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The expression of aromatase in gonadotropes is regulated by estradiol and GnRH in a manner that differs from the regulation of LH

Abbreviated title: regulation of aromatase expression in rat pituitary

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Guillaume Galmiche, Nicolas Richard, Sophie Corvaisier, Marie-Laure Kottler have nothing to declare.

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ABSTRACT

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 (OVX). Administration of E2 in OVX receiving either GnRH agonist or GnRH antagonist clearly demonstrated that E2 also reduces *cyp19* expression at the pituitary level. The selective ERα ligand, PPT and the selective ERβ ligand, DPN both mimic the E2 effects. By contrast, PPT reduces LHβ expression while DPN does not. In addition, using transient transfection assays in a LβT2 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 LHβ and *cyp19* expression.

INTRODUCTION

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, NADPH-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 a same protein regardless of the promoter used [2].

Estradiol (E2) is considered as the critical determinant of plasma gonadotropin levels in female 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 (T) significantly suppressed LHβ mRNA and serum LH levels in wild-type castrates while they had no significant effect on castrated estrogen receptor-alpha knockout mice (αERKO), 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 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 T.

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 immuno-localization of the P450arom protein. Second, we examined the total aromatase mRNA expression during the estrous cycle and assessed the effects of ovariectomy (OVX) 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 & METHODS

Animals

Mature female Sprague-Dawlay 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 (BW) 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 (i.p., 100mg/kg and 4mg/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 h 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 Estrous. 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 (s.c.) every 2 days for 3 weeks with 10µg 17β-

estradiol (Sigma, L'Isle d'Abeau, France). Rats (n=7 per group) were divided into 2 groups: ovariectomized, and ovariectomized plus E2 supplementation, and were killed three weeks later.

Experiment 3. The purpose of this experiment was to determine the isoform of ER used by estrogens to regulate the expression of the *cyp19* gene. A selective ERα agonist, propyl pyrazole triol (PPT), and an ERβ agonist, diarylpropionitrile (DPN) were obtained from Tocris Cookson Ltd. (Avonmouth, UK). OVX rats (n=5 per group) were randomly assigned to 3 groups: PPT, DPN, or PPT + DPN. 3 weeks after ovariectomy, rats were injected daily (s.c.) for 3 days with 1 mg of PPT, 1 mg of DPN, or 1 mg of PPT + 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-Trp^6-GnRH, Triptorelin L.P. 3 mg, kindly provided by IPSEN Pharma Biotech, Signes, France). Animals (n=7 per groups) were randomly assigned to 3 groups: intact, OVX and OVX plus E2 supplementation. 3 weeks after ovariectomy, OVX and intact rats were injected daily (s.c.) for 5 days with 100µg of antagonist before being sacrificed, as adapted from previous studies [19, 20]. The long-acting GnRH agonist (75 µg) was administered once (i.m.) on OVX and intact rats, as previously described [21], three weeks 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 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 then rinsed with PBS 1X, dehydrated in ethanol, and embedded in paraffin. Tissue blocks were serially sectioned at 3µm and sections were mounted onto Super Frost Plus slides (Menzel-Glaser/CML, Nemours, France) before being air-dried at 37 C. Sections were made in the rostro-caudal 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) then retrotranscribed into a reaction mixture (40µl) containing 100 U M-MLV Reverse Transcriptase (Amersham Pharmacia Biotech, Orsay, France), 1X 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 2µg 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 reactions, 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, LHβ, 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 Corporation, Bedford, U.S.A.) 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 LHβ 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'-CCATTTGGCTTTGGGCCC-3'), in a total volume of 30µl reaction mixture while LHβ mRNA was quantified using the SYBR Green Universal PCR Master Mix 2X (Applied Biosystems), in a total volume of 20µl. Samples were heated 10 min at 95 C, followed by 40 cycles of 15 sec at 95 C then 1min 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 reactions were performed in duplicate and a reagent blank prepared using the RT blank was included with each plate to detect contamination by genomic DNA.

Estradiol production

Serum E2 levels were measured by using a radioimmunoassay kit (ESTRADIOL, DiaSorin, France) validated for use in rats (Table. 1). The limit of detection was 5pg/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 x g, then the supernatants were ultra-centrifuged at 4 C for 60 min at 150000 x g. Protein concentration in the supernatant was measured with the Bradford micro-method. Finally, samples were frozen at -80 C until Western blotting.

100 μg of the microsomal proteins were loaded into each lane, 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, then incubated for 2 h at room temperature with a rabbit polyclonal antibody directed against rat P450arom (kindly provided by Dr. Carretero, University of Salamanque, Spain) at 1/1000 dilution in PBS supplemented with 3% nonfat dried milk. After being washed in TBS (Tris 10 mM, NaCL 15 mM, pH 7.4) for 10 min, the membrane was incubated for 90 min at room temperature with a rabbit immunoglobulin 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 H_2O, then immersed in 100 mM citrate buffer (pH 6.0), and finally heated in a standard microwave for 2x5 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, non-specific 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 anti-rat FSHβ (NIDDK) at a 1/1000 dilution, rabbit polyclonal anti-rat prolactin [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 immunoglobulins associated with a streptavidin-fluorescein complex (RPN 1232, Amersham Pharmacia Biotech). Dual fluorescence labeling was then tested on a same section with the rabbit polyclonal antibody against rat P450arom *plus* mouse monoclonal antibody against rat LHβ (kindly provided by Dr. Counis, University of Paris VI, France) at a 1/300 dilution. Texas Red-conjugated secondary antibodies directed against mouse immunoglobulins were subsequently used to reveal anti-LHβ monoclonal antibody. After rinsing with PBS 1X, sections were cover-slipped with Vectashield Mounting Medium (Vector 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, 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

LβT2 cells, generously provided by Dr. P. Mellon (La Jolla, CA), were maintained in monolayer cultures in high glucose Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (Invitrogen, Life Technologies) and penicillin/streptomycin (Sigma) at 37 C in humidified 5% CO_2. For transient transfection, cells were plated in 24-well plates at a density of $3x10^5$ cells/well the day before transfection. On the day of transfection, Lipofectamine 2000 reagents (Invitrogen, Life Technologies; 2µl/well) was used as according to manufacturer's instructions to cotransfect 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 hours. Transfected cells were washed once in DMEM with no phenol red (Sigma) and serum, then treated with either a GnRH agonist (Triptorelin acetate; IPSEN Pharma Biotech), E2 (Sigma), or a combination of Triptorelin *plus* E2. After 6h or 24 hours, *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 µl/well lysis buffer. *Firefly* luciferase assays were performed by adding 100 µl luciferase assay reagent to 20 µl 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). 100 µl 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 metestrus groups for *in vivo* studies or untreated cells for *in vitro* studies, then expressed as the mean values ± 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, Illinois, USA).

RESULTS

Pituitary aromatase is expressed in gonadotrope cells from two cyp19 transcripts

Cyp19 transcripts with their specific untranslated first exon were isolated by RT-PCR. PCR reactions 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-bp and 140-bp which fit the predicted sizes of the amplified sequences for brain and gonadal subtypes, respectively (Fig. 1, A). No PCR product was obtained in control reactions in the absence of reverse transcriptase.

Microsomal proteins were then extracted from rat pituitaries 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. 1, B). 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* (PI) or in the *pars nervosa* (PN) (only shown for P450arom and LH; Fig. 2, D1-3). 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 immunostaing. Examination of two consecutive sections indicated that P450arom and FSH were localized in the same cells (Fig. 2, A1-2). However, only certain cells were positive for the two proteins. Thanks to a specific mouse monoclonal

antibody against LHβ, we tested colocalization using two different immunolabeling methods. Results clearly showed that P450arom and LHβ 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, B1-2, C1-2).

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 -metestrus, proestrus and estrus- using real time RT-PCR (Fig. 3). Quantification was performed by amplifying the total aromatase mRNA with exons IX-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, though not significantly.

Estradiol 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 4-A shows that OVX resulted in a significant increase in the pituitary expression of total aromatase mRNA compared to metestrus, whereas E2 supplementation (Table 1) completely reversed the effects of OVX. These variations paralleled those observed for LHβ expression.

Estradiol down-regulates the expression of the aromatase gene in the rat pituitary via ERα and ERβ

To determine the contribution of estrogen receptors (alpha and beta isoforms) in E2 regulatory action on *cyp19* gene expression, 3-week OVX rats were injected over 3 days with the selective ERα ligand, PPT, the potency-selective agonist of ERβ, DPN, or the combined administration of PPT + 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 to OVX values, at a level close to that observed in metestrus (Fig. 4, B). The responses to the combined administration of PPT and DPN differed significantly from those caused by the administration of PPT alone (Fig. 4, B). By contrast, PPT alone or in combination with DPN -but not DPN alone- reversed the effects of OVX on LHβ mRNA expression (Fig. 4, B).

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. 4, C) or a long-acting GnRH agonist, Triptorelin (Fig. 4, D). 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 days did not significantly suppress total aromatase mRNA levels, compared to metestrus values (Fig. 4, C). By contrast, Cetrorelix prevented the increase of total aromatase mRNA levels observed after ovariectomy (Fig. 4, C). E2 administration (Table 1) amplified the decrease of *cyp19* expression in a manner similar to that observed for LHβ in OVX rats. (Fig. 4, C).

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 to metestrus values (Fig. 4, D). Similarly, in OVX rats, this treatment did not modify total aromatase mRNA levels while we observed the well-known desensitization of LHβ, compared to OVX rats alone (Fig. 4, D). Again, E2 (Table 1) reduced aromatase and LHβ mRNA expression (Fig. 4, D).

GnRH stimulates aromatase promoters PII and PI.f activity and estradiol reduces GnRH-dependent activities in LβT2 cells

To examine the direct effect of GnRH and estradiol on aromatase expression, LβT2 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 6h stimulated promoter PII activity in a dose dependent manner compared with the basal activity (7 fold and 18 fold respectively Fig. 5, A). 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 fold and 1.5 fold respectively, Fig. 5, B). Interestingly, when cells were treated for 24h with GnRH (10^{-7}M), the stimulation amplitude was lower for promoter PII (18 fold to 10-fold, Fig. 5, A &C) but was higher for promoter PI.f (1.5 fold to 2.4 fold, Fig. 5, B & D) compared with a 6 h treatment. While 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 6h (data not shown) as well as at 24 h (Fig. 5, C& D).

DISCUSSION

Both approaches used in our study - dual fluorescence labeling using LHβ and P450arom antibodies and evidence of an up-regulation of aromatase gene expression by GnRH which 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 P1.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 subtypes are found in low concentrations in the pituitary gland, we were unable to determine which was mainly expressed. Using immunolabeling with anti-LHβ, -FSHβ and -P450arom antibodies, we observed that aromatase was expressed in cells which expressed LH and, to a lesser extent, FSH proteins, but not PRL or GH. We can not, 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 the estrous cycle, its lowest level occurring in the morning of proestrus when maximum 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 [16], who showed that aromatase labelling 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. In order 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 LHβ 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 LβT2 cells, a cell line that closely resembles a differentiated gonadotrope. As expected, GnRH markedly increased PII in a dose dependant manner, and to a lesser extent, PI.f aromatase promoters.

Studies exploring cellular mechanisms involved in the LHβ 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 LHβ, GnRH receptor and free α-subunit (αGSU) transcription activation [33-37]. Taking these data into consideration, the presence of SF-1 in gonadotropes which 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 LHβ expression. Indeed long-acting GnRH agonist depresses LHβ in OVX using a post-receptor 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 NOS [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 characterized.

Estradiol levels in OVX receiving GnRH antagonist *plus* E2 were much higher than with any other treatment. Thus, we cannot conclude as to whether the decrease in aromatase mRNA levels is due to a direct pituitary effect or to 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 LβT2 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 estrogen receptors (ERs). Previous studies have demonstrated that the pituitary expresses both ERα and ERβ isoforms, with higher levels for ERα than ERβ [40-42] and a co-expression of both

isoforms in the rat gonadotropes [41]. In our study, activation of ERα by the selective ligand PPT and to lesser extent, activation of ERβ 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 OVX and E2 supplementation, except that ERβ activation by the selective ligand DPN was unable to restore LHβ mRNA levels. Concerning LH, this result is consistent with previous reports showing that estrogen-induced LHβ regulation is heavily dependent upon the actions of ERα, as only αERKO or αβERKO female mice exhibited elevated LHβ gene expression, but not βERKO 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 LHβ genes are not related to a cell-specific effect but suggest the role of specific transcription factors. Our PCR analyses showed that *cyp19* gene is under the control of promoter II and promoter I.f. However, to date, no high affinity estrogen receptor binding sites have been identified in these promoters, in spite of the fact that *cyp19* expression is negatively regulated by both potent ERα and the ERβ agonists. It is well known that ERs can modulate the transcription from promoters that lack typical ERE, using alternative response elements to which ERs are not bound or specific intracellular factors recruited by ERα and/or ERβ [44]. For example, estrogens have been found to stimulate either the neurotensine or interleukin gene expression in spite of the lack of ERE 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 intra-cellular 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 LHß 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 PKC [50]. In this model, the highest levels of phosphoCREB (pCREB) coincided with the maximal induction of endogenous *cyp19* gene [30]. It is known that ovariectomy increases pCREB in the pituitary while 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 while 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 LH surge. Indeed, in ewe, a combination of increased GnRH pulse frequency and estrogen leads to a pCREB response in gonadotrope cells [52].

The converging signalling of both pathways and concerted action of GnRH and E2 at the pituitary level are involved in the timing and initiation of LH-surge. At proestrus, the E2 circulating level is high, the number of gonadotrope cells that stained for ERα/ERβ increases [53], GnRH-R are up-regulated [54], and GnRH stimulates ERs transactivation [55]. The LH surge that is mainly dependant 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 signalling. Accordingly, Kazeto & Trant studies [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 LHβ 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 LβT2 cells, and negatively controlled by chronic exposure to E2 via ERs. We also provide evidence for the involvement of both common and specific intracellular factors that could account for dissociated variations of LHβ and *cyp19* expression.

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FIGURE LEGENDS

Fig. 1. Detection of the aromatase transcripts by RT-PCR (A) and P450 arom 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 µg microsomal protein. Testis was used as a positive control.

Fig. 2. Colocalization of aromatase with FSH (A1-2), GH (B1-2), PRL (C1-2) by single immunofluorescence staining and with LH (D/E/F1-3) 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, F3). The *arrows* show positive cells for aromatase, which are also positive for FSH (A1-2) or LH (D/E/F 1-3). 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 D1-3). 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.

Fig. 3. Expression of pituitary aromatase mRNA in adult (90-day 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 LHβ quantification was used as control. Values without common notations (a, b) differ significantly ($P < 0.01$). Values are represented as the mean ± SEM.

Fig. 4. Expression of aromatase and LHβ mRNA in pituitaries from 3-wk OVX rats treated with estradiol-17β alone (A) or with the ERα-selective ligand PPT, the potency-selective ERβ agonist DPN and 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 LHβ 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 ± SEM. Values without common notations (a, b, c) differ significantly ($P < 0.01$).

Fig. 5. Effects of a GnRH agonist, Triptorelin (Trip) and 17β-estradiol (E2) on PII and PI.f promoter activity. LβT2 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). GnRH dose-response study of aromatase PII promoter (A) or PI.f (B) treated for 6h. Effect of E2/GnRH cotreatment (C and D). Cells were stimulated with E2 (10^{-5} M) and Triptorelin (10^{-7}M) for 24h before harvesting. Results are expressed as the fold induction over the basal activity value and are the mean ± SEM of three independent experiments in triplicate. Values without common notations (a, b, c) differ significantly ($P < 0.01$).

TABLES

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 ± 3.76	abundant leukocytes	≤ 5
Proestrus	249.2 ± 10.90	Proestrus epithelial Nucleated cells	34 ± 4.15[a]
Estrus	ND	Yes	≤ 5
OVX	248.9 ± 9.81	No	≤ 5
OVX+E2	234.3 ± 5.38	Yes	80.6 ± 14.3[b]
ER agonists			
PPT	207.2 ± 6.75	Yes	≤ 5
DPN	206.4 ± 21.13	No	≤ 5
PPT + DPN	217.6 ± 17.21	Yes	≤ 5
Cetrorelix			
Intacts	264.0 ± 10.99	No	≤ 5
OVX	304.0 ± 13.97	No	≤ 5
OVX+E2	258.0 ± 7.55	Yes	547.5 ± 99.63[b]
triptorelin			
Intacts	243.1 ± 4.66	No	6.92 ± 0.94[b]
OVX	247.3 ± 9.30	No	≤ 5
OVX+E2	248.6 ± 7.13	Yes	32.11 ± 4.74[b]

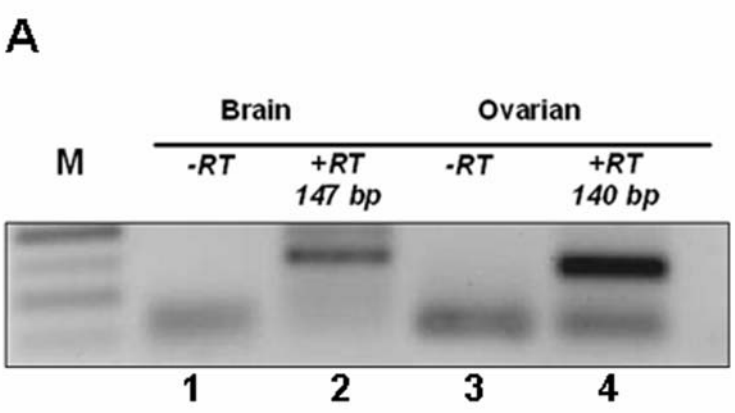
* Values are expressed as the mean ± SEM at last five determinations per group
[a] Indicates significant differences from corresponding Metestrus group (P<0.001)
[b] Indicates significant differences from corresponding OVX group (P<0.001)
ND, Not Done

Table 2. Primers used in this study

Target mRNA	Primer sequence	product (bp)	Temperature	References
Total Aromatase	AROT-F: 5'- TTT ACC CTT GAA AAC TTT GAG AAG AAC -3' **Exon 9** AROT-R: 5'- GTA ACC AGG ACA ACT TTC ATC ATC AC -3' **Exon 10**	122	60	M33986
Brain aromatase	ARO1f-F: 5'- TAA AAG ATG GCA CAC ACA AAG AGT-3' **Exon I.f** ARO1f-R: 5'- GCC TCA GAA GGA AAA ATGTAA A-3' **Exon 2**	147	57	Kato *et al.,* 1997
Ovarian aromatase	AROov-F: 5'- CTG TCC ATT CCA GCA CCC TTA -3' **Exon II** AROov-R: 5'- TTC CAA AAA CAT CTT GTG CTA TTT TG -3' **Exon 2**	140	55	M33986
LHβ	LH-F: 5' - GTA GGG AAG GTA TCA AGA ATG G - 3' **784 805** LH-R: 5' - TTG ACG TCC ACC AGT TAG G - 3' **915 - 933**	149	60	J00749
Actin	ACT-F: 5'- CAA CCG TGA AAA GAT GAC CCA G - 3' **423 - 444** ACT-R: 5'- ATG GGC ACA GTG TGG GTG AC - 3' **565 - 575**	153	60	NM 031144

FIGURES

FIGURE 1



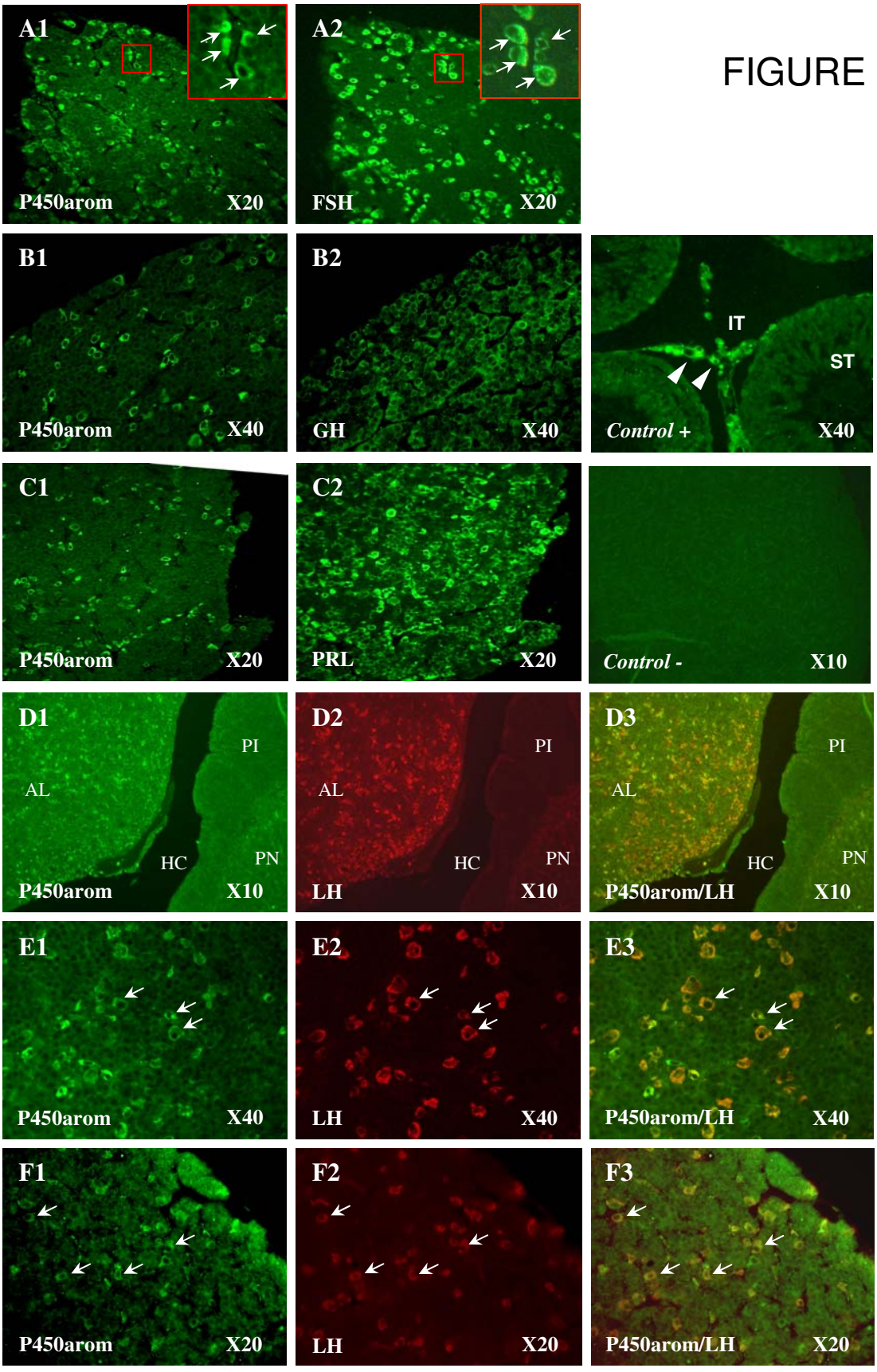

A1
P450arom
X20
A2
FSH
X20
B1
P450arom
X40
B2
GH
X40
IT
ST
Control +
X40
C1
P450arom
X20
C2
PRL
X20
Control -
X10
D1
PI
AL
HC
PN
P450arom
X10
D2
PI
AL
HC
PN
LH
X10
D3
PI
AL
HC
PN
P450arom/LH
X10
E1
P450arom
X40
E2
LH
X40
E3
P450arom/LH
X40
F1
P450arom
X20
F2
LH
X20
F3
P450arom/LH
X20

FIGURE 2

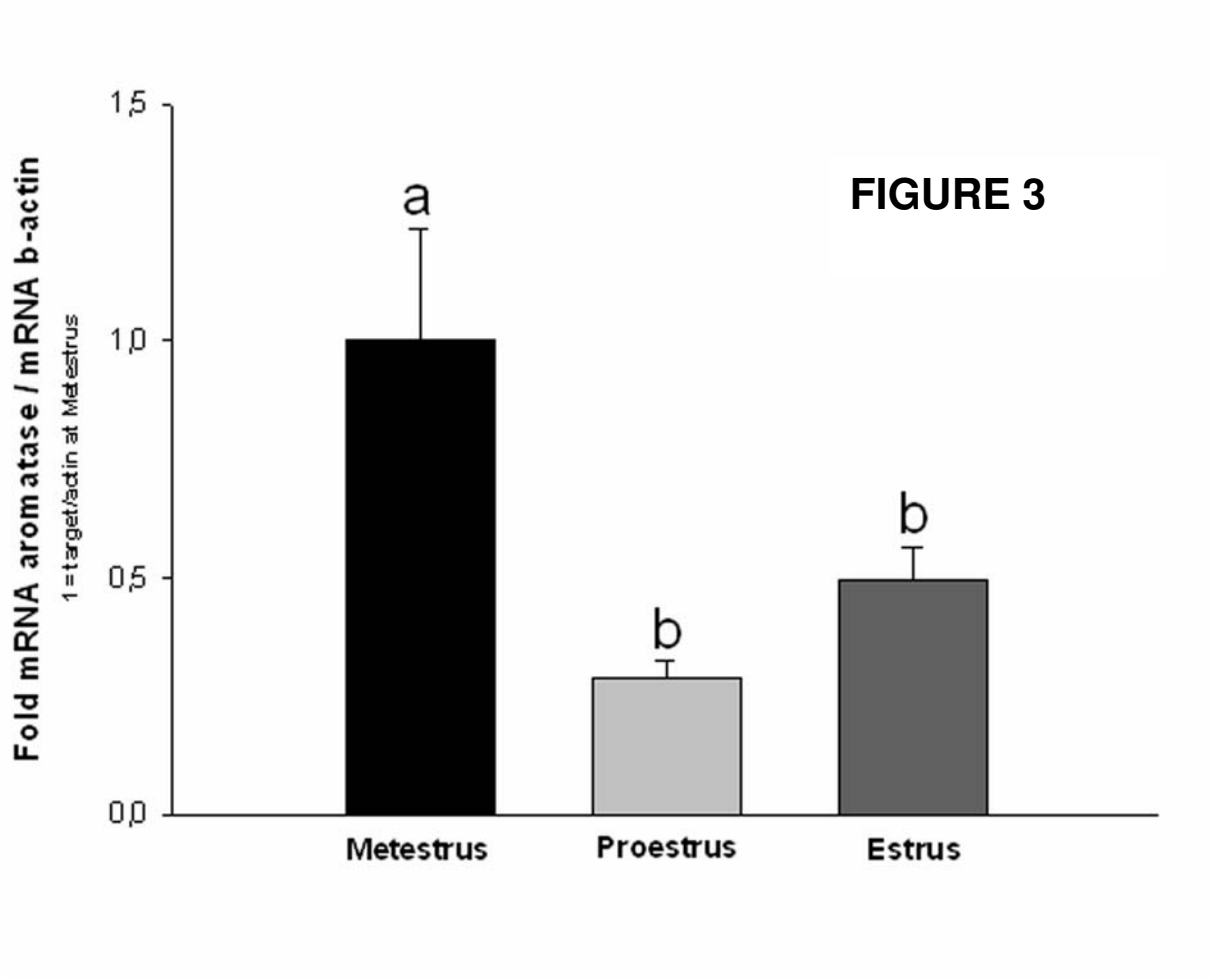
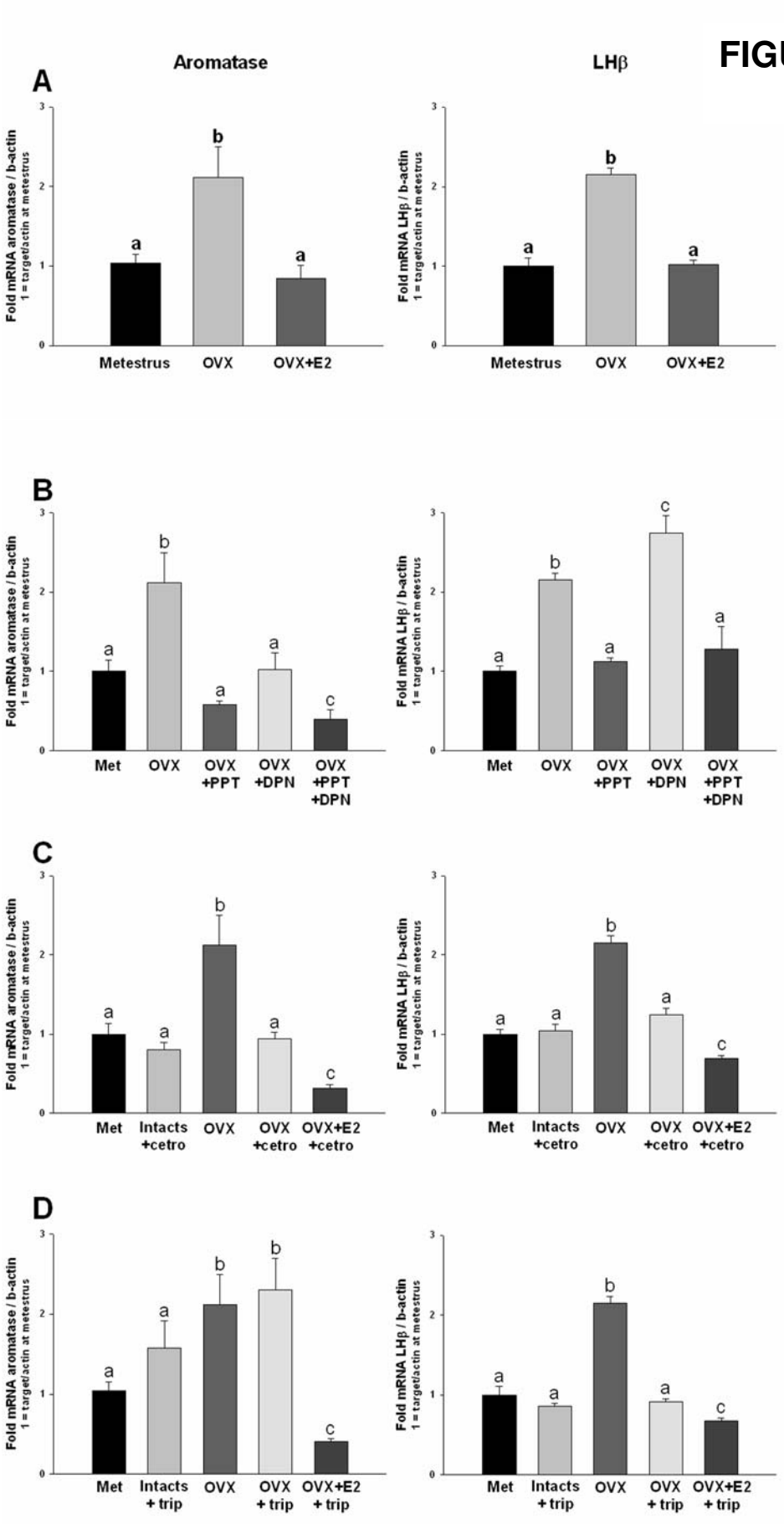

Fold mRNA aromatase / mRNA b-actin
1 = target/actin at Metestrus
1,5
1,0
0,5
0,0
a
b
b
Metestrus
Proestrus
Estrus

FIGURE 3

FIGURE 4


Aromatase
LHβ
A
Fold mRNA aromatase / b-actin
1 = target/actin at metestrus
Fold mRNA LHβ / b-actin
1 = target/actin at metestrus
a
b
a
Metestrus
OVX
OVX+E2
B
a
b
a
a
c
Met
OVX
OVX +PPT
OVX +DPN
OVX +PPT +DPN
a
b
a
c
a
C
a
a
b
a
c
Met
Intacts +cetro
OVX
OVX +cetro
OVX+E2 +cetro
a
a
b
a
c
D
a
a
b
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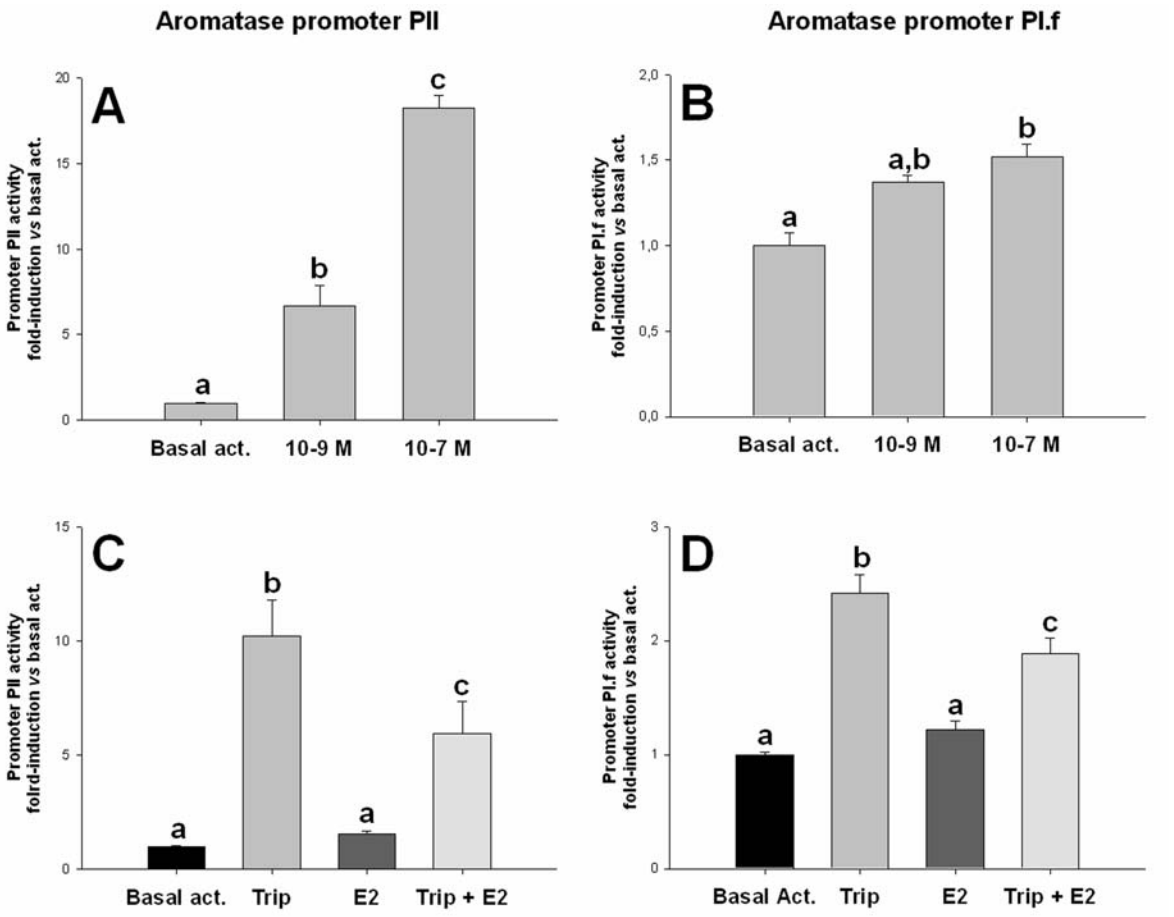
c
Met
Intacts + trip
OVX
OVX + trip
OVX+E2 + trip
a
b
a
c

FIGURE 5


Aromatase promoter PII
A
Promoter PII activity
fold-induction vs basal act.
20
15
10
5
0
a
b
c
Basal act.
10-9 M
10-7 M
Aromatase promoter PI.f
B
Promoter PI.f activity
fold-induction vs basal act.
2,0
1,5
1,0
0,5
0,0
a
a,b
b
Basal act.
10-9 M
10-7 M
C
Promoter PII activity
fold-induction vs basal act.
15
10
5
0
a
b
a
c
Basal act.
Trip
E2
Trip + E2
D
Promoter PI.f activity
fold-induction vs basal act.
3
2
1
0
a
b
a
c
Basal Act.
Trip
E2
Trip + E2