

The Expression of Aromatase in Gonadotropes Is Regulated by Estradiol and Gonadotropin-Releasing Hormone in a Manner that Differs from the Regulation of Luteinizing Hormone

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The role of estrogens is dual: they suppress basal expression of gonadotropins and enhance GnRH responsiveness at the time of the LH surge. Estrogens are synthesized by cytochrome P450 aromatase (P450arom), encoded by the *cyp19* gene. We focused on the *cyp19* gene in rat and showed that it is expressed in gonadotropes through promoters PII and PI.f, using RT-PCR and dual fluorescence labeling with anti-P450arom and -LH antibodies. Real-time PCR quantification revealed that aromatase mRNA levels varied during the estrous cycle and were significantly increased after ovariectomy. This effect is prevented by estradiol (E2) as well as GnRH antagonist administration, suggesting that GnRH may mediate the steroid effect. Interestingly, the long-acting GnRH agonist that induces LH desensitization does not modify aromatase expression in ovariectomized rats. Administration of E2 in ovariectomized rats receiving either GnRH ag-

onist or GnRH antagonist clearly demonstrated that E2 also reduces *cyp19* expression at the pituitary level. The selective estrogen receptor- α ligand propyl pyrazole triol and the selective estrogen receptor- β ligand diarylpropionitrile both mimic the E2 effects. By contrast, propyl pyrazole triol reduces LH β expression whereas diarylpropionitrile does not. In addition, using transient transfection assays in an L β T2 gonadotrope cell line, we provided evidence that GnRH agonist stimulated, in a dose-dependant manner, *cyp19* promoters PII and PI.f and that E2 decreased the GnRH stimulation. In conclusion, our data demonstrate that GnRH is an important signal in the regulation of *cyp19* in gonadotrope cells. Both common and specific intracellular factors were responsible for dissociated variations of LH β and *cyp19* expression. (*Endocrinology* 147: 4234–4244, 2006)

THE REGULATION OF the anterior pituitary is achieved by the cell-specific and combined actions of central, peripheral, and local factors. It is established that estrogens are involved in the modulation of the hypothalamic-pituitary-gonadal axis. However, it is difficult to delineate the overall contribution of each site of action, particularly at the level of the pituitary gland. The biosynthesis of estrogens from androgens is catalyzed by the aromatase complex, which consists of cytochrome P450 aromatase (P450arom) and a flavoprotein, reduced nicotinamide adenine dinucleotide phosphate-cytochrome P450 reductase (1). The P450arom is encoded by a single copy gene, *cyp19*, composed of nine coding exons (exons II–X). The *cyp19* gene expression is regulated by multiple tissue-specific promoters producing alternate 5'-untranslated exons I that are then spliced onto a common 3'-splice acceptor site in the exon II upstream of the translation start site (2). However, all transcripts contain an

identical open reading frame and encode the same protein regardless of the promoter used (2).

Estradiol (E2) is considered as the critical determinant of plasma gonadotropin levels in females by completing an endocrine feedback loop on the hypothalamus, affecting the pattern of GnRH release, and on the pituitary gland, suppressing the GnRH-stimulated LH secretion (3–5). Besides endocrine action, E2 is also reported to act in an auto- or paracrine manner to influence certain functions. In rodent brains, previous studies provide evidence of local estrogen production in the thalamus and hypothalamus of mouse (6) and in the hypothalamus and amygdala of adult rats (7). In the ovary, estrogens enhance FSH action on granulosa cells (8), inhibit androgen synthesis in thecal cells (9), and attenuate apoptosis (10). Finally, previous studies using *in vitro* and *in vivo* models have demonstrated that *in situ* aromatase is involved in local estrogen production in both bone (11) and breast cancer (12–14).

Several studies suggest a role of aromatase in the pituitary. Both E2 and testosterone significantly suppressed LH β mRNA and serum LH levels in wild-type castrates, whereas they had no significant effect on castrated estrogen receptor- α knockout (α ERKO) mice, suggesting that androgens must be metabolized to be effective (3). When the action of endogenous E2 was suppressed by an aromatase inhibitor in early and midpubertal boys, the LH amplitude and the

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Abbreviations: CREB, cAMP response element-binding protein; DPN, diarylpropionitrile; E2, estradiol; ER, estrogen receptor; α ERKO, ER α knockout; GnRH-R, GnRH receptor; OVX, ovariectomized; P450arom, cytochrome P450 aromatase; pCREB, phospho-CREB; PPT, propyl pyrazole triol; SF-1, steroidogenic factor-1.

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GnRH-induced LH response increased, whereas LH pulse frequency did not change (15). Taken together, these data suggest that the normal regulation of the gonadotrope function requires the local aromatization of testosterone.

The intriguing presence of aromatase immunostaining in the pituitary (16) led us to question the exact location of its secretion and the mechanism of action of gonadal steroids. We report the results of *in vivo* and *in vitro* experiments designed to localize and study the expression of aromatase in the female rat pituitary. First, we characterized the aromatase transcripts expressed in the pituitary and carried out an immunolocalization of the P450arom protein. Second, we examined the total aromatase mRNA expression during the estrous cycle and assessed the effects of ovariectomy and steroid supplementation. We were also interested in establishing whether GnRH and E2 may alter *cyp19* gene expression *in vivo* in female rats and *in vitro* using reporter gene assays.

Materials and Methods

Animals

Mature female Sprague-Dawley rats were purchased from University of Caen, France. Animals were maintained under standard laboratory conditions (12-h light, 12-h dark cycle) and housed individually with water available *ad libitum*. Body weights were monitored before and during the experiments on a weekly basis and were maintained equal between groups by control of food intake. All procedures were in accordance with the instructions of the Ministère de l'Agriculture et de la Pêche-Service Santé Animale (France).

Ovariectomy and vaginal smears

Ovaries were removed from female rats under anesthesia by injection of a ketamine-midazolam cocktail (ip, 100 mg/kg and 4 mg/kg, respectively) at random stages of the estrous cycle. All animals were housed individually after surgery. The estrous cycle and the estrogenic treatment were checked daily by examining vaginal smears between 0900 and 1000 h and measuring serum E2 (Table 1).

Experimental design

Experiment 1. The expression of the *cyp19* gene in the female rat pituitary was studied across the estrous cycle. Rats were distributed into three

categories according to the phase of the estrous cycle, as determined by vaginal smears ($n = 6$ per group): metestrus, proestrus, and estrus. Only animals exhibiting two consecutive normal estrous cycles were included.

Experiment 2. The purpose of this experiment was to examine the effects of castration and E2 supplementation on pituitary aromatase mRNA. The treatment paradigm we used enabled measures to be taken at a time when the negative feedback of estrogens was operational. Ovariectomized (OVX) rats were injected (sc) every 2 d for 3 wk with 10 μ g 17 β -estradiol (Sigma, L'Isle d'Abeau, France). Rats ($n = 7$ per group) were divided into two groups, OVX and OVX plus E2 supplementation, and were killed 3 wk later.

Experiment 3. The purpose of this experiment was to determine the isoform of estrogen receptor (ER) used by estrogens to regulate the expression of the *cyp19* gene. A selective ER α agonist, propyl pyrazole triol (PPT), and an ER β agonist, diarylpropionitrile (DPN) were obtained from Tocris Cookson Ltd. (Avonmouth, UK). OVX rats ($n = 5$ per group) were randomly assigned to three groups: PPT, DPN, or PPT plus DPN. Three weeks after ovariectomy, rats were injected daily (sc) for 3 d with 1 mg PPT, 1 mg DPN, or 1 mg PPT plus DPN, as adapted from previous studies (17, 18).

Experiment 4. The purpose of this experiment was to determine the effect of E2 at the pituitary or hypothalamic level using either a GnRH antagonist (Cetrorelix acetate, kindly provided by Serono, Boulogne, France) or a GnRH agonist (D-Trp⁶-GnRH, Triptorelin 3 mg, kindly provided by IPSEN Pharma Biotech, Signes, France). Animals ($n = 7$ per group) were randomly assigned to three groups: intact, OVX, and OVX plus E2 supplementation. Three weeks after ovariectomy, OVX and intact rats were injected daily (sc) for 5 d with 100 μ g antagonist before being killed, as adapted from previous studies (19, 20). The long-acting GnRH agonist (75 μ g) was administered once (im) on OVX and intact rats, as previously described (21), 3 wk before being killed.

Tissue collection

After each experiment, rats were weighed, anesthetized with ether, and killed by decapitation. Blood samples (3–5 ml) were obtained from each animal through a cardiac catheter. The blood was immediately centrifuged in dry tubes, and the serum was stored at -20 C until E2 evaluation. Pituitaries were dissected immediately after death, flash frozen in liquid nitrogen, and stored at -80 C for subsequent RNA extraction. For immunohistochemistry, pituitaries from male and female rats were carefully dissected and immediately fixed in 4% paraformaldehyde for 24 h and then rinsed with $1 \times$ PBS, dehydrated in ethanol, and embedded in paraffin. Tissue blocks were serially sectioned at 3 μ m, and sections were mounted onto Super Frost Plus slides (Menzel-Gla-

TABLE 1. Body weight, vaginal cornification, and serum E2 levels in the different experimental groups

Groups	Body weight (g)	Vaginal cornification	Serum E2 (pg/ml)
Metestrus	235.6 \pm 3.76	Abundant leukocytes	≤ 5
Proestrus	249.2 \pm 10.90	Proestrus epithelial, nucleated cells	34 \pm 4.15 ^a
Estrus	ND	Yes	≤ 5
OVX	248.9 \pm 9.81	No	≤ 5
OVX + E2	234.3 \pm 5.38	Yes	80.6 \pm 14.3 ^b
ER agonists			
PPT	207.2 \pm 6.75	Yes	≤ 5
DPN	206.4 \pm 21.13	No	≤ 5
PPT + DPN	217.6 \pm 17.21	Yes	≤ 5
Cetrorelix			
Intact	264.0 \pm 10.99	No	≤ 5
OVX	304.0 \pm 13.97	No	≤ 5
OVX + E2	258.0 \pm 7.55	Yes	547.5 \pm 99.63 ^b
Triptorelin			
Intact	243.1 \pm 4.66	No	6.92 \pm 0.94 ^b
OVX	247.3 \pm 9.30	No	≤ 5
OVX + E2	248.6 \pm 7.13	Yes	32.11 \pm 4.74 ^b

Values are expressed as the mean \pm SEM of at least five determinations per group. ND, Not done.

^a Significant differences from corresponding metestrus group ($P < 0.001$).

^b Significant differences from corresponding OVX group ($P < 0.001$).

ser/CML, Nemours, France) before being air dried at 37°C. Sections were made in the rostrocaudal direction from all regions of the gland.

RT-PCR analysis

Total RNA was extracted using the Trizol reagent kit (Invitrogen, Life Technologies, Cergy Pontoise, France) and then retrotranscribed into a reaction mixture (40 μ l) containing 100 U Moloney murine leukemia virus reverse transcriptase (Amersham Pharmacia Biotech, Orsay, France), 1 \times reaction buffer (Amersham), 0.2 mM deoxynucleotide triphosphates (Promega, Charbonnières, France), 50 pmol oligo(dT) primers (Invitrogen, Life technologies), 20 U ribonuclease inhibitor (Promega), and 2 μ g total RNA. The reaction was carried out at 37°C for 60 min then at 95°C for 5 min. Primers for the gonadal-specific first exon II and for the brain-specific exon I.f were designed using Primer Express Software (version 2.0, Applied Biosystems) and synthesized by Life Technologies (Table 2). After PCR, amplified fragments were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

Quantification of the transcripts

Construction of standard curves. We constructed by RT-PCR specific standard curves for aromatase, LH β , and β -actin to determine the amount of each transcript in the different tested samples. After being visualized on 1.5% agarose gel, specific bands were gel purified by Ultrafree-DA (Millipore Corp., Bedford, MA) according to the manufacturer's recommendations, and the identity of each PCR product was confirmed by sequencing (CEQ DTCS Quick Start Kit; Beckman Coulter, France). Standard curves were then generated by 10-fold serial dilution.

Real-time PCR analysis. Relative levels of total aromatase mRNA were examined by real-time PCR, using an ABI Prism 7000 Sequence Detector System (Applied Biosystems, Courtaboeuf, France) according to the manufacturer's directions. The sequences of the primers selected for total aromatase analyses were located on exons IX–X. This allowed us to assess all aromatase variants, independently from the tissue-specific exon I inserted in the mRNA. Because our experimental manipulations are known to induce dramatic changes in the functioning of the gonadotropic axis, we also monitored LH β mRNA expression as a control. Total aromatase mRNA was quantified using the TaqMan Universal PCR Master Mix (Applied Biosystems) and a TaqMan specific probe (5'-CCATTGGCTTGGGCC-3'), in a total volume of 30 μ l reaction mixture whereas LH β mRNA was quantified using the SYBR Green Universal PCR Master Mix 2 \times (Applied Biosystems), in a total volume of 20 μ l. Samples were heated 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C then 1 min at 60°C or 64°C. For all samples, the quantification of the β -actin gene was used as the endogenous control for the normalization of initial RNA levels. PCR were performed in duplicate and a reagent blank prepared using the RT blank was included with each plate to detect contamination by genomic DNA.

E2 production

Serum E2 levels were measured by using a RIA kit (ESTRADIOL, DiaSorin, Antony, France) validated for use in rats (Table 1). The limit

of detection was 5 pg/ml of serum. All samples were run in duplicate in the same assay. The intra- and interassay coefficients of variation were 4 and 11%, respectively.

Western blot analysis

For microsomal protein extraction, pituitaries and testes were crushed and homogenized in phosphate buffer (0.1 M, pH 7.4; Sigma) containing a protease inhibitor cocktail tablet (Roche Applied Science, Meylan, France). Samples were first centrifuged at 4°C for 5 min at 8000 \times g, and then the supernatants were ultracentrifuged at 4°C for 60 min at 150,000 \times g. Protein concentration in the supernatant was measured with the Bradford micromethod. Finally, samples were frozen at –80°C until Western blotting.

The microsomal proteins (100 μ g) were loaded into each lane and then separated on a 12% polyacrylamide denaturing gel, and proteins were blotted onto nitrocellulose membranes (Amersham Pharmacia Biotech) for 60 min at 15 V using a semidry transfer system (OWL, Illkirch, France). The membrane was blocked for 90 min at room temperature in PBS supplemented with 3% nonfat dried milk and then incubated for 2 h at room temperature with a rabbit polyclonal antibody directed against rat P450arom (kindly provided by Dr. Carretero, University of Salamanca, Spain) at 1/1000 dilution in PBS supplemented with 3% nonfat dried milk. After being washed in TBS (10 mM Tris, 15 mM NaCl, pH 7.4) for 10 min, the membrane was incubated for 90 min at room temperature with a rabbit Ig antibody at 1/4000 dilution in PBS supplemented with 3% nonfat dried milk. The antibody-protein complexes were revealed using the enhanced chemiluminescence visualization system (ECL; Amersham Pharmacia Biotech).

Fluorescence immunohistochemistry

Male and female pituitary sections were deparaffinized in toluene and rehydrated through a graded ethanol series, washed in H₂O, then immersed in 100 mM citrate buffer (pH 6.0), and finally heated in a standard microwave twice for 5 min at 750 W and 5 min at 550 W. The container holding the sections was removed and allowed to cool for 20 min at room temperature. After a brief wash in PBS, nonspecific binding sites were blocked with 3% BSA in PBS for 60 min. We first tested immunostaining in adjacent sections with rabbit polyclonal antibody against rat P450arom at a 1/500 dilution, rabbit polyclonal antirat FSH β (NIDDK) at a 1/1000 dilution, rabbit polyclonal antirat prolactin (PRL) (22) at a 1/1000 dilution, or rabbit polyclonal antisynthetic human GH (NIDDK, no. IC-4, AFP-1613102481) at a 1/500 dilution. After overnight incubation at 4°C, polyclonal antibodies were revealed with a biotinylated secondary antibody directed against rabbit Ig associated with a streptavidin-fluorescein complex (RPN 1232; Amersham Pharmacia Biotech). Dual fluorescence labeling was then tested on the same section with the rabbit polyclonal antibody against rat P450arom plus mouse monoclonal antibody against rat LH β (kindly provided by Dr. Counis, University of Paris VI, France) at a 1/300 dilution. Texas Red-conjugated secondary antibodies directed against mouse Ig were subsequently used to reveal anti-LH β monoclonal antibody. After rinsing with 1 \times PBS, sections were coverslipped with Vectashield mounting medium (Vector

TABLE 2. Primers used in this study

Target mRNA	Primer sequence (5'–3')	Product (bp)	Temperature	Ref.
Total aromatase				
Forward	TTT ACC CTT GAA AAC TTT GAG AAG AAC, exon 9	122	60	M33986
Reverse	GTA ACC AGG ACA ACT TTC ATC ATC AC, exon 10			
Brain aromatase				
Forward	TAA AAG ATG GCA CAC ACA AAG AGT, exon I.f	147	57	Kato <i>et al.</i> (24)
Reverse	GCC TCA GAA GGA AAA ATGTAA A, exon 2			
Ovarian aromatase				
Forward	CTG TCC ATT CCA GCA CCC TTA, exon II	140	55	M33986
Reverse	TTC CAA AAA CAT CTT GTG CTA TTT TG, exon 2			
LH β				
Forward	GTA GGG AAG GTA TCA AGA ATG G, 784–805	149	60	J00749
Reverse	TTG ACG TCC ACC AGT TAG G, 915–933			
Actin				
Forward	CAA CCG TGA AAA GAT GAC CCA G, 423–444	153	60	NM 031144
Reverse	ATG GGC ACA GTG TGG GTG AC, 555–575			

Laboratories, Inc., Burlingame, CA). Exclusion of the primary antibody was run as negative control to confirm the specificity of the immunostaining, and rat testis sections were used as positive control for aromatase staining. Sections were viewed using fluorescence microscopy (Axioskop 2 plus; Carl Zeiss, Le Pecq, France), and digital images were collected using a Zeiss AxioCam digital camera and the AxioVision image analysis system (version 4.1; Carl Zeiss).

Reporter plasmids used in transfection studies

The construct containing region -1037 to $+94$ of the rat aromatase gene promoter PII, inserted upstream of the *Firefly* luciferase gene into the pGL2-Basic vector (Promega), was generously provided by Dr. M. McPhaul and has been described previously (23). The 1029-bp fragment of the rat aromatase gene promoter PI.f inserted upstream of the *Firefly* luciferase gene into the pGL3-Basic vector (Promega) was isolated in our laboratory (unpublished data) and displays a nucleotide sequence homologous to that registered in the rat genome resource data bank of the National Center for Biotechnology Information.

Transfection experiments

L β T2 cells, generously provided by Dr. P. Mellon (La Jolla, CA), were maintained in monolayer cultures in high-glucose DMEM (Sigma) supplemented with 10% fetal bovine serum (Invitrogen, Life Technologies) and penicillin/streptomycin (Sigma) at 37 C in humidified 5% CO₂. For transient transfection, cells were plated in 24-well plates at a density of 3×10^5 cells per well the day before transfection. On the day of transfection, Lipofectamine 2000 reagents (Invitrogen, Life Technologies; 2 μ l/well) was used according to the manufacturer's instructions to co-transfect cells with the pGL2-promoter construct (823 ng/well) or the pGL3-promoter (720 ng/well) and the *Renilla* luciferase pRL-TK expression vector (Promega; 15 ng/well) for 24 h. Transfected cells were washed once in DMEM with no phenol red (Sigma) and serum and then treated with either a GnRH agonist (Triptorelin acetate; IPSEN Pharma Biotech), E2 (Sigma), or a combination of Triptorelin plus E2. After 6 or 24 h, *Firefly* and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). Cells were washed once with PBS (pH 7.4) and harvested by adding 100 μ l/well lysis buffer. *Firefly* luciferase assays were performed by adding 100 μ l luciferase assay reagent to 20 μ l cell extract, and luminescence was measured with a 2-sec delay and a 10-sec measurement in a Mithras LB 940 microplate reader (Berthold Technologies, Bad Wildbad, Germany). One hundred microliters of Stop & Glo reagent were then added, and *Renilla* luciferase activity was determined under the same conditions. The *Firefly* luciferase data were corrected for transfection efficiency with *Renilla* luciferase activity.

Statistical analysis

All results are expressed as a ratio to a calibrator that was chosen to be the metestrous groups for *in vivo* studies or untreated cells for *in vitro* studies and then expressed as the mean values \pm SEM. Statistical analysis was performed using one-way ANOVA, and differences between groups were determined by the Fisher protected least significant difference test (SigmaStat for Windows, version 3.1; SPSS Inc., Chicago, IL).

Results

Pituitary aromatase is expressed in gonadotrope cells from two *cyp19* transcripts

The *cyp19* transcripts with their specific untranslated first exon were isolated by RT-PCR. PCR were individually performed with the following primer sets: ARO1f-F/ARO1f-R for the brain-specific exon I.f and AROov-F/AROov-R for the gonadal-specific first exon II (Table 2). After electrophoresis of PCR products, we observed two specific bands of 147 and 140 bp that fit the predicted sizes of the amplified sequences for brain and gonadal subtypes, respectively (Fig. 1A). No PCR product was obtained in control reactions in the absence of reverse transcriptase.

Microsomal proteins were then extracted from rat pi-

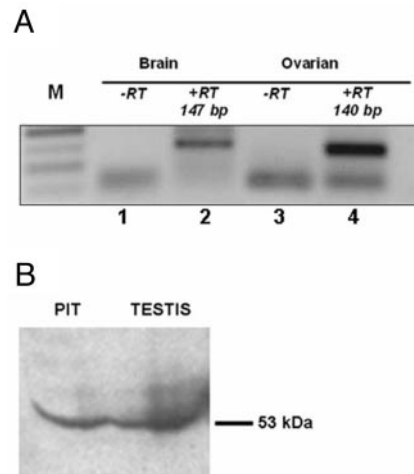


FIG. 1. Detection of the aromatase transcripts by RT-PCR (A) and P450arom protein by Western blot (B) in the rat pituitary. A, PCR products were generated with primer sets ARO1f-F/ARO1f-R for brain transcript (lane 2, 147 bp) and AROov-F/AROov-R for ovarian transcripts (lane 4, 140 bp) on 1.5% agarose gel: -RT, water blank controls (lanes 1 and 3); M, marker lane. B, P450arom protein was analyzed by Western blot from 100 μ g microsomal protein. Testis was used as a positive control.

uitaries and testis and were analyzed by Western blot to detect the P450arom proteins encoded by the aromatase transcripts. A single band with apparent molecular mass of 53 kDa was visualized both in the testis and the pituitary (Fig. 1B). The size of this protein is consistent with the predicted size.

Finally, immunohistochemistry was used to identify the cell type that synthesized aromatase (Fig. 2). Male pituitary sections were immunostained to adequately visualize the aromatase protein (16). Findings were then confirmed on female pituitary sections. Whatever the antibody used, the immunostaining was restricted to the anterior lobe, and no immunostaining was found in the pars intermedia or in the pars nervosa (only shown for P450arom and LH; Fig. 2, D1–D3). A positive labeling was detected in the cytoplasmic compartment but not in nuclei. Immunolabeling was undetectable when primary antibodies were omitted (Fig. 2, control -). A positive immunoreaction to P450arom was detected in Leydig cells within the interstitial tissue (Fig. 2, control +), thus confirming the specificity of the aromatase immunostaining. Examination of two consecutive sections indicated that P450arom and FSH were localized in the same cells (Fig. 2, A1 and A2). However, only certain cells were positive for the two proteins. Thanks to a specific mouse monoclonal antibody against LH β , we tested colocalization using two different immunolabeling methods. Results clearly showed that P450arom and LH β immunostaining colocalized within the same cells both in males (Fig. 2, D3 and E3) and females (Fig. 2, F3). P450arom was not found in the cells showing positive immunostaining for PRL and GH (Fig. 2, B1, B2, C1, and C2).

Aromatase gene expression is regulated across the estrous cycle

Total aromatase mRNA expression was examined in the rat pituitary at three stages of the estrous cycle, metestrous, proestrous, and estrus, using real time RT-PCR (Fig. 3).

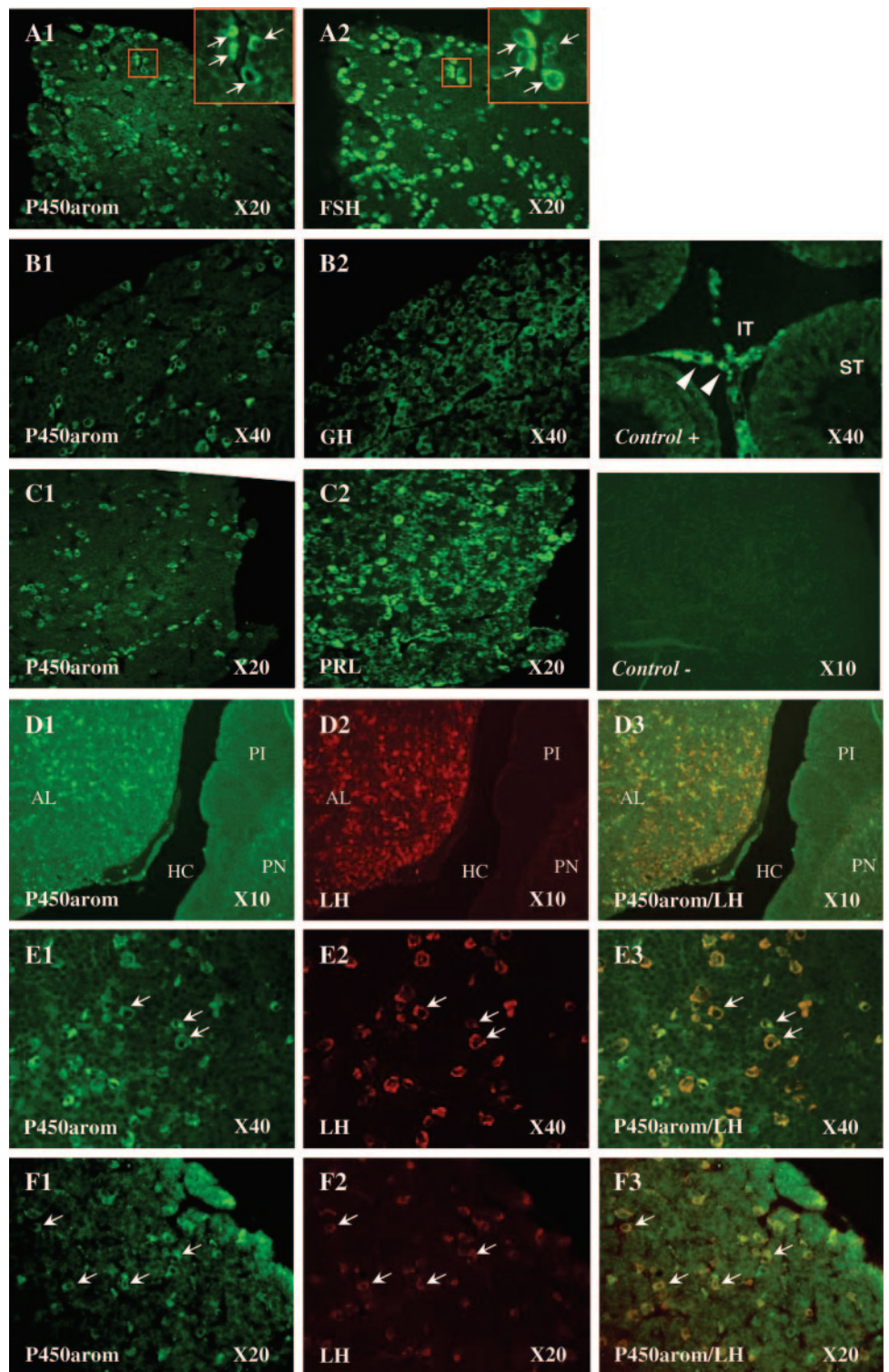


FIG. 2. Colocalization of aromatase with FSH (A1 and A2), GH (B1 and B2), PRL (C1 and C2) by single immunofluorescence staining and with LH (D, E, and F1–F3) by double immunofluorescence staining, in male (A–E) and female (F) rat pituitary sections. Fluorescent immunohistochemistry was carried out using fluorescein for aromatase, FSH, GH, and PRL (green), and Texas Red for LH (red). Dually labeled cells stand out in yellow/orange when images are merged (D3, E3, and F3). The arrows show positive cells for aromatase, which are also positive for FSH (A1 and A2) or LH (D, E, and F1–F3). These immunofluorescent data show that aromatase is expressed in gonadotrope cells but not in GH or PRL cells in the rat. Whatever the antibody used, immunostaining was found to be restricted to the anterior lobe (AL) but absent in the pars intermedia (PI) or in the pars nervosa (PN) (only shown in D1–D3). No specific staining was detected in the absence of primary antibody (Control –). A positive immunoreactivity to P450arom was detected in Leydig cells within the interstitial tissue (IT) (arrowheads, Control +). HC, Hypophysial cleft; ST, seminiferous tubule.

Quantification was performed by amplifying the total aromatase mRNA with exons IX and X. The regularity of the estrous cycle was checked by measuring E2 levels and examining vaginal smears (Table 1). Figure 3 demonstrates that aromatase mRNA expression changed according to

the estrous cycle stage. Aromatase mRNA levels were significantly higher during metestrus than during estrus and proestrus. We also observed that aromatase mRNA levels were lower in proestrus than in estrus, although not significantly.

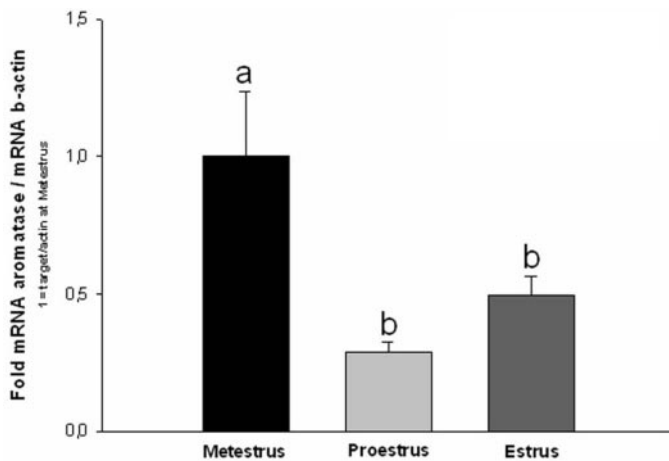


FIG. 3. Expression of pituitary aromatase mRNA in adult (90-d-old) rats across the estrous cycle. Transcripts were measured by real-time RT-PCR using appropriate primers and normalized to β -actin mRNA levels. Data are expressed as fold change vs. metestrus group. The LH β quantification was used as control. Values without common letters (a and b) differ significantly ($P < 0.01$). Values are represented as the mean \pm SEM.

E2 decreases aromatase gene expression in the pituitary

Based on the above data, we set up an experiment designed to test the effect of steroid removal by ovariectomy and E2 replacement. The potency of E2 supplementation was checked by measuring E2 levels and by examining vaginal smears (Table 1). Figure 4A shows that ovariectomy resulted in a significant increase in the pituitary expression of total aromatase mRNA compared with metestrus, whereas E2 supplementation (Table 1) completely reversed the effects of ovariectomy. These variations paralleled those observed for LH β expression.

E2 down-regulates the expression of the aromatase gene in the rat pituitary via ER α and ER β

To determine the contribution of ER (α and β isoforms) in E2 regulatory action on *cyp19* gene expression, 3-wk OVX rats were injected over 3 d with the selective ER α ligand, PPT, the potency-selective agonist of ER β , DPN, or the combined administration of PPT plus DPN. The potency of the treatment was checked by vaginal smears (Table 1). PPT administration and to a lesser extent DPN administration resulted in a significant decrease of aromatase mRNA compared with OVX values, at a level close to that observed in metestrus (Fig. 4B). The responses to the combined administration of PPT and DPN differed significantly from those caused by the administration of PPT alone (Fig. 4B). By contrast, PPT alone or in combination with DPN, but not DPN alone, reversed the effects of ovariectomy on LH β mRNA expression (Fig. 4B).

Dual regulation of the aromatase gene expression by estrogens at pituitary and hypothalamic levels

To highlight the E2 effect at the pituitary or hypothalamic level on aromatase mRNA expression, OVX and intact rats were treated with a GnRH antagonist, Cetrorelix (Fig. 4C) or a long-acting GnRH agonist, Triptorelin (Fig. 4D). The potency of different treatments was checked by measuring E2

levels and by examining vaginal smears (Table 1). In intact female rats (Table 1), daily administration of Cetrorelix for 5 d did not significantly suppress total aromatase mRNA levels compared with metestrus values (Fig. 4C). By contrast, Cetrorelix prevented the increase of total aromatase mRNA levels observed after ovariectomy (Fig. 4C). E2 administration (Table 1) amplified the decrease of *cyp19* expression in a manner similar to that observed for LH β in OVX rats (Fig. 4C).

In intact female rats (Table 1), the single administration of Triptorelin, a long-acting GnRH agonist, did not significantly increase the total aromatase mRNA expression compared with metestrus values (Fig. 4D). Similarly, in OVX rats, this treatment did not modify total aromatase mRNA levels, although we observed the well-known desensitization of LH β , compared with OVX rats alone (Fig. 4D). Again, E2 (Table 1) reduced aromatase and LH β mRNA expression (Fig. 4D).

GnRH stimulates aromatase promoters PII and PI.f activity, and E2 reduces GnRH-dependent activities in L β T2 cells

To examine the direct effect of GnRH and E2 on aromatase expression, L β T2 cells were transfected with reporter genes containing aromatase promoters PII or PI.f. We found that GnRH agonist treatment at 10^{-9} or 10^{-7} M for 6 h stimulated promoter PII activity in a dose-dependent manner compared with the basal activity (7- and 18-fold, respectively; Fig. 5A). By contrast, the stimulation of promoter PI.f by GnRH is less important, with no significant difference between 10^{-9} and 10^{-7} M (1.4- and 1.5-fold, respectively; Fig. 5B). Interestingly, when cells were treated for 24 h with GnRH (10^{-7} M), the stimulation amplitude was lower for promoter PII (18- to 10-fold; Fig. 5, A and C) but was higher for promoter PI.f (1.5- to 2.4-fold; Fig. 5, B and D) compared with a 6-h treatment. Although treatment with E2 alone did not cause any significant response on promoter PII and PI.f activities, whatever the doses used (10^{-5} to 10^{-9} M, data not shown), the combined incubation of E2 (10^{-5} M) with Triptorelin (10^{-7} M) resulted in a negative effect on GnRH-stimulated promoter PII and PI.f activities at 6 h (data not shown) as well as at 24 h (Fig. 5, C and D).

Discussion

Both approaches used in our study, dual fluorescence labeling using LH β and P450arom antibodies and evidence of an up-regulation of aromatase gene expression by GnRH that exclusively targets gonadotrope cells in the anterior pituitary, clearly demonstrated the expression of aromatase in gonadotrope cells.

RT-PCR analysis indicates that aromatase is synthesized from two previously described transcripts in the rat pituitary, the gonadal-specific first exon II under the control of promoter PII and the brain-specific exon I.f under the control of promoter PI.f. In rodent brains, the brain subtype is the major transcript in the thalamic-hypothalamic areas of the mouse (6) and in the hypothalamus and amygdala of adult rats (7), although in both cases, low amounts of transcripts containing the gonadal subtype may also be present in these regions (6, 24). However, because brain and gonadal sub-

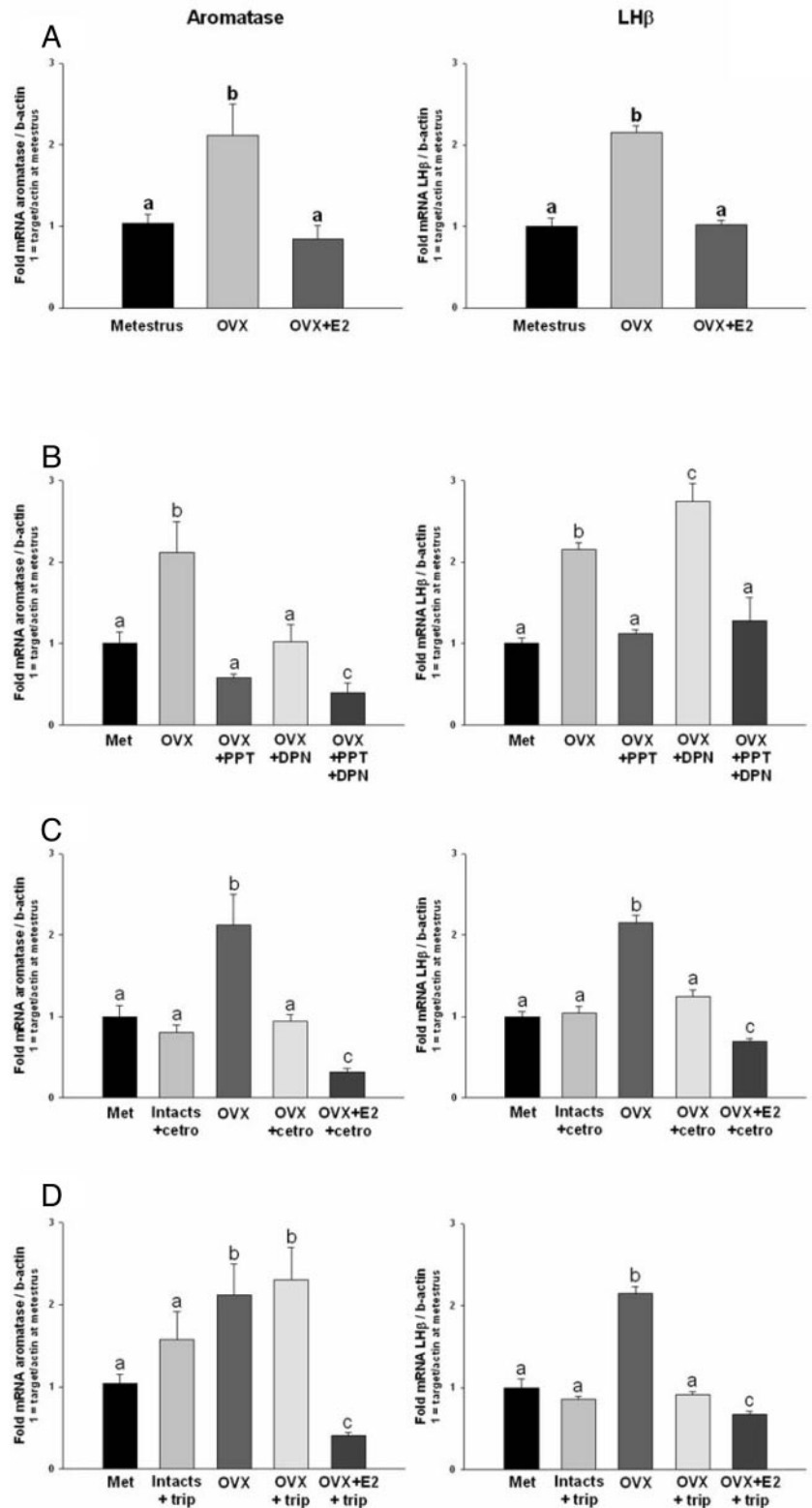


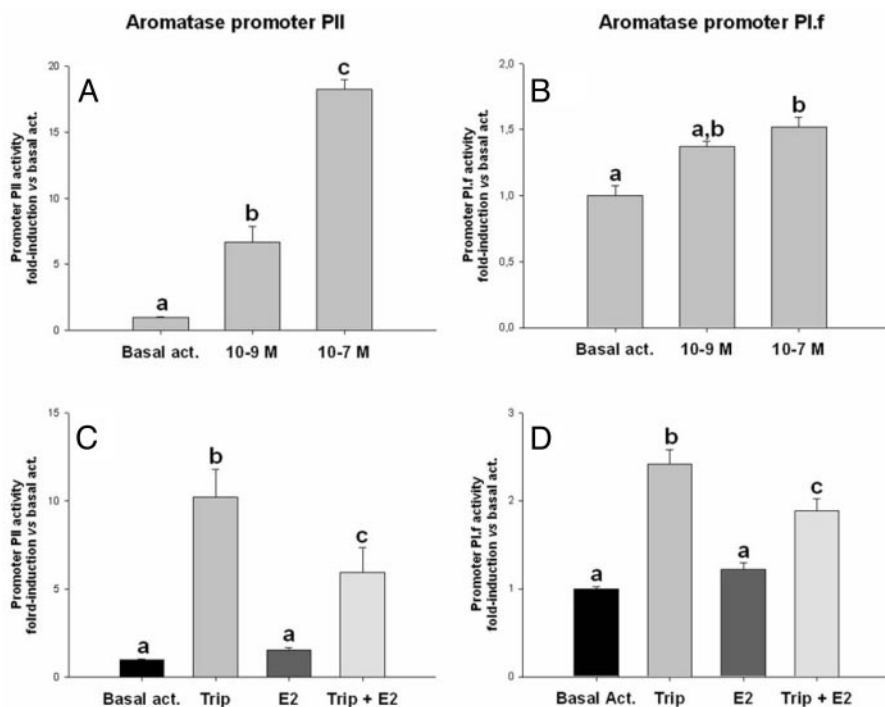
FIG. 4. Expression of aromatase and LH β mRNA in pituitaries from 3-wk OVX rats treated with E2 alone (A) or with the ER α -selective ligand PPT, the potency-selective ER β agonist DPN, and a combination of PPT plus DPN (B), the GnRH antagonist Cetrorelix (C), or the long-acting GnRH agonist Triptorelin (D). Pituitary mRNA levels of the targets in 3-wk OVX rats and control females at the morning of metestrus are also presented. The LH β quantification was presented as a control. Transcripts were measured by real-time RT-PCR using appropriate primers and normalized to β -actin mRNA levels. Data are expressed as fold change *vs.* metestrus group. Values are represented as the mean \pm SEM. Values without common letters (a–c) differ significantly ($P < 0.01$).

types are found in low concentrations in the pituitary gland, we were unable to determine which was mainly expressed. Using immunolabeling with anti-LH β , -FSH β , and -P450arom antibodies, we observed that aromatase was expressed in cells that expressed LH and, to a lesser extent, FSH

proteins, but not PRL or GH. We cannot, however, exclude the possibility that aromatase is present in other cell types such as ACTH and TSH cells.

Our analyses using real-time PCR showed for the first time that the level of P450arom mRNA significantly varied during

FIG. 5. Effects of a GnRH agonist, Triptorelin (Trip), and 17 β -estradiol (E2) on PII and PI.f promoter activity. L β T2 cells were transiently transfected with a construct containing region –1037/+94 of the rat aromatase gene promoter PII (on the left, A and C) or with a construct containing region –1029/+40 of the rat aromatase gene promoter PI.f (on the right, B and D). A and B, GnRH dose-response study of aromatase PII promoter (A) or PI.f (B) treated for 6 h; C and D, effect of E2/GnRH cotreatment. Cells were stimulated with E2 (10^{-5} M) and Triptorelin (10^{-7} M) for 24 h before harvesting. Results are expressed as the fold induction over the basal activity value and are the mean \pm SEM of three independent experiments in triplicate. Values without common letters (a–c) differ significantly ($P < 0.01$).



the estrous cycle, its lowest level occurring in the morning of proestrus when maximal levels of estrogens were observed, just before the LH surge occurring in the afternoon. The effects of castration and steroid replacement recorded on aromatase mRNA levels concord with the histochemical data of Carretero *et al.* (16), who showed that aromatase labeling varied in intensity across the cycle. These results support a direct action of gonadal steroids on the pituitary to negatively control the expression of aromatase.

Numerous studies have demonstrated that E2 acts on the hypothalamic level to modify both GnRH pulse frequency and amplitude (3) and directly on gonadotropes to modify either the number of GnRH receptors (GnRH-R) (25, 26), intracellular responses to GnRH (26), or transcriptional activity of gonadotropin subunit genes (27). Therefore, the exact mechanism by which the steroid exerts its effect on *cyp19* gene expression is difficult to ascertain. To separate the pituitary gland from endogenous GnRH secretion, we designed a protocol blocking GnRH-induced gonadotropin secretion. We found that administering a GnRH antagonist to OVX rats prevents the increase of *cyp19* gene transcription. The main mechanism of action of GnRH antagonists was thought to be based on a competitive occupancy of GnRH-R and the counteraction of the stimulatory effect of endogenous GnRH (28). Thus, GnRH antagonists do not directly influence gene expression at the pituitary but do exert their suppressive effects by counteracting the up-regulation caused by GnRH. Accordingly, a significantly greater reduction in *cyp19* as well as in LH β gene expressions by Cetrorelix was observed in OVX rats, which have higher GnRH concentration in the pituitary portal vessels than in controls. Thus, our experimental procedure clearly establishes that GnRH exerts a positive regulation of *cyp19* gene expression in OVX rats. As an additional strategy to detect the direct

effect of GnRH on *cyp19* expression, specific aromatase promoters PII or PI.f driving reporter gene were introduced into L β T2 cells, a cell line that closely resembles a differentiated gonadotrope. As expected, GnRH markedly increased PII in a dose-dependent manner and, to a lesser extent, PI.f aromatase promoters.

Studies exploring cellular mechanisms involved in the LH β and *cyp19* gene regulation have identified several common key factors such as the steroidogenic factor-1 (SF-1) and the cAMP response element-binding protein (CREB). In the gonads, promoter II activity of *cyp19* is regulated by FSH and a cAMP-dependent signaling mechanism giving rise to an interaction between the gonadal promoter II with the transcription factors SF-1 and CREB, both in humans and in rats (29–31). SF-1, which is selectively expressed in the gonadotrope population (32), was also shown to be crucial for LH β , GnRH-R, and free α -subunit (α GSU) transcription activation (33–37). Taking these data into consideration, the presence of SF-1 in gonadotropes that is consequently linked to GnRH stimulation implies that GnRH may affect aromatase expression.

The variations of mRNA levels under administration of a long-acting GnRH agonist causing homologous desensitization provides evidence that the intracellular mechanisms responsible for GnRH activation of aromatase expression differ from those governing regulation and desensitization of LH β expression. Indeed, long-acting GnRH agonist depresses LH β in OVX rats using a postreceptor phenomenon (21), whereas it does not modify aromatase expression. Similarly, a dissociated regulation of transcriptional stimulation and mRNA stability was shown for the α -subunit (38) and for nitric oxide synthase (39), illustrating the fact that elements under GnRH regulation in gonadotrope cells each respond

to desensitization with distinct characteristics using specific mechanisms that remain to be characterized.

Estradiol levels in OVX rats receiving GnRH antagonist plus E2 were much higher than with any other treatment. Thus, we cannot conclude whether the decrease in aromatase mRNA levels is a direct pituitary effect or a result of the total inhibition of GnRH input. However, administration of E2 to long-acting GnRH-agonist-treated animals clearly demonstrated that E2 also acts directly on the pituitary by negatively controlling aromatase expression. When L β T2 cells were treated with E2, the basal activities of aromatase promoters PII and PI.f remained unchanged. However, E2 reduced the GnRH induction of promoters PII and PI.f activities according to our *in vivo* observations.

It is well established that estrogen-induced changes are mainly mediated via ER. Previous studies have demonstrated that the pituitary expresses both ER α and ER β isoforms, with higher levels for ER α than ER β (40–42) and a coexpression of both isoforms in the rat gonadotropes (41). In our study, activation of ER α by the selective ligand PPT and, to a lesser extent, activation of ER β by the selective ligand DPN were able to mimic the effect of estrogen supplementation in OVX rats, thus suggesting the involvement of ER pathways in the regulation of the *cyp19* gene. The responses of pituitary aromatase expression in the different experimental groups were closely paralleled by changes in pituitary LH mRNA levels after ovariectomy and E2 supplementation, except that ER β activation by the selective ligand DPN was unable to restore LH β mRNA levels. Concerning LH, this result is consistent with previous reports showing that estrogen-induced LH β regulation is heavily dependent upon the actions of ER α , because only α ERKO or $\alpha\beta$ ERKO female mice exhibited elevated LH β gene expression, but not β ERKO female mice (43).

In our study, we found that aromatase and LH colocalized within the same gonadotrope cells. Therefore, the differences observed between the regulation of *cyp19* and LH β genes are not related to a cell-specific effect but suggest the role of specific transcription factors. Our PCR analyses showed that the *cyp19* gene is under the control of promoter II and promoter I.f. However, to date, no high-affinity ER binding sites have been identified in these promoters, despite the fact that *cyp19* expression is negatively regulated by both potent ER α and the ER β agonists. It is well known that ER can modulate the transcription from promoters that lack typical estrogen response elements, using alternative response elements to which ER are not bound or specific intracellular factors recruited by ER α and/or ER β (44). For example, estrogens have been found to stimulate either the neurotensin or interleukin gene expression despite the lack of estrogen response element motifs in these promoters (45, 46). The regulation of the *cyp19* gene by E2 also appeared to be cell type dependent, reinforcing the hypothesis that specific intracellular factors are implied. Indeed, E2 inhibited *cyp19* gene expression in germ cells (47), whereas in Leydig cells, E2 enhanced it in a dose- and time-related manner (48). Thus, alternate mechanisms such as transcriptional interference via protein-protein interactions may be the molecular basis for the inhibitory functions of estrogens and could explain differences between the regulation of *cyp19* and LH β gene expression.

It has been reported that the regulation of *cyp19* gene expression in rat gonads mainly depends on SF-1 and CREB content. CREB contains several consensus phosphorylation sites for various kinases, in particular protein kinase A (49) and protein kinase C (50). In this model, the highest levels of phospho-CREB (pCREB) coincided with the maximal induction of endogenous *cyp19* gene (30). It is known that ovariectomy increases pCREB in the pituitary, whereas E2 treatment dramatically decreases pCREB content via a mechanism linked to the GnRH signaling pathway (51). Thus, the increase of CREB phosphorylation in the gonadotrope cells could be responsible for GnRH positive regulation of *cyp19* expression, whereas the decrease in CREB phosphorylation could be responsible for E2 negative regulation. However, the relevance of E2-induced change in pCREB has not yet been analyzed in the context of the initiation of the LH surge. Indeed, in the ewe, a combination of increased GnRH pulse frequency and estrogen leads to a pCREB response in gonadotrope cells (52).

The converging signaling of both pathways and concerted action of GnRH and E2 at the pituitary level are involved in the timing and initiation of the LH surge. At proestrus, the E2 circulating level is high, the number of gonadotrope cells that stained for ER α /ER β increases (53), GnRH-R are up-regulated (54), and GnRH stimulates ER transactivation (55). The LH surge that is mainly dependent on the increase of GnRH input occurs in the afternoon of the proestrus, and we clearly demonstrated that GnRH enhanced aromatase expression. Thus, our results lead us to hypothesize that aromatase expression could be enhanced during the LH surge to amplify E2 signaling. Accordingly, Kazeto and Trant (56) have recently shown in catfish that the preovulatory induction of the CYP19A2 gene by E2 is similar to the pattern of gene expression for LH β in the pituitary. This may underlie some degree of redundancy within the control of the LH surge, a key component of reproductive hormone synthesis.

In conclusion, we have shown that P450arom is synthesized by gonadotrope cells from two different transcripts carrying the gonadal-specific first exon II and the brain-specific exon I.f. We report for the first time that *cyp19* gene expression is positively regulated by GnRH *in vivo* in the rat pituitary gland and *in vitro* in L β T2 cells and negatively controlled by chronic exposure to E2 via ER. We also provide evidence for the involvement of both common and specific intracellular factors that could account for dissociated variations of LH β and *cyp19* expression.

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