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Authors: Singh, Surya P., Wolfe, Andrew, Ng, Yewade, DiVall, Sara A., Buggs, Colleen, et al.

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## Impaired Estrogen Feedback and Infertility in Female Mice with Pituitary-Specific Deletion of Estrogen Receptor Alpha (ESR1)<sup>1</sup>

Surya P. Singh,<sup>3,6</sup> Andrew Wolfe,<sup>2,3,4</sup> Yewade Ng,<sup>4</sup> Sara A. DiVall,<sup>4</sup> Colleen Buggs,<sup>7</sup> Jon E. Levine,<sup>5</sup> Fredric E. Wondisford,<sup>4</sup> and Sally Radovick<sup>4</sup>

Divisions of Pediatric Endocrinology and Metabolism,<sup>4</sup> Johns Hopkins University School of Medicine, Baltimore, Maryland

Department of Neurobiology and Physiology,<sup>5</sup> Northwestern University, Evanston, Illinois

Department of Biochemistry,<sup>6</sup> Banaras Hindu University, Varanasi (UP), India

Department of Pediatric Endocrinology,<sup>7</sup> Baylor College of Medicine, Houston, Texas

### ABSTRACT

Mice lacking estrogen receptor alpha in the pituitary gonadotroph (PitEsr1KO) were generated to determine the physiologic role of pituitary estrogen signaling in the reproductive axis. PitEsr1KO female mice are subfertile or infertile and have elevated levels of serum luteinizing hormone (LH) and LH beta subunit gene expression, reflecting a lack of estrogen negative feedback effect on the gonadotroph. While serum LH values are elevated in PitEsr1KO mice, the degree of elevation is much less than that observed in ESR1-null mice, indicating that the hypothalamus must also have an important role in estrogen negative feedback. PitEsr1KO mice also demonstrate a defect in estrogen positive feedback, as surge LH values and estrous cyclicity are absent in these mice. Although sex steroid feedback in the reproductive axis is thought to involve discrete anatomic regions that mediate either a positive or negative estrogen effect, PitEsr1KO mice demonstrate novel evidence that localizes both estrogen positive feedback and estrogen negative feedback to the gonadotroph, which suggests that they may be mechanistically related.

anterior pituitary, Cre/loxP, estradiol, estradiol receptor, gonadotropin-releasing hormone, luteinizing hormone, ovary, ovulatory cycle

### INTRODUCTION

Neuroendocrine control of ovulation is mediated by pulsatile secretion of the decapeptide gonadotropin-releasing hormone (GnRH), which binds to receptors on gonadotrophs. This results in synthesis and secretion of luteinizing hormone (LH), which then directs gonadal steroidogenesis. Estradiol (E2) in turn exerts negative feedback action at the level of the

hypothalamus and pituitary to alter GnRH and LH synthesis and secretion. In rodent models, estrogen secretion results in an abrupt release of GnRH in proestrus and an LH surge that triggers ovulation. Thus, both positive and negative feedback actions of estrogen on GnRH and LH secretion are important for the physiologic control of reproduction.

After detailed investigations of animal models, controversies still exist as to the effect of estrogen on the central reproductive axis, the mechanism of the effect of estrogen (direct vs. indirect), the level or levels of estrogen regulation (hypothalamic, pituitary, or both), and which isoforms of estrogen receptor (ER) (ESR1 [also known as ER $\alpha$ ] and/or ESR2 [also known as ER $\beta$ ]) mediate signaling. *Esr1* and *Esr2* knockout (KO) mouse models have been developed to define the physiologic effects of E2 signaling through its cognate receptors. *Esr1* KO mice display significant gonadal defects and impaired feedback regulation of the neuroendocrine axis marked by elevated levels of LH and E2 [1, 2]. Compared with wild-type (WT) mice, the phenotype of *Esr2* KO mice was reported to be much milder, with subfertility and mild elevations in LH, until a recent study by Antal et al. reported infertility [3–6]. These models have defined ESR1 as the predominant receptor isoform that conveys negative feedback regulation to the central reproductive axis [7] and support a predominant role for ESR1 in feedback regulation of the central reproductive axis. Cell-specific KO of *Esr1* has recently been used to define more precisely the level of E2 regulation of the reproductive axis. Using a neuron-specific *Esr1* KO mouse model, estrogen positive feedback has been shown to require brain ESR1 [8].

To determine the cell-specific effects of ESR1 at the level of the gonadotroph, our laboratory developed a new *Esr1* floxed mouse model in which exon 3, which encodes the DNA-binding domain of ESR1, was flanked by loxP sites. We have shown that deletion of exon 3 using this model results in a lack of any detectable ESR1 protein [9]. These floxed animals were then bred to animals in which Cre recombinase expression is under the control of the mouse common glycoprotein  $\alpha$  subunit promoter. This promoter has been shown to target the gonadotroph and direct the expression of Cre recombinase to the anterior pituitary [10, 11]. The resultant mice, referred to as PitEsr1KO, demonstrate a loss of ESR1, specifically in the anterior pituitary.

The PitEsr1KO females had irregular estrous cycles punctuated by prolonged periods of disorganized cycling. This resulted in either infertility or subfertility marked by a decrease in the number of litters and size of the litters. The serum LH and E2 values were elevated compared with those in control littermates; however, the elevation in both values was lower

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<sup>2</sup>Correspondence: Andrew Wolfe, Division of Pediatric Endocrinology, Johns Hopkins University School of Medicine, Baltimore, MD 21287. FAX: 410 502 7580; e-mail: Awolfe3@jhmi.edu

<sup>3</sup>These authors contributed equally to this work.

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than that in mice with a complete KO of *Esr1*. In addition, the degree of elevation tracked with the severity of the reproductive defect, with infertile female mice having a greater elevation in serum LH vs. subfertile female mice. The ovaries of *PitEsr1*KO mice were larger, with disrupted architecture, and displayed few or no corpora lutea vs. WT animals. In addition to the defect in estrogen negative feedback, *PitEsr1* KO female mice have evidence of blunted or absent LH surge activity. These findings demonstrate for the first time (to our knowledge) a close relationship between estrogen negative and positive feedback in the pituitary and suggest that these regulatory pathways may not be dissociable *in vivo*.

## MATERIALS AND METHODS

### Transgene Construction

To generate the *Cga*-Cre transgene (Fig. 1A), a 2.1-kilobase (kb) fragment containing a nuclear localization signal, the bacteriophage P1 gene Cre recombinase, and the beta-actin polyadenylation signal was excised from pML78. This fragment was subcloned downstream of the *Cga* promoter (−5000 to +43) in pGEM7Zf+. A 7.0-kb *Cga*-Cre fragment was generated by digestion of the *Cga*-Cre plasmid, which reduced the *Cga* promoter to 4.5 kb.

### Generation, Genotyping, and Breeding of Transgenic Mice

Purified DNA was microinjected into zygotes from C57BL/6J × SJL parents. Embryos were transferred to postcoitum pseudopregnant CD1 females. Genomic DNA was prepared from tail biopsies of all progeny born and screened for the presence of the transgene. Mice with the *Cga*-Cre transgene (*Tg(Cga-cre)**IAwo*) were identified by PCR using oligonucleotides that amplify a 150-base pair (bp) product spanning the junction of the *Cga* promoter and the Cre coding sequence (5′-ACATTGTTCCCTCAGATCG-3′ and 5′-ATG TGAGCGAGTAACAACCCGTCGGATTCT-3′). Reactions proceeded for 30 cycles of denaturation at 92°C for 15 sec, annealing at 60°C for 45 sec, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. All reactions were performed under standard conditions using approximately 100–200 ng of genomic DNA, 0.5 pmol/μl primers, 2.5 mM MgCl<sub>2</sub>, and 0.02 U/μl Taq DNA polymerase per reaction. Transgenic founders and their progeny were bred to C57BL/6J mice to establish and maintain lines. Three lines were generated with the *Cga*-Cre transgene.

### Generation and Genotyping of “Floxed” *Esr1* Mice by Homologous Recombination

The cloned mouse *Esr1* fragment was obtained from BAC clones of the *Esr1* locus obtained from the Microarray and Genomics Facility at Roswell Park Cancer Institute (Buffalo, NY) and used to generate a targeting construct in which exon 3 of the mouse *ESR1* gene was flanked by loxP sites, or “floxed” [12]. Homologous recombination was determined by using an EcoRI digestion of embryonic stem (ES) cell DNA. After ES cell selection was achieved, the Neo cassette was removed from the mutant allele *in vivo*. To that effect, a self-excising ACN [tACE-Cre/Neo] cassette was used that included a testes-specific promoter tACE [angiotensin-converting enzyme] driving the expression of Cre recombinase. The tACE-Cre fragment is adjacent to a Neo marker gene [13], and the entire tACE-Cre/Neo cassette is flanked by two loxP sites. Retaining a Neo cassette *in vivo* has been shown to have unpredictable consequences on the phenotype of mice, including generation of a hypomorphic allele with a wide range of phenotypic abnormalities [14, 15] and misregulation of adjacent genes [16]. The tACE present in the ACN cassette has been shown to specifically activate Cre recombinase activity in the testes (sperm cells) but not in other tissues [13]. Thus, self-excision of the ACN cassette in the testis of chimeric male mice resulted in sperm cells transmitting the mutant allele that contains the floxed *ESR1* gene, lacking the ACN cassette, to the F1 progeny.

The ES cells heterozygous for the allele containing the integrated homologous recombinant were injected into WT mouse blastocysts. The resultant mice were chimeric for WT host-derived and recombinant ES-derived cells. Male chimeric mice transmitted one of two alleles, WT or homologous recombinant, through their sperm cells. Two types of deletions are expected to occur in the testes of chimeric males: type 1 deletion, in which both the ACN and exon 3 fragments are deleted (*Esr1*<sup>tm1.1Awo</sup>), and type 2 deletion, in which only the ACN cassette (and not exon 3) is deleted (*Esr1*<sup>tm1.2Awo</sup>). In the type 2

deletion, the mutant allele will be marked by two loxP sites flanking exon 3 of the *Esr1* gene and will not contain the ACN cassette.

Southern blot analysis of tail DNA obtained from the F1 progeny was performed to identify heterozygous F1 progeny with either a type 1 or type 2 deletion. F1 mice heterozygous for the type 1 deletion were bred. Generalized homozygous *Esr1* KO mice served as controls in these experiments. F1 mice heterozygous for the type 2 deletion were selected through Southern blot and PCR analysis. Heterozygote floxed *Esr1* mice (*Esr1*<sup>+/tm1.2Awo</sup>) were bred to homozygosity to generate homozygous floxed *Esr1* mice (*Esr1*<sup>tm1.2Awo/tm1.2Awo</sup>). The F2 *Esr1*<sup>tm1.2Awo/tm1.2Awo</sup> animals were backcrossed with the pure C57BL/6 strain to generate a more homogeneous background in which to perform the analyses. The floxed *Esr1* mice were genotyped using primers flanking both loxP sites (sense 5′-AGGCTTTGTCTCGCTTCC-3′ and antisense 5′-CCAAGGAGAACAGAGAGACTTACTAG-3′).

### Generation of *PitEsr1*KO Mice Using a Cell Type-Specific Cre/loxP Binary System

Heterozygote pituitary KO mice (*Tg(Cga-cre)**IAwo*, *Esr1*<sup>+/tm1.2Awo</sup>) mice were bred to *Esr1*<sup>tm1.2Awo/tm1.2Awo</sup> animals, generating pituitary *Esr1* KO (*PitEsr1*KO) mice. To confirm a KO of *Esr1* in the pituitary, PCR analysis on pituitary DNA obtained from *PitEsr1*KO mice was performed using the genotyping oligonucleotide primers that flank exon 3 and the loxP sites (as already described).

### Reagents

Most of the common chemicals used were obtained from Sigma Chemical Co. (St. Louis, MO). The LH beta (LHB) polyclonal antibody was obtained from the National Hormone and Peptide Program, National Institutes of Health (Bethesda, MD), and anti-ESR1 rabbit antibody was obtained from Millipore (Billerica, MA). Estrous cycling was performed using a Diff-Quick stain kit from IMEB Inc. (San Marcos, CA).

### Animal Care

All animal procedures were performed according to the Johns Hopkins University protocol approved by the animal care and use committee. All mice used in the experiments had mixed CD1/129SVJ genetic backgrounds.

### Fertility Testing

Six-wk-old female mice of varying genotypes (WT, *Esr1*<sup>+/+</sup>; heterozygote KO, *Tg(Cga-cre)**IAwo*, *Esr1*<sup>+/tm1.2Awo</sup>; and *PitEsr1*KO, *Tg(Cga-cre)**IAwo*, *Esr1*<sup>tm1.2Awo/tm1.2Awo</sup>) were housed with WT males for a total of 6 mo. Females were monitored daily for vaginal plugs. The date of birth and size of litters for each female were recorded. Pups were removed after delivery.

### Ovarian Function and Histology

Vaginal lavage was performed to document estrous cyclicity. Ovariectomy was performed as previously described [17], and the mice were allowed to recover for 10 days. Animals were anesthetized with ketamine/xylazine. Animals were perfused using 4% cold paraformaldehyde. The ovary and pituitary were then transferred to 10% formalin. Brains were transferred to 30% sucrose solution in 0.1 M phosphate buffer to dehydrate. On the next day, ovaries and pituitaries were washed twice with 1× PBS and transferred to 70% ethanol overnight at 4°C. After dehydration, the ovary and pituitary were processed for paraffin embedding, and 6-μm sections were cut using a microtome for hematoxylin-eosin (H&E) staining (ovary) and for immunostaining (pituitary).

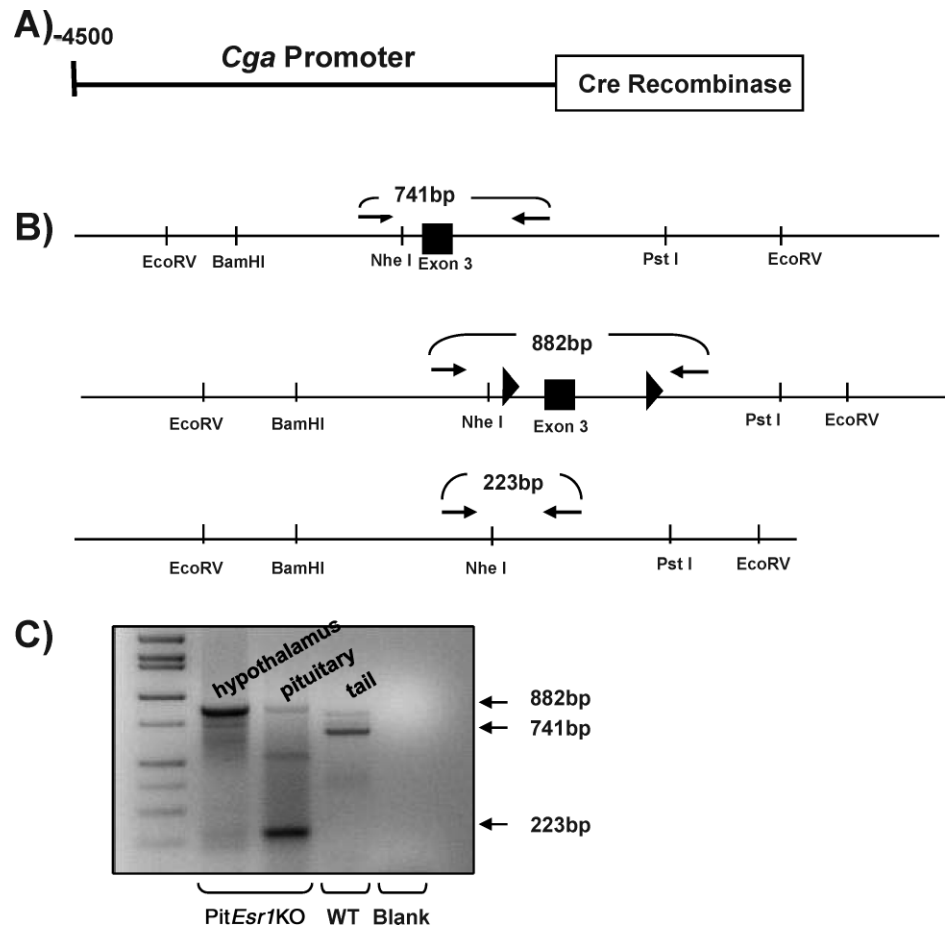
### Immunohistochemistry of the Pituitary

Six-micrometer coronal sections were deparaffinized and serially rehydrated in ethanol. After antigen retrieval, gonadotrophs were labeled using an LHB-specific antibody, and ESR1-expressing cells were labeled using an ESR1-specific antibody (rabbit antiserum C1355; Millipore), followed by Cy3 and fluorescein isothiocyanate-conjugated secondary antibodies (Jackson Laboratories, Bar Harbor, ME) [18].

### Hormone Determinations

Blood was collected from the mandibular vein either from a terminal bleed or in survival studies in mice lightly anesthetized with isoflurane. Serum was

FIG. 1. Construct used to target the *Esr1* locus. **A)** Schematic of the transgene used to produce the *Tg(Cga-cre)1Awo*. A 4500-bp murine common  $\alpha$  promoter fragment was fused to the Cre recombinase gene. **B)** The targeting construct used to generate *Esr1* floxed (*Esr1*<sup>tm1.2Awo/tm1.2Awo</sup>) mice. The loxP sites were inserted in intronic regions surrounding exon 3, and a self-excising neomycin cassette, which was used for clonal selection, was inserted just downstream of the 3' loxP site in intron 3. This was removed in the F1 generation. **C)** To display the specificity of the conditional KO, DNA was extracted from pituitary and hypothalamic tissue of *PitEsr1* KO mice. An agarose gel was run of PCR products performed with primers specifically designed to amplify the mouse *Esr1* floxed gene. A 223-bp band is shown in the pituitary of the floxed *Esr1* KO. An 882-bp floxed band is shown in the hypothalamus of the *PitEsr1* KO mouse. Genomic DNA extracted from the tail of a WT mouse was used as a positive control for the size of the WT control (741 bp). A PCR reaction containing no DNA was used as a negative control (Blank).



obtained after centrifugation for radioimmunoassay (RIA) of LH and 17- $\beta$ -E2 by the Northwestern University Radioimmunoassay Core Facility under the direction of Dr. John Levine, National Institute of Diabetes and Digestive and Kidney Diseases (Bethesda, MD) antiserum and standards (rLH-RP-3 standard/rLH-S-11 antibody) were used for LH measurements. The LH assay sensitivity was 0.2 ng/ml. An E2 double-antibody RIA kit (Diagnostic Products Corp., Los Angeles, CA) containing antibodies and standards was used for E2 RIAs. The E2 assay sensitivity was 2.0 pg/ml. Total thyroxine (T4) was measured using an ELISA kit (Alpha Diagnostic International, San Antonio, TX).

For the Luminex (Luminex Corporation, Austin, TX) system analysis, serum obtained from cycling WT and *PitEsr1* KO mice was stored at  $-20^{\circ}\text{C}$  until the assay was performed. Because of the small samples of blood obtained from the mice during the study, only single measurements were performed using 8  $\mu\text{l}$  of serum per individual time point for each animal. The LH was analyzed using xMap technology (Millipore) with the rat pituitary panel. A standard curve was generated using 5-fold serial dilutions of the LH/follicle-stimulating hormone (FSH) standard cocktail provided by the vendor. Standards and samples were incubated with the antibody-coated beads on a microplate shaker overnight at  $4^{\circ}\text{C}$  and washed three times using a vacuum manifold apparatus. Detection antibody was then added to the wells and incubated on a microplate shaker at room temperature for 30 min. Streptavidin-phycoerythrin solution was then added, and an additional 30-min incubation at room temperature was performed using the microplate shaker. Plates were then washed three times, and sheath fluid was added to each well. Beads were resuspended on the microplate shaker for 5 min. Plates were then read on the Luminex 200IS system using xPonent software (Luminex Corporation). Data were analyzed using five-parameter logistic curve fitting. The limit of detection for the LH assay was 80 pg/ml, and the intraassay coefficient of variation was between 9% and 6.3%.

### Quantitative Real-Time PCR

Total RNA was obtained from pituitary and hypothalamic tissue using Trizol (Invitrogen, San Diego, CA) extractions as previously described [19, 20]. Two micrograms of RNA was reverse transcribed (iScript cDNA synthesis kit; BioRad, Hercules, CA) to produce cDNA. The cDNA obtained from 50 ng of total RNA was used in each reaction. Twenty-five-microliter PCR reactions were performed using the IQ SybrGreen Supermix (BioRad). Reactions were

measured using the MyiQ quantitative real-time PCR machine (BioRad). The following primer sets were used: mouse *Gnrh1* (sense 5'-CCCTTTGACTTT CACATCC-3' and antisense 5'-GGGTTCTGCCATTTGATCCAC-3'), *Esr1* (sense 5'-CTTCAGTGCCAAACAGCCT-3' and antisense 5'-GACAGT CTCTCTCGGCCAT-3'), *Esr2* (sense 5'-GCCAACCTCCTGATGCTTC TTT-3' and antisense 5'-TTGTACCCTCGAAGCGTGTGA-3'), *Lhb* (sense 5'-CAGTCTGCATCACCTTCACCA-3' and antisense 5'-GGT AGGTGCACACTGGCTGA-3'), and a ribosomal 18S (*Rn18s*) control (sense 5'-TGGTTGATCTGCCAGTAG-3' and antisense 5'-CGACCAAAGGAA CCATAACT-3'). The PCR conditions were optimized to generate >95% PCR efficiency, and only those reactions between 95% and 105% efficiency were included in subsequent analyses. The cycle threshold ( $C_t$ ) was obtained for each sample. A corrected  $C_t$  (delta  $C_t$ ) was calculated by subtracting the RN18S  $C_t$  from the unknown sample  $C_t$  for each sample. Relative differences from the control sample were then calculated using the following formula: fold change =  $2^{(\text{control delta } C_t \text{ minus sample delta } C_t)}$ . The PCR products were also analyzed by gel electrophoresis.

### Statistical Analysis

All results are expressed as the mean  $\pm$  SEM. Statistical significance was assessed by one-way or two-way ANOVA using the GraphPad Prism 5 program (San Diego, CA), with  $P < 0.05$  considered significant. Post hoc analysis was conducted using Tukey multiple comparison test or Bonferroni post test.

## RESULTS

### Generation of *PitEsr1* KO Mice

*PitEsr1* KO mice were obtained by breeding *Esr1* floxed mice (*Esr1*<sup>tm1.2Awo/tm1.2Awo</sup>) to mice expressing Cre recombinase in the anterior pituitary (*Tg(Cga-cre)1Awo*) (Fig. 1). Mice were born in the expected mendelian frequency and were of normal size and weight. *Esr1*<sup>tm1.2Awo/tm1.2Awo</sup> and *Tg(Cga-cre)1Awo* animals were identified using published methods

[21]. The resulting mice were tested for the presence of a pituitary-specific KO using a PCR strategy. Figure 1C shows the results of a PCR analysis of DNA from several tissues in these animals. Note that *PitEsr1*/KO mice had a smaller band (223 bp) in pituitary DNA due to the deletion of the third exon of *Esr1* in this tissue. A weak-intensity floxed band (882 bp) was also observed in this PCR reaction, presumably representing the intact floxed allele in non-*Cga*-expressing pituitary cells. However, the smaller band was not observed in hypothalamic tissue or tail DNA from the *PitEsr1*/KO and WT mice, which contained bands consistent with the floxed and WT alleles, respectively (Fig. 1C), or in cortex, cerebellum, liver, or gonads (data not shown). These data indicate the specificity of the recombination event. Although this analysis demonstrated the tissue-specific KO, it was unable to show the extent of ESR1 in the pituitary because of the heterogeneous nature of cells in the mRNA sample.

#### Absence of ESR1 in Gonadotrophs of *PitEsr1*/KO Mice

Immunohistochemistry for LHB and ESR1 in the pituitary of control mice demonstrates colocalization of LHB and ESR1 (Fig. 2A, merged image, upper right panel). These results demonstrate that about 20% of cells could be identified as gonadotrophs, of which about 50% contain ESR1 [22]. There was an increase in the number of LH-staining cells in WT vs. *PitEsr1*/KO mice (Fig. 2A, center panels). As expected, ESR1 is absent from the gonadotrophs of *PitEsr1*/KO mice (Fig. 2A, merged image, lower right panel), while it is present in non-gonadotroph cells of the pituitary (Fig. 2A, lower left panel), thereby establishing the specificity of the assay. Examination of >30 pituitary sections failed to reveal any colocalization of LHB and ESR1 in two subfertile or two infertile *PitEsr1*/KO mice. These findings validate the deletion of *Esr1* in pituitary gonadotrophs. *Esr1* mRNA levels were measured using quantitative RT-PCR in both WT and *PitEsr1*/KO mice, as shown in Figure 2B. There was a statistically significant reduction in *Esr1* mRNA levels in the pituitary of *PitEsr1*/KO vs. control mice consistent with the immunohistochemistry results.

#### Pituitary *ESR2* Expression Was Not Affected by the Lack of *ESR1*

Because *ESR2* has also been shown to be expressed in the pituitary [22–24], *Esr2* mRNA levels were determined using quantitative RT-PCR in both WT and *PitEsr1*/KO mice (Fig. 2B). There was no difference in *Esr2* mRNA levels in the pituitary between *PitEsr1*/KO and control mice.

#### Infertility in Female *PitEsr1*/KO Mice

*PitEsr1*/KO female mice and age- and sex-matched control mice were tested for fertility with proven fertile control male mice. After 6 mo of breeding, *PitEsr1*/KO female mice revealed two distinct phenotypes, infertile and subfertile. Five of nine *PitEsr1*/KO females were infertile, and the remaining four females were subfertile (Fig. 3A). Subfertile mice had a significantly decreased litter size (5.9 pups/litter vs. 9.8 pups/litter in control animals) and an increased number of days between litters (31 days vs. 21 days in control animals) (Fig. 3B). The difference in days between litters for the subfertile *PitEsr1*/KO mice did not achieve statistical significance because of a large variability of intervals, ranging from 42 days to 21 days. Vaginal plugs were frequently detected in infertile *PitEsr1*/KO females, suggesting that the infertility was not caused by any impairment of sexual behavior in the female KO

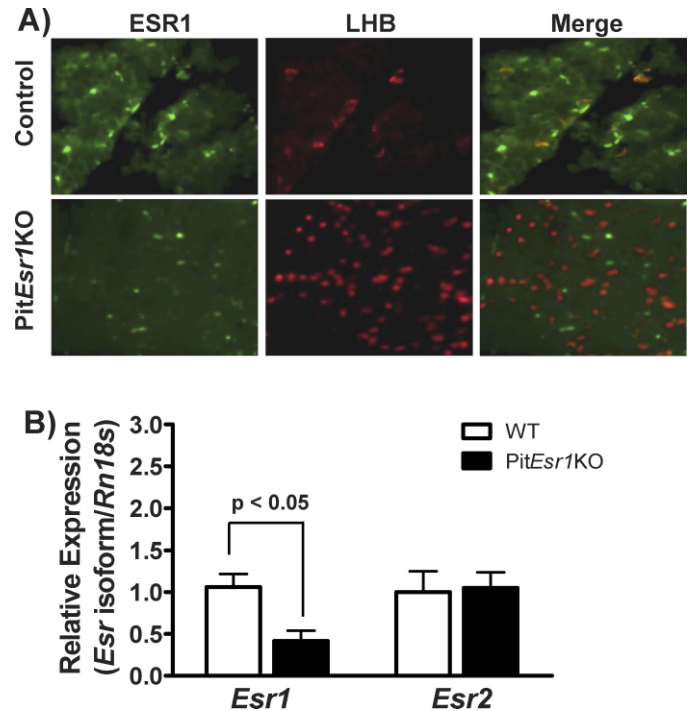


FIG. 2. ESR1 expression is abolished in LH-expressing cells in the pituitary of female *PitEsr1*/KO mice. **A**) Double immunofluorescence for ESR1 (green), LHB (red), or both (yellow) in the pituitaries of control (top row) or *PitEsr1*/KO (bottom row) mice. No LH-expressing cells were found to coexpress ESR1 in the *PitEsr1*/KO mice (bottom right panel). Original magnification  $\times 100$ . **B**) Total RNA was extracted from pituitary tissue of *PitEsr1*/KO mice. The RNA from the pituitary of WT mice was used as a control. Quantitative real-time PCR was performed using primers specifically designed to amplify mouse *Esr1* and mouse *Esr2*. Data are expressed as the mean  $\pm$  SEM (n = 6).

mice. Control mice display fertility parameters similar to those of WT mice in the colony. Male *PitEsr1*/KO mice had normal fertility as judged by test matings with known fertile females (data not shown).

#### Disrupted Ovarian Histology in *PitEsr1*/KO Mice

Hematoxylin-eosin staining of ovarian sections from WT control mice showed follicles in different stages of development and the presence of multiple corpora lutea (Fig. 4A). In contrast, ovarian sections from subfertile and infertile *PitEsr1*/KO mice had few and no corpora lutea, respectively; both displayed an increase in interstitial space compared with that in control mice. Also seen were atretic follicles and primary and secondary follicles that failed to reach to the periphery of the ovary. As shown in Figure 4B, the average number of corpora lutea was significantly less in subfertile *PitEsr1*/KO vs. control mice (four vs. 14). Among 10 ovaries that were evaluated, a single corpus luteum was found in the ovary of an infertile female *PitEsr1*/KO mouse.

#### Hormone Levels in *PitEsr1*/KO Mice

The mean nonsurge serum LH value in WT female mice with normal fertility was 0.2 ng/ml, whereas the mean values of subfertile and infertile *PitEsr1*/KO mice were significantly higher (0.4 ng/ml and 0.8 ng/ml, respectively) (Fig. 3C). These values are somewhat lower than previously reported LH values obtained in the same assay [25]. The mean E2 level at 800 h in WT female mice with normal fertility was 2.5 pg/ml, whereas



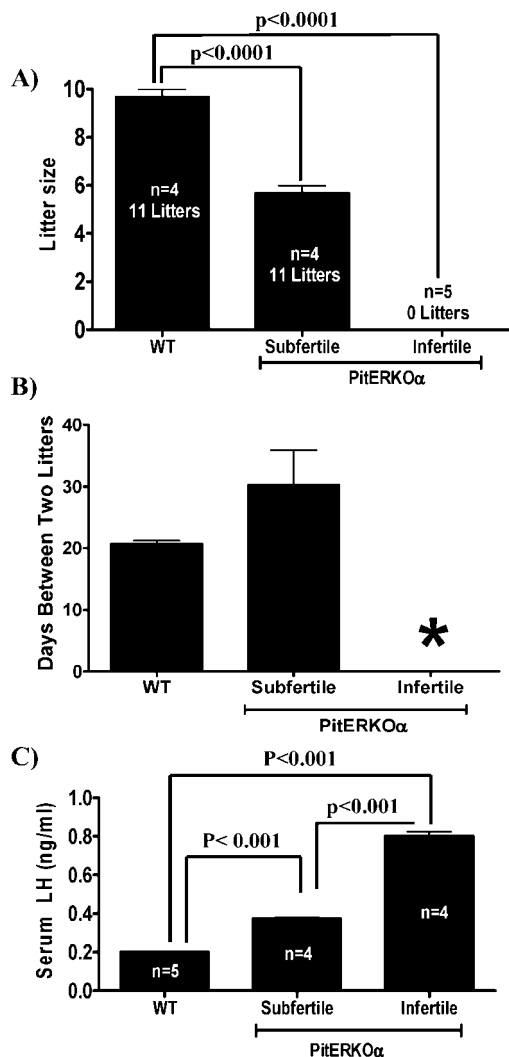


FIG. 3. *PitEsrlKO* mice exhibit impaired fertility. **A)** Litter size was assessed in WT control mice and *PitEsrlKO* mice. Data from subfertile and infertile *PitEsrlKO* are shown. **B)** Time between litters of WT control mice and *PitEsrlKO* mice. The asterisk indicates that no litters were observed in *PitEsrlKO* mice. **C)** The LH levels are elevated in *PitEsrlKO* female mice. The LH levels (in ng/ml) are indicated for WT controls and subfertile and infertile *PitEsrlKO* female mice. The number of mice analyzed for each group is indicated. Significance relative to the other groups is indicated by brackets.

subfertile and infertile *PitEsrlKO* mice demonstrated significantly elevated E2 levels (Fig. 5A). Unlike serum LH values, there was no difference in the degree of E2 elevation between subfertile and infertile *PitEsrlKO* mice. Because the *Cga* promoter should also target the pituitary thyrotroph, we determined the T4 levels in these mice. Serum T4 levels were not significantly different from those of control mice in any of the *PitEsrlKO* animals (data not shown).

#### Estrogen Feedback Regulation of *Lhb* Subunit and *Gnrh1* mRNA in *PitEsrlKO* Mice

*Lhb* and *Gnrh1* mRNA levels were measured in infertile *PitEsrlKO* mice and compared with those in control animals. Consistent with a pituitary defect in estrogen negative feedback, basal *Lhb* mRNA levels were >3-fold elevated in *PitEsrlKO* mice vs. control mice (Fig. 5C, white bars). After gonadectomy, *Lhb* subunit mRNA levels in *PitEsrlKO* mice

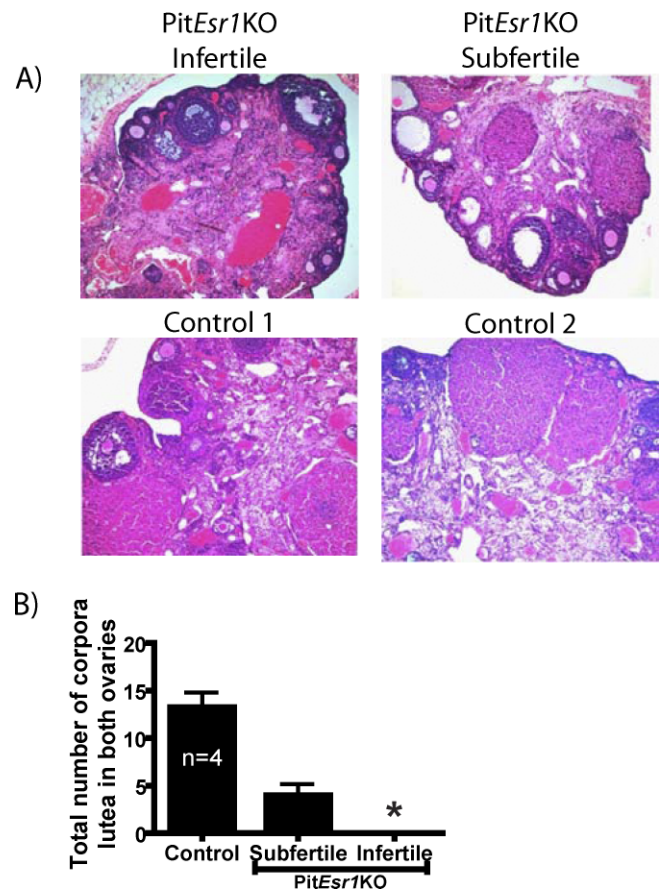


FIG. 4. Mouse ovarian morphology is disrupted in *PitEsrlKO* mice. **A)** The H&E-stained ovarian sections are shown from infertile and subfertile *PitEsrlKO* mice and from two representative WT controls. *PitEsrlKO* ovaries exhibit increased numbers of atretic follicles and cysts and reduced numbers of corpora lutea compared with those in WT ovaries. Original magnification  $\times 100$ . **B)** Counts of corpora lutea in paired ovaries of *PitEsrlKO* and WT control females. The asterisk indicates that only one corpus luteum was observed in a *PitEsrlKO* ovary.

were elevated to the same degree as that seen in the WT mice; however, the fold change was reduced because of an elevation in baseline values. In contrast, hypothalamic *Gnrh1* mRNA levels were not statistically different between *PitEsrlKO* mice and control mice (data not shown).

#### Lack of Estrous Cyclicity in *PitEsrlKO* Mice

Over 8 consecutive days, the estrous cycle stage was evaluated in the morning by vaginal cytology, and afternoon blood samples were obtained for serial serum LH measurements in a multiplex assay system. Figure 6 shows data obtained from one representative control animal and two representative *PitEsrlKO* mice. In control mice, we observed 4- to 5-day estrous cycles as assessed by vaginal cytology, and these correlated with LH surge levels (Fig. 6A). Surge levels reached 7 ng/ml in control mice, and nonsurge levels were measured at approximately 80 pg/ml, which is the limit of detectability in this assay. In *PitEsrlKO* mice, disorganized estrous cyclicity was always observed, and it was at times difficult to definitively assess the stage. Basal LH levels were higher in *PitEsrlKO* mice, averaging about 0.2 ng/ml, which is roughly 4-fold higher than those in control mice, and no definitive surge LH values were obtained. Peak LH levels in

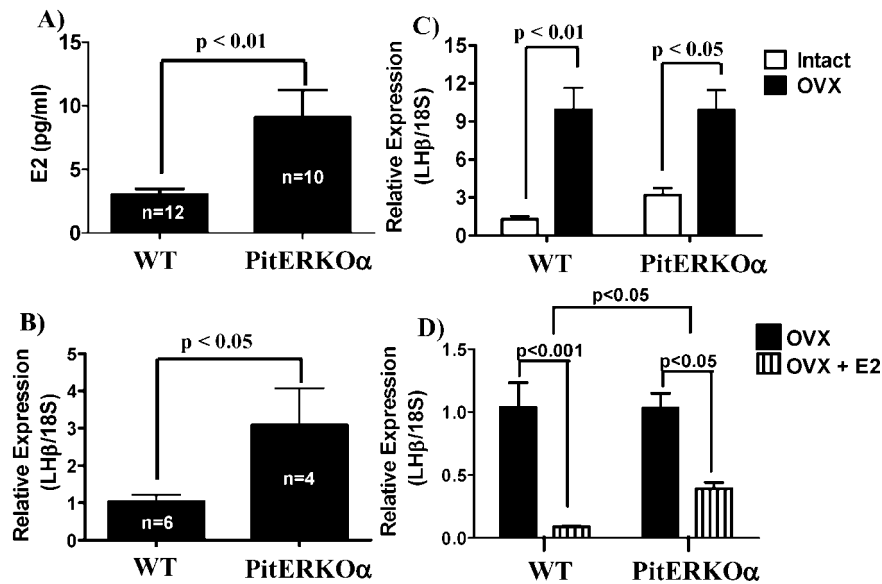


FIG. 5. Negative feedback is impaired in the *PitEsr1*KO female mice. **A)** Serum E2 levels are elevated in *PitEsr1*KO female mice compared with those in WT controls. **B)** Total RNA was extracted from pituitary tissue of WT and *PitEsr1*KO mice. Quantitative real-time PCR was performed using primers specifically designed to amplify mouse *Lhb* and corrected for *Rn18s* content. **C)** Total RNA was extracted from pituitary tissue of WT and *PitEsr1*KO mice that were ovariectomized (OVX) or sham ovariectomized (Intact). Data are expressed as the mean  $\pm$  SEM (n = 6–11). **D)** Total RNA was extracted from pituitary tissue of WT and *PitEsr1*KO mice that were ovariectomized (OVX) or ovariectomized and administered E2 (OVX+E2). Data are expressed as the mean  $\pm$  SEM (n = 6–11). Brackets above adjacent bars indicate significant differences between the two groups. The bracket at the top of **D** denotes a significant difference in the response of the two genotypes to E2.

*PitEsr1*KO mice reached approximately 0.4 ng/ml, or about 18-fold lower than those in control animals.

## DISCUSSION

Neuroendocrine control of ovarian function is mediated by the pulsatile secretion of GnRH into the hypophysial portal vasculature [26, 27]. The GnRH decapeptide binds to GnRH receptors on the plasma membrane of gonadotrophs, which then directs the synthesis and secretion of the gonadotropins LH and FSH into the peripheral circulation to control ovarian steroidogenesis and folliculogenesis. Ovarian steroid secretion in turn exerts homeostatic feedback actions that alter GnRH and gonadotropin secretions. Throughout most of the rodent estrous cycle, E2 exerts negative feedback actions on GnRH and LH secretion until (on the afternoon of proestrus) elevated follicular E2 secretions evoke an abrupt release of a preovulatory GnRH surge and hence an LH surge, which triggers ovulation [28, 29]. Thus, both negative and positive feedback actions of E2 are critically important in the physiologic control of cyclic hormone secretions and ovulatory cyclicity.

Generation and study of both *Esr1* and *Esr2* KO mouse models have made important contributions to our understanding of estrogen action in the reproductive axis [2, 4]. Both male and female *Esr1* KO mice are infertile, although the *Esr2* KO mice in conflicting studies have been reported to exhibit normal fertility in males and subfertility in females [30–36] or infertility in both sexes [5]. Findings in physiologic studies [7, 37, 38] of *Esr1* KO mouse models suggest that estrogen negative feedback occurs primarily through ESR1. From these models, it is unclear whether the level of feedback is hypothalamic, pituitary, or both, as ESR1 is absent from all tissues. Moreover, *Esr1* KO mice have a severe gonadal

phenotype that could be owing to either a direct effect of a loss of ESR1 from the ovary or an indirect effect due to an alteration of gonadotropin secretion. Hence, the role of ESR1 in estrogen feedback regulation of the pituitary gonadotroph is complicated by the potential sites of estrogen action and nature of feedback in the reproductive axis.

The Cre/loxP system in transgenic mice makes it possible to examine the effects of gene deletion in a specific cell type or tissue [39–43]. Two studies [8, 44] have used this technology to study the effects of *Esr1* deletion in the brain and pituitary. In the first study [8], *Esr1* was deleted from neurons using the *Camk2a* promoter, resulting in a defect in estrogen-mediated positive feedback, which the authors concluded was indirectly mediated by neurons that contact the GnRH neuron. Although the authors ascertained that estrogen positive feedback was located in the hypothalamus, it was not possible to determine whether ESR1 also had a role in hypothalamic negative feedback because of the design of the study. In contrast, *Esr1* was deleted from the anterior pituitary in a second study [44]. These authors determined that *PitEsr1*KO mice had impaired estrous cyclicity and infertility, suggesting that pituitary *Esr1* KO might have impaired estrogen positive feedback. Curiously, these mice had apparently normal numbers of corpora lutea and serum LH levels. Thus, whether estrogen positive feedback is located at the level of the pituitary is unclear from this study, and the locus of estrogen negative feedback remains to be defined. The complete infertility observed in that study differs from our observation of infertility and subfertility. This may be owing to several causes. Although similarly constructed, the floxed *Esr1* and *Cga*-Cre mice used in the present study were of different origin from those used by Gieske et al. [44]. In addition, fertility assessment by Gieske et al. was performed for 3 mo vs. 6 mo in the present study. It is possible that subfertility may have been observed if Gieske et al. had

TABLE 1. Summary of *Esr1* KO models and their effect on the reproductive axis.\*

Location	Serum LH	Serum E <sub>2</sub>	Ovary	E <sub>2</sub> positive feedback	E <sub>2</sub> negative feedback	Reference no.
Generalized	Very high	Very high	Absent CL	Absent	Very abnormal	[7, 26, 27]
Neuron	NR	NR	Absent CL	Absent	?	[8]
Anterior pituitary	Normal	NR	Normal CL	?	Normal	[33]
Anterior pituitary	High	High	Reduced/absent CL	Absent	Abnormal	Current study

\* NR, not reported in the basal state; CL, corpora lutea.

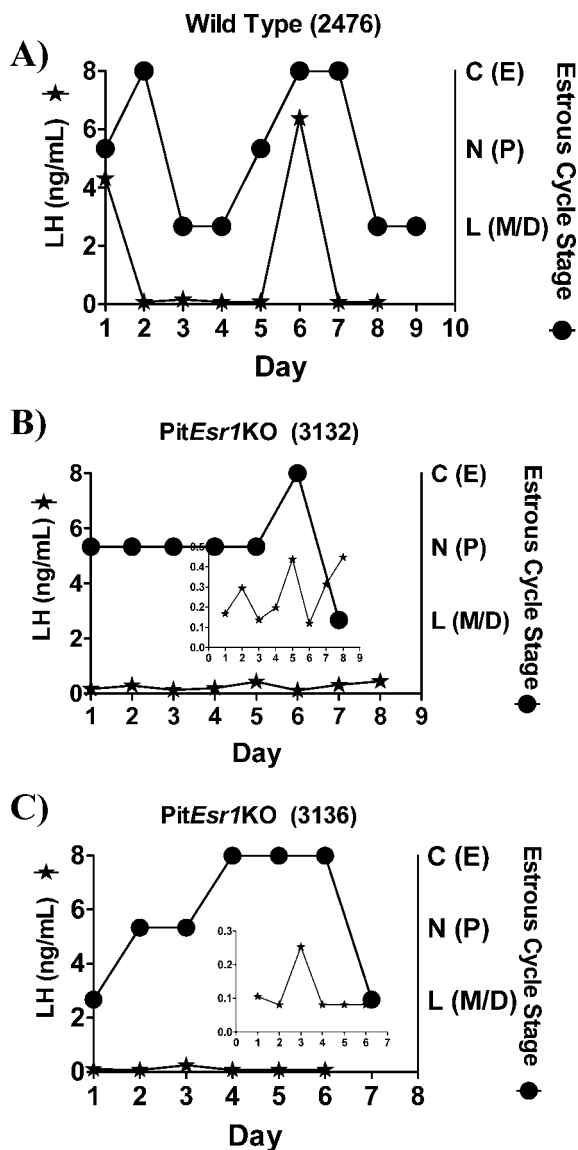


FIG. 6. Estrous cycle stage and daily LH levels from WT and *PitEsr1KO* mice. The LH levels (left axis) and estrous cycle stage as assessed by vaginal cytology (right axis) are shown in a representative WT mouse (A) and two representative *PitEsr1KO* mice (B and C). The LH levels (in ng/ml) are represented by black stars. The insets in the *PitEsr1KO* mice graphs (B and C) show LH levels (in ng/ml) in a reduced scale so that the small changes in LH can be visualized. Estrous stage is indicated by black circles as either C (primarily cornified epithelium, estrous [E]), N (primarily nucleated epithelium, proestrous [P]), or L (primarily leukocytic cells, metestrous/diestrous [M/D]).

extended their fertility assessment. Table 1 summarizes the female reproductive phenotypes of the complete *Esr1* KO, brain *Esr1* KO, and pituitary *Esr1* KO models. In addition, it emphasizes the differences observed in our study vs. the study by Gieske et al.

Given these conflicting results, we report herein on the generation and characterization of a mouse model lacking *Esr1* in the pituitary gonadotroph (*PitEsr1KO*). We developed a floxed *Esr1* mouse in which exon 3 was flanked by loxP elements. Production of a complete *Esr1* KO mouse using a beta-actin Cre mouse demonstrated a complete lack of ESR1 immunoreactivity and almost completely abolished expression of ESR1 target genes [9]. *PitEsr1KO* mice were generated by

crossing the floxed *Esr1* mouse with a mouse expressing Cre recombinase under the control of a 4.5-kb mouse common glycoprotein  $\alpha$  subunit promoter (CGA) (Fig. 1). This promoter fragment has been previously shown to confer high levels of hormonally regulated expression in the pituitary gonadotroph [10, 11]. Study of the *PitEsr1KO* mouse model revealed both subfertility and infertility in female mice (Fig. 3); male mice had normal fertility. Differences in fertility were not due to the extent of ESR1 loss in the anterior pituitary, as immunohistochemistry revealed a complete lack of ER in pituitary gonadotrophs regardless of the fertility phenotype (Fig. 2A). About 20% of the pituitary cells, either from WT or *PitEsr1KO* mice, were immunopositive for LHB, which is similar to the numbers reported in the literature for rodents [22–24, 45]. Somewhat greater numbers of LHB immunopositive cells were observed in pituitaries obtained from *PitEsr1KO* mice, although this difference was not statistically significant (data not shown). As expected based on the deletion of *Esr1* from the gonadotrophs, the amount of *Esr1* mRNA was decreased in pituitaries harvested from *PitEsr1KO* mice (Fig. 2B). It is unlikely that a compensatory increase in gonadotroph ESR2 could explain the differences in fertility, as ESR2 was found in few (<10%) gonadotrophs [22], and no difference in total pituitary *Esr2* mRNA was observed between *PitEsr1KO* and WT mice (Fig. 2B). Differences in fertility corresponded to the ovarian phenotype, in which a reduced number of corpora lutea was noted in subfertile female mice (Fig. 4), and only one corpus luteum was found in the infertile mice. A few hemorrhagic cystic follicles were also noted in infertile *PitEsr1KO* mice but not to the extent found in complete *Esr1KO* mice. These data suggest that follicular development is partially arrested in subfertile and completely arrested in infertile *PitEsr1KO* mice, in contrast to previous findings [33].

Results in our *PitEsr1KO* mouse model differ from those in the study by Gieske et al. [44] in several other aspects. First, we demonstrate the mechanism by which pituitary ESR1 loss causes infertility; we definitively show that *PitEsr1KO* mice lack LH surge activity. Using a novel experimental design, we measured daily afternoon LH values from small-sample blood volumes (using a multiplex ligand assay system) and directly correlated LH measurements with the cycle stage. Data from WT mice demonstrate clear evidence of LH surge activity, which was totally absent in the *PitEsr1KO* mice (Fig. 6, B and C). The insets in Figure 6, B and C, suggest that the GnRH neuron may still exhibit pulsatile secretion in *PitEsr1KO* mice, as indicated by differences in daily LH secretion, albeit at low levels. Thus, in contrast to previous findings [8], estrogen positive feedback is also clearly located in part at the pituitary level.

Second, we demonstrate a defect in E2 negative feedback in *PitEsr1KO* mice. Nonsurge serum LH levels were significantly elevated in both subfertile and infertile *PitEsr1KO* mice (Fig. 3C), although infertile animals had a greater elevation in serum levels. Basal serum E2 levels (Fig. 5A) and pituitary LHB mRNA levels (Fig. 5B) were also elevated consistent with the serum LH levels. However, hypothalamic *Gnrh1* mRNA levels were unchanged, despite the mild elevation in E2 levels in *PitEsr1KO* mice (data not shown). Although serum LH values were not as elevated as those found in complete *Esr1* KO mice, they were nonetheless significantly elevated (2- to 4-fold) in *PitEsr1KO* female mice compared with those in WT animals. Clearly, other pathways may regulate the central reproductive axis in response to estrogen [34, 35]. When *PitEsr1KO* mice were ovariectomized and administered estrogen, *Lhb* mRNA levels were incompletely suppressed (Fig. 5D), which is also consistent with a defect in estrogen negative feedback



regulation in *PitEsr1*/KO mice. Thus, *PitEsr1*/KO mice demonstrate a defect in estrogen negative feedback at the level of the gonadotroph that is associated with absent LH surges and impaired or absent fertility. Given that both estrogen positive and negative feedback is disrupted in the *PitEsr1*/KO mice, these feedback mechanisms cannot be dissociated in the pituitary.

Thus, the physiologic role of the endogenous ESR1 signaling pathway in pituitary gonadotrophs has been defined using *PitEsr1*/KO mice. *PitEsr1*/KO female mice are subfertile or infertile and have elevated levels of serum LH and *Lhb* gene expression, reflecting the lack of estrogen negative feedback on the gonadotroph. Although serum LH values are elevated in *PitEsr1*/KO mice, the degree of elevation is much less than that observed in *Esr1* KO mice, indicating that the hypothalamus must also have an important role in estrogen negative feedback. Estrogen levels are also modestly elevated in *PitEsr1*/KO mice, but *Gnrhl* mRNA levels in the hypothalamus were not altered. Correlation between estrous cyclicity and serum LH values demonstrates that *PitEsr1*/KO mice also have a defect in estrogen positive feedback, as surge LH values are not observed in these mice. Therefore, *PitEsr1*/KO mice demonstrate a defect in both estrogen negative and positive feedback on the reproductive axis in the setting of preserved hypothalamic estrogen signaling. As in the pituitary, estrogen feedback in the central reproductive axis may be discrete neither in location nor in the direction of its effect.

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