Estrogen receptor (ESR) 2 partially offsets the absence of ESR1 in gonadotropes of pituitary-specific *Esr1* knockout female mice

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Abstract

Estrogen receptor 1 and 2 (ESR1 and 2) mediate estrogen (E) action on gonadotrope function. While much is known about the effects of ESR1 on the gonadotrope, there is still some controversy regarding the effects of ESR2. To investigate the role of ESR2 in the gonadotrope, 45-day-old female mice of two different genotypes were used: wild type (WT) and pituitary (gonadotropes and thyrotropes)-specific *Esr1* knockout (KO). All mice were ovariectomized (OVX) and 15 days later injected over 3 days with 2.5 µg 17β-estradiol (E₂), 0.2 mg of the selective ESR1 or 2 agonists, propylpyrazole triol and diarylpropionitrile, respectively, or 0.1 ml oil. The day after treatment, anterior pituitary glands were dissected out for evaluation of gonadotrope ultrastructural morphology and pituitary immunohistochemical expression of progesterone receptor (*Pgr* (*Pr*)). Blood was collected and serum LH levels were assessed. Activation of ESR1 in WT mice resulted in the following: i) uterine ballooning and vaginal cornification, ii) negative feedback on LH secretion, iii) increased number of homogeneous (functional) gonadotropes, and iv) pituitary *Pgr* expression ($35.9 \pm 2.0\%$ of pituitary cells). Activation of ESR1 in KO mice induced normal uterine, vaginal, and LH secretion responses, but failed to increase the number of functional gonadotropes, and induced significantly lower *Pgr* expression ($21.0 \pm 3.0\%$ of pituitary cells) than in WT mice. Whilst activation of ESR2 had no significant effects in WT mice, it doubled the number of functional gonadotropes exhibited by KO mice injected with oil. It is concluded that E₂ exerted its action in KO mouse gonadotropes via ESR2.

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Introduction

Gonadotrope function in rodents and other species (Schwartz 2000) is dependent on the activation of estrogen receptors (ESRs) by ovarian-derived estrogen (E; Fink 1988). In its negative feedback mode, E maintains LH secretion at low levels during estrus, metestrus, diestrus, and proestrus morning in rats and mice (Fink 1988). This action is evident in the decreasing effect of exogenous E on serum LH levels in ovariectomized (OVX) animals. However, the relative contribution of the pituitary to the inhibitory feedback by gonadotrope ESR remains unclear (Fink 1988, Messinis 2006).

In its positive feedback mode, E acts on the hypothalamus–pituitary axis (Goodman & Knobil 1981, Parker 1995), triggering a surge of the hypothalamic decapeptide GnRH1 and greatly augmenting pituitary responsiveness to GnRH1 (Fink 1988, 2000). At the

pituitary level, the effects of E on the gonadotrope are exerted through activation of the complete gonadotrope ESR orchestra (Alonso *et al.* 2006). Rat and mouse gonadotropes express both ESR1 and 2 isoforms (Wilson *et al.* 1998, Sánchez-Criado *et al.* 2005, Gieske *et al.* 2008). The actions of ESR1 are much better known (Parker 1995) than those of ESR2, which remain elusive (Pettersson & Gustafsson 2001, Imamov *et al.* 2005, Koehler *et al.* 2005, Sugiyama *et al.* 2010). The role played by each ESR isoform in the gonadotrope is currently being studied, *in vivo* and *in vitro*, in OVX rats injected with the selective ESR1 and 2 agonists, propylpyrazole triol (PPT) and diarylpropionitrile (DPN), respectively (Sánchez-Criado *et al.* 2004, Garrido-Gracia *et al.* 2007, 2008), and in global or ESR isoform-specific knockout (KO) mice (Dupont *et al.* 2000, Hewitt & Korach 2003, Gieske *et al.* 2008, Singh *et al.* 2009, Kim *et al.* 2011).

In the rat, E withdrawal by OVX induces gonadotrope hypertrophy, disorganization of membrane-enclosed intracellular organelles, lack of expression of gonadotrope progesterone receptor (*Pgr* (*Pr*)), and elevated serum LH levels (Sánchez-Criado *et al.* 2004, 2005), and a 3-day E treatment reverses the effects of OVX resulting in proestrus-like rats (Legan & Tsai 2003). Both in rats (Sánchez-Criado *et al.* 2005) and in mice (Turgeon & Waring 1994), the absence of ligand-dependent or -independent activation of gonadotrope E-dependent PGR results in the reduction of GNRH1 self-priming and LH surge. Activation of ESR2 by DPN treatment in OVX rats has similar but milder effects to activation of ESR1 with PPT, including i) shrinkage of hypertrophied gonadotropes, ii) reorganization of disrupted intracellular organelle morphology, and iii) induction of *Pgr* expression in the gonadotrope without eliciting GNRH1 self-priming. The only exception is the lack of effect of ESR2 activation on LH secretion (Sánchez-Criado *et al.* 2004). However, main weakness of these results is that PPT and DPN are not totally selective (Sun *et al.* 1999, Stauffer *et al.* 2000, Meyers *et al.* 2001, Harris *et al.* 2002), and OVX rat gonadotropes express both ESR1 and 2 (Sánchez-Criado *et al.* 2005). Pituitary-specific *Esr* KO mice appear to be an excellent model for determining the effects of pituitary ESR1 or ESR2 activation in the complete absence of ESR2 or ESR1, respectively.

This study investigated the effects of specific ESR1 and 2 activation on gonadotrope ultrastructural morphology and pituitary immunohistochemical (IHC) *Pgr* expression in OVX wild-type (WT) and pituitary (gonadotrope and thyrotrope)-specific *Esr1* KO mice (Gieske *et al.* 2008). The effects of ESR agonists (17 β -estradiol (E_2), PPT, and DPN) on uterus and vagina and on LH release were also evaluated.

Results

Vaginal smear cytology and uterine ballooning in OVX WT and KO mice

Oil-injected female OVX mice of both WT and KO genotypes displayed absence of uterine ballooning, and vaginal smears exhibited infiltration mainly by leukocytes. Both WT and KO OVX females injected with E_2 and PPT displayed intense uterine ballooning and nucleated and/or cornified epithelial cells on vaginal smears. Finally, OVX WT and KO females injected with DPN displayed neither uterine ballooning nor nucleated or cornified cells on vaginal smears.

Pituitary *Esr2* expression in OVX WT and OVX KO mice

Gonadotropes, identified by IHC expression of *Lhb* (β LH) (reddish cytoplasm), expressed *Esr2* (orange nuclei) in both OVX WT (Fig. 1A) and KO (Fig. 1B) genotypes.

Figure 1 Co-localization of ESR2 in gonadotropes of WT (A) and KO (B) oil-injected OVX mice. Nuclear expression of *Esr2* (orange color) is seen in gonadotropes identified by cytoplasmic expression of *Lhb* (reddish color, thin black arrows in A and B). Nuclear expression of *Esr2* is also seen in pituitary cells other than gonadotropes (white arrows). Negative nuclei are also visible (thick arrows). Double consecutive ABC methods. See Materials and Methods section for technical details.

Identification, number, and size of gonadotropes in OVX WT and KO mice: effects of ER ligand treatments

Gonadotropes were identified ultrastructurally as described in Materials and Methods section (Fig. 2). Gonadotropes of OVX WT and KO mice accounted for 11.2 ± 0.3 and $10.1 \pm 0.3\%$, respectively, of the total number of pituitary cells; the mean diameter of gonadotropes in OVX WT and KO mice was 11.0 ± 0.9 and $10.1 \pm 0.5 \mu\text{m}$, respectively. No differences in the number and size of gonadotropes were found between OVX WT and KO mice or among the different ESR ligand treatments (results not shown).

Ultrastructural features of gonadotropes in OVX WT and OVX KO mice: effects of ESR ligands

Ultrastructurally, two different types of gonadotropes were distinguished in OVX WT and KO mice:

Figure 2 Anterior pituitary cell types identified by key ultrastructural features. A gonadotrope (LH/FSH) with rounded, eccentric nuclei and homogeneous, moderately electron-dense cytoplasm containing scarce, rounded secretory granules of slightly varying size is visible close to a blood capillary (asterisk). Lactotropes (PRL) had rounded to elongated nuclei and dark cytoplasm; secretory granules varied considerably in size and shape. The single corticotrope (ACTH) observed displayed the lowest nuclear and cytoplasmic electron density. Somatotropes (GH), the most numerous cells, had large, rounded secretory granules of fairly homogeneous size, located peripherally in the cytoplasm. Finally, the thyrotrope (TSH) had rounded nuclei with moderately electron-dense cytoplasm and scarce secretory granules of homogeneous size. See Materials and Methods section for additional details. Pituitary section from an OVX WT mouse injected with oil.

homogeneous (H) and vesiculated/degenerated (V/D) (Fig. 3). H gonadotropes (Fig. 3A) had rounded, eccentric euchromatic nuclei with one or two nucleoli. The moderately electron-dense cytoplasm was homogeneous and contained abundant, slightly dilated RER cisternae with low density, uniform content, well-developed Golgi complex(es), and abundant dense matrix mitochondria. Scarce rounded, electron-dense secretion granules varying in size from 200 to 400 nm were observed in the proximity of the cytoplasmic membrane, either singly or in small clusters. The most striking feature of V/D gonadotropes (Fig. 3B and C) was the disorganization of membrane-enclosed intracellular organelles, including regular or irregular dilatation of empty RER cisternae, giving the cytoplasm a vesiculated or vacuolated appearance; Golgi complex and secretory granules were either small or absent. Varying number of

mitochondria was observed. Some V/D gonadotropes (Fig. 3C) displayed irregular nuclei with highly condensed chromatin and a dilated envelope.

Although in different proportions, all groups of OVX WT and KO mice exhibited both types of gonadotropes

Figure 3 Representative micrographs of gonadotrope subtypes: homogeneous (A), vesiculated (B), and degenerated/vacuolated (C) found in OVX WT and KO mice injected with oil, E₂, PPT, or DPN. See Fig. 2 for detailed description of gonadotropes.

Figure 4 Percentage of homogeneous gonadotropes in OVX WT and KO mice injected with oil, E₂, PPT, or DPN (see the legend of Fig. 2 and Materials and Methods section for morphological and treatment details). ^a $P < 0.05$ vs corresponding OVX WT treatment group; ^b $P < 0.05$ vs corresponding genotype oil-injected mice. ANOVA followed by Student–Newman–Keuls multiple range test.

(Fig. 4). OVX WT mice injected with oil vehicle exhibited $40.6 \pm 0.7\%$ of H gonadotropes compared with $25.9 \pm 0.6\%$ in OVX KO mice ($P < 0.05$; Fig. 4). Differences were also found in the effects of ESR ligands. Treatment of OVX WT mice with E₂ and PPT increased ($P < 0.05$) the percentage of H gonadotropes to 54.0 ± 0.9 and $56.8 \pm 1.2\%$, respectively, and, accordingly, decreased that of V/D gonadotropes. DPN treatment also prompted a less marked increase ($P < 0.05$) in the percentage of H gonadotropes to $47.0 \pm 0.8\%$. In OVX KO mice, E₂ and DPN had similar effects on gonadotrope morphology; the percentage of H gonadotropes increased significantly to 42.0 ± 0.7 and $43.0 \pm 0.8\%$, respectively ($P < 0.05$). By contrast, PPT exerted no effect compared with oil treatment in OVX KO mice.

Pituitary *Pgr* expression in OVX WT and KO mice: effects of ESR ligand treatments

Pituitaries from OVX mice of both genotypes injected with oil vehicle did not express PGR (Figs 5A and 6). Treatment with E₂ or the selective ESR1 agonist PPT (Figs 5B, C and 6), but not the selective ESR2 agonist DPN, induced *Pgr* expression in both OVX genotypes (Fig. 6). However, the percentages of PGR-positive cells in the pituitaries of OVX WT mice treated with E₂ and PPT were 38.4 ± 2.6 and $33.3 \pm 2.2\%$, respectively, while

Figure 5 PGR immunohistochemistry of the pituitary of OVX WT and KO mice injected with oil, E₂, PPT, or DPN (see the Materials and Methods section for details of treatments). (A) Representative examples of the lack of *Pgr* expression in WT+oil, WT+DPN, KO+oil, or KO+DPN OVX mice. (B) *Pgr* expression in the pituitaries of WT+PPT or +E₂ OVX mice. (C) *Pgr* expression in the pituitaries of KO+PPT or +E₂ OVX mice.

Figure 6 Percentage of positive PGR pituitary cells in OVX WT and KO mice injected with oil, E₂, PPT, or DPN (see Materials and Methods section for details of treatments). ^a $P < 0.05$ vs corresponding OVX WT treatment groups; ^b $P < 0.05$ vs corresponding genotype oil-injected mice. ANOVA followed by Student–Newman–Keuls multiple range test.

percentages were significantly lower (23.0 ± 4.9 and $18.9 \pm 2.5\%$, respectively) in OVX KO mice (Fig. 6).

Effects of ESR ligands on serum LH concentrations in OVX WT and KO mice

In comparison with oil-injected OVX WT and KO mice, treatment with DPN had no effect on serum LH concentrations in OVX mice of either OVX genotype (Fig. 7). By contrast, treatment with E₂ and PPT significantly decreased serum LH concentrations in both genotypes. No differences in serum LH levels were found between WT and KO OVX mice (Fig. 7) for any of the treatments.

Discussion

The results indicated that selective activation of ESR2 in OVX KO mice increased the number of homogeneous gonadotropes of normal appearance, but had no effect

on *Pgr* expression, LH secretion, or peripheral target E-dependent tissues. In addition, the results indicated similar *Esr2* expression in gonadotropes of both OVX KO and WT mice. Other authors report that only gonadotropes and thyrotropes from pituitaries of OVX KO mice lack *Esr1* (Gieske *et al.* 2008, Singh *et al.* 2009). Moreover, the selective lack of *Esr1* expression in gonadotropes was confirmed in the present study by: i) the lack of effect of PPT on the number of homogeneous gonadotropes in OVX KO mice, ii) the similar response, in both mouse genotypes, of uterus and vagina to E₂ and PPT but not to DPN treatment (present results, Harris *et al.* 2002, Frasier *et al.* 2003, Hewitt & Korach 2003), iii) the negative feedback of ESR1 activation on LH secretion in both OVX WT and KO mice, and iv) significantly ($P < 0.05$) lower expression of *Pgr* in OVX KO than in OVX WT mice. Taken in conjunction, these

Figure 7 Serum LH concentrations (ng/ml) in OVX WT and KO mice injected with oil, E₂, PPT, or DPN (see Materials and Methods section for details of treatment). No differences were found between LH levels in OVX WT and KO mice. ^a $P < 0.05$ vs corresponding genotype oil-injected mice; ^b $P < 0.05$ vs DPN-injected OVX KO mice. ANOVA followed by the Student–Newman–Keuls multiple range test.

findings confirmed, though only in part, the adequacy of the model used to study the effects of specific activation of ESR2 on mouse gonadotropes. Also, in contrast with our original hypothesis, results indicated the existence of striking pituitary differences between rats and mice.

The present findings contrast with those obtained by the authors in rats, in which ESR2 replaced most ESR1 action including shrinkage of OVX-induced hypertrophy of gonadotropes, reorganization of membrane-enclosed intracellular organelles, and gonadotrope *Pgr* expression (Sánchez-Criado *et al.* 2006). Gonadotrope size in OVX KO mice was similar to that of OVX WT mice both in controls and in ESR ligand-treated animals. In the rat, a time-dependent hypertrophy takes place after OVX even to the extent of forming signet-ring cells from 35 days onward (Garner & Blake 1981), reversible after ESR1 activation (Gordon *et al.* 2009). No signet-ring gonadotropes were observed at the time of the study in the pituitaries of either OVX WT or KO mice lacking ESR1 activity. In addition, E₂ and PPT treatment of OVX WT mice did not reduce the size of gonadotropes, as occurs in the rat (Sánchez-Criado *et al.* 2006). These findings suggest that OVX-induced E withdrawal was not followed, under the current conditions, by gonadotrope hypertrophy.

Differences in gonadotrope morphology were observed between OVX KO and WT mice; the former had significantly fewer H gonadotropes than the latter. These are considered to be the functional gonadotropes found in proestrus (Garner & Blake 1981, Sánchez-Criado *et al.* 2005). Moreover, activation of ESR2 with either E₂ or DPN increased the number of H gonadotropes in OVX WT and KO mice while activation of ESR1 with PPT increased the number of H gonadotropes only in OVX WT mice exclusively. The finding that pituitaries of OVX KO mice still exhibited H gonadotropes, together with the absence of gonadotrope hypertrophy, may suggest that endogenous E played a role in OVX KO mice gonadotrope biology through ESR2 activation by inducing the reorganization of membrane-enclosed intracellular organelles, mostly RER cisternae and Golgi complexes, thus increasing the pool of homogeneous gonadotropes at the expense of vesiculated/degenerated gonadotropes. This gonadotrope recruitment would then be capable of response to GNRH1 stimulation under the E-positive feedback mode (Smith *et al.* 1984). In the rat, OVX-induced disorganization of membrane-enclosed intracellular organelles is also reversed by DPN treatment in the absence of ESR1 activation (Sánchez-Criado *et al.* 2006, Garrido-Gracia *et al.* 2008). However, although these effects on gonadotrope morphology were also seen in OVX WT and KO mice, the absence of effect of OVX on gonadotrope size at the time of the study suggests that gonadotrope morphology in the mouse is not under such a strong estrogenic control as it is in the rat.

The present results also showed that pituitary cells did not express PGR in the absence of ESR1 activation (OVX WT and KO mice whether injected with oil or DPN). This confirms the strong ESR1 dependency of *Pgr* expression (Fox *et al.* 1990, Szabo *et al.* 2000). Again, the situation is dissimilar to that of the OVX rat because ESR2 activation does induce *Pgr* expression (Sánchez-Criado *et al.* 2004, 2006). In OVX WT mice, activation of ESR1 by E₂ induced *Pgr* expression in $38.4 \pm 2.6\%$ of pituitary cells, while the average proportion of pituitary PGR-positive cells in the rat is about 10% (Sánchez-Criado *et al.* 2005, 2006, Garrido-Gracia *et al.* 2008). This difference is most likely due to the fact that in mice both gonadotropes and lactotropes express PGR (Turgeon *et al.* 2001). Interestingly, a decrease in the proportion of PGR-positive pituitary cells by about 15% was recorded when comparing activation of ESR1 by E₂ in OVX WT ($38.4 \pm 2.6\%$) and KO ($23.0 \pm 4.9\%$) mice (present results), suggesting that activation of ESR1 in lactotropes (Turgeon *et al.* 2001) induces *Pgr* expression in OVX KO mice. Though possible, it is unlikely that *Pgr* is expressed in some gonadotropes in the absence of *Esr1* expression in KO mice as a result of indirect activation. E-dependent gonadotrope *Pgr* expression is an absolute requisite for GNRH1 self-priming in the rat (Turgeon & Waring 1994, Sánchez-Criado *et al.* 2006) and therefore for a full preovulatory LH surge (Fink 1988). Mouse gonadotropes also display GNRH1 self-priming and P-potential of GNRH1 secretion (Turgeon & Waring 1994). PGR is necessary for E-induced signals leading to the LH surge in mice (Chappell *et al.* 1999). Whereas activation of ESR2 with DPN induced *Pgr* expression but not GNRH1 self-priming in the OVX rat (Sánchez-Criado *et al.* 2004), DPN had no effect on *Pgr* expression in OVX mice (present results). However, *Pgr* KO mice still exhibit GNRH1 self-priming (Turgeon & Waring 2001) indicating that, in the mouse, PGR is not strictly necessary for amplifying the GNRH1 signal. It has been demonstrated that GNRH1 self-priming and *Pgr* expression have been shown to be dissociated phenomena (Sánchez-Criado *et al.* 2004, 2005).

As observed in rats (Sánchez-Criado *et al.* 2004), administration of E₂ and PPT, but not DPN, reduced serum LH levels in OVX WT and KO mice, showing that ESR1, but not ESR2, mediates E-negative feedback on LH secretion in mice (Dorling *et al.* 2003, present results). This suggests that the pituitary can be considered as a passive gland, depending on GNRH1 for LH secretion. Furthermore, the inhibitory effects of E on both hypothalamic *Gnrh1* mRNA levels and LH secretion are reportedly absent in global *Esr1* KO mice (Wersinger *et al.* 1999, Dorling *et al.* 2003). Since GNRH1-secreting neurons do not express *Esr1* (Shivers *et al.* 1983), the question of the supra-pituitary level at which E₂ and PPT activate ESR1 arises. It seems reasonable to assume that ESR-mediated critical negative feedback is not exerted on GNRH1 neurons directly but rather transynaptically

through modulation of interneuron afferents from several hypothalamic E-sensitive presynaptic neurons expressing *Esr1* both in rats (Levine 2000, Messinis 2006, Wintermantel *et al.* 2006) and in mice (Yaghmaie *et al.* 2010).

In summary, from the parameters so far studied in the present experiments, the only role played by ESR2 on the reproductive axis of *Esr1* pituitary (gonadotrope and thyrotrope)-specific KO mice was to revert the deleterious effects of the absence of ESR1 activity (from conception to sacrifice) on mouse gonadotrope morphology. Moreover, the absence of effects of OVX on gonadotrope size, and of ESR2 activation on *Pgr* expression at the time of the study, points to a mild E control of gonadotrope biology in the mouse. These findings confirm earlier data indicating differences in the gonadotrope biology between rats and mice (Abbot *et al.* 1986, Turgeon *et al.* 1999, 2001, Turgeon & Waring 2001, 2006) and suggest that these two rodent species have adopted different reproductive strategies, which are further evidenced in the different response to pheromonal stimuli (Sánchez-Criado *et al.* 1989).

Materials and Methods

Animals and general conditions

All mice used in this study were of C57BL/6 genetic background. Forty-five-day-old *Esr1^{fl/fl}* with WT phenotype and pituitary-specific α GSU-Cre:*Esr1^{fl/fl}* mice, referred to here as KO mice, were used (Gieske *et al.* 2008). KO mice were generated following a previously published strategy (Dupont *et al.* 2000, Gieske *et al.* 2008). Sibling WT mice were used as controls. KO mice thus lacked *Esr1* only in gonadotropes and thyrotropes (Gieske *et al.* 2008). Animal handling procedures were followed in accordance with the University of Kentucky Animal Care and Use Committee.

Surgery and treatments

WT ($n=13$) and KO mice ($n=13$) were bilaterally OVX under isoflurane inhalation at a mean 45 days of age. Fifteen days post-OVX, mice ($n=3$ /group) were injected (s.c.) over three consecutive days with either 2.5 μ g E_2 (Sigma), 0.2 mg of the selective ESR2 agonist DPN (Tocris, Ellisville, MO, USA; Meyers *et al.* 2001), or 0.2 mg of the selective ESR1 agonist PPT (Tocris; Stauffer *et al.* 2000). Controls ($n=4$) were administered 0.1 ml olive oil by s.c. injection. The day after the third injection, animals were killed and right atrial blood was collected by cardiac puncture to determine serum LH concentrations. The anterior pituitary was dissected out and divided into two halves. One half was used for ultrastructural evaluation of gonadotrope morphology and the other for evaluation of *Esr2*, *Lhb*, and *Pgr* expression using IHC. The presence of uterine ballooning and vaginal smear cytology was also recorded.

Preparation of pituitaries for electron microscopy

One half of each dissected pituitary was further cut into two pieces, and tissues were fixed for 2 h at 4 °C in 2.5% glutaraldehyde (Ladd Research, Williston, VT, USA) in 0.1 M cacodylate (EMS, Hatfield, PA, USA) buffer prepared on the day of use. The tissue was washed four times in 5% buffered sucrose in 0.1 M cacodylate for 5 min each wash and then left in this solution for 3 days, at 4 °C. Following incubation, tissue was infiltrated with 1% osmium (Ladd Research) in 0.1 M cacodylate buffer for 1.5 h at 4 °C and then washed twice with 5% buffered sucrose in 0.1 M cacodylate for 5 min each. Tissues were then dehydrated in a graded series of alcohols (50–70–80–90–100%-absolute ethanol) for 5 min per solution. Samples were treated with propylene oxide twice for 15 min each and then infiltrated with 50% propylene oxide/50% eponate resin (47% LX-112, 30% DDSA, 20% NMA, and 0.013% DMP (Ladd Research)) overnight under a 60-W bulb. Vials were left uncovered to allow propylene oxide to evaporate. The following day, samples were washed twice in 100% eponate resin for 1 h each time. Samples were embedded in BEEM capsules in 100% eponate resin and heated at 60 °C in an enclosed oven for 2 days. Thick sections and thin sections were prepared by the University of Kentucky Electron Microscopy and Imaging Centre. Tissue was thick sectioned (1–2 μ m) and stained with toluidine blue for examination under light microscopy. Selected areas were thin sectioned on a Reichert Ultracut E microtome at 60–80 nm, mounted on copper mesh grids, and stained with uranyl acetate and lead citrate.

Preparation of pituitaries for IHC

The second half of each pituitary was placed in 10% buffered formalin for 1.5 h. Tissues were then dehydrated in alcohol (70%, 80%, 90%, 95%, 100%) and ethanol/xylene series (ethanol 100%, 50%:50%, 30%:70%, 100% xylene x 2). Each wash lasted 20 min. Tissues were subjected to soft paraffin infiltration for 30 min at 60 °C, followed by two washes in hard paraffin for 30 min each, and then placed in paraffin blocks and allowed to cool on ice before cassettes were prepared for sectioning.

Ultrastructural and morphometric examination

Gonadotropes were identified and differentiated from other anterior pituitary cell types by the ultrastructural characteristics of the nucleus (rounded and eccentric), cytoplasm (homogeneous and moderately electron dense), secretory granules (scarce, rounded, and with varying slightly in size), and by their pituitary localization (often close to blood capillaries) (Tougard & Tixier-Vidal 1988, Gartner & Hiatt 2007, Garrido-Gracia *et al.* 2008). The other pituitary cell types were easily recognized by their ultrastructural characteristics in i) lactotropes (rounded to elongated nuclei and dark cytoplasm with secretory granules of highly heterogeneous size and shape), ii) corticotropes (displaying the lowest nuclear and cytoplasm electron density), iii) somatotropes (the most abundant cells, with large, rounded secretory granules of fairly homogeneous

size located peripherally in the cytoplasm), and iv) thyrotropes (rounded nuclei with moderately electron dense cytoplasm and scarce secretory granules of variable uniform size but varying in distribution). The percentage of gonadotropes was evaluated under electron microscopy in two grids/mouse. Within each grid, 10 squares $\times 100 \mu\text{m}$ each were analyzed ($200\,000 \mu\text{m}^2$ in total). Gonadotrope size was evaluated on the same surface area of $200\,000 \mu\text{m}^2$ /mouse. Gonadotrope nuclei were delineated with a digital pen to obtain first the perimeter and later the mean diameter. Around five to seven gonadotropes were counted and measured per grid square. This examination was carried out independently by two evaluators and differences were solved by consensus after joint reassessment.

IHC of pituitary PGR

The commercial mouse monoclonal anti-human PGR receptor clone PR10A9 (Immunotech, Marseille, France), diluted 1:15 000, and the avidin–biotin–peroxidase complex (ABC) IHC technique were used as described previously (Sánchez-Criado *et al.* 2004). Three consecutive tissue sections per case were immunostained. Images were captured ($\times 40$ microscope lens) from four randomly selected neighboring, non-overlapping fields of each immunostained tissue section. The number of PGR-positive and -negative cells was counted with a digital pen tablet (Volito2; Wacom Europe GmbH, Krefeld, Germany). The percentage of PGR-positive cells was calculated using Image-Pro Plus 4.5. A minimum of 1000 anterior pituitary cells were counted per tissue section (3000 per case).

Co-localization of ESR2 and Lhb in pituitary cells

For the simultaneous IHC co-localization of ESR2 and LHB in a single tissue section, individual immunostainings with each antibody were performed consecutively. Polyclonal rabbit anti-LHB (AbDSerotec, Dusseldorf, Germany), diluted 1:200, was incubated for 18 h at 4°C after three 10 min washes in PBS; the ABC method (Vector Laboratories, Burlingame, CA, USA) was applied following manufacturer's recommendations and the immune reaction was developed with the chromogen DAB. Next, tissue sections were subjected to high-temperature antigen retrieval by incubation with 0.01 M citrate buffer, pH 6.0, at 95°C for 3 min in a water bath for ESR2 detection. After cooling down at room temperature for 20 min, endogenous peroxidase activity was blocked with 3% hydrogen peroxidase in methanol for 30 min at room temperature. Polyclonal rabbit anti-human ESR2 antibody (Upstate Biotechnology-Millipore, Billerica, MA, USA), diluted 1:40, was incubated for 18 h at 4°C . After three 10 min washes in PBS, the ABC method was applied following manufacturer's recommendations. This immune reaction was developed with the chromogen Novared (Vector Laboratories). Nuclei were counterstained with Harris hematoxylin.

Measurement of serum LH concentrations

IRMA of serum LH levels was performed by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis core (NICHD (SCCPRR) Grant U54-HD28934,

University of Virginia, VA, USA). All samples were tested in the same assay. The intra-assay coefficient of variation was 3.1% and assay sensitivity was 0.07 ng/ml. Serum LH concentrations were expressed as nanogram per milliliter.

Statistical analysis

All data had normal distribution and homogeneous variance. In accordance, statistical analysis was performed by two-way ANOVA to test for significant differences among groups (Hardyck & Petrinovich 1976). Where significant differences existed, ANOVA was followed by the Student–Newman–Keuls multiple range test. Significance was considered at the 0.05 level.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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