary, our data indicate that E₂/ER α actions are critical to the negative modulation of LH synthesis and secretion, but less important to the regulation of FSH synthesis and secretion in female mice, and both of these actions occur primarily at the level of the hypothalamus. Conversely, the ability of E₂ to enhance the GnRH response of wild type, but not α ERKO gonadotropes in culture indicates that the positive influence of E₂ on gonadotropin secretion is also ER α mediated, but occurs at the level of the pituitary. Thus, ER α is responsible for aspects of both negative and positive feedback effects of E₂ on LH synthesis and secretion.

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