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Presence of Immunoreactive Gonadotropin Releasing Hormone (GnRH) and Its Receptor (GnRHR) in Rat Ovary During Pregnancy

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Abstract

The present study aims at quantification of gonadotropin releasing hormone (GnRH) by radioimmunoassay, relative expression of its mRNA by real-time PCR accompanied by its cellular localization in the rat ovary by immunonohistochemistry (IHC) during different time points of pregnancy. To determine the involvement of endogenous ovarian GnRH in receptor mediated local autocrine/paracrine functions within the ovary, the cell specific localization of the classical receptor for GnRH (GnRHR) in the ovary by IHC and expression pattern of its mRNA were studied during pregnancy. Receptor expression during each time point within the ovary was reconfirmed by Western blot analysis accompanied by densitometric analysis of the signal intensity. Results reveal that the content of ovarian GnRH reaches its maximum on Day 20. The densitometric analysis of GnRHR receptor expression from Western blot study exhibits a decreasing trend by Day 20. Presence of GnRH and GnRHR mRNA in the ovary indicates the local synthesis of both ligand and receptor in the rat ovary. Differential expression of GnRH/GnRHR in the corpus luteum throughout pregnancy strengthens the hypothesis of the involvement of ovarian GnRH in local ovarian functions by receptor-mediated mechanisms. The expression of GnRH and GnRHR in the atretic antral follicles is indicative of the possible involvement of this decapeptide in processes like follicular atresia. The expression of GnRH/GnRHR in the nonatretic antral follicles and their oocytes requires further indepth investigation. Collectively, this study for the first time reveals the presence of endogenous ovarian GnRH/GnRHR supporting their possible involvement in local autocrine/paracrine functions during pregnancy.

Keywords

GnRH; GnRHR; ovary; pregnancy; rats

INTRODUCTION

Gonadotropin releasing hormone (GnRH) was identified originally as a hypothalamic decapeptide which stimulates pituitary gonadotropes to synthesize and release gonadotropins

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(Burgus et al., 1972). The notion that multiple GnRH ligand and receptor forms are distributed in a wide range of vertebrate tissues playing diverse physiological functions has been widely accepted (Millar, 2003). In mammals like rats and human, GnRH and its receptor (GnRHR) have been reported to be synthesized in a myriad of tissues outside their classical site of production such as the ovary (Schirman-Hildesheim et al., 2005), oviduct (Sengupta et al., 2007), endometrium, placenta (Khodr and Siler Khodr, 1978), mammary glands (Palmon et al., 1994). The exact functional significance of the GnRH ligand receptor complex of extra-hypothalamic origin in mammals remains unknown.

Previously, the rat ovarian extract was found to be biologically active in stimulating secretion of gonadotropins from rat pituitary cell culture (Ying et al., 1981). Later, GnRH or GnRH-like peptide was quantified in the rat ovary (Aten et al., 1986; Schirman-Hildesheim et al., 2005) and human ovary (Aten et al., 1987). Detection of rat ovarian GnRH mRNA, cloning of its DNA followed by sequencing, exhibited a 244 base pair sequence identity between hypothalamic and ovarian GnRH in rats (Oikawa et al., 1990). Later, this decapeptide was reported to be produced particularly by the ovarian granulosa cells during the rat estrous cycle (Oikawa et al., 1990; Clayton et al., 1992; Schirman-Hildesheim et al., 2005) where it was suggested to play an autocrine/paracrine role regulating functions like oocyte maturation (Dekel and Shalgi, 1987), follicular selection, atresia (Billig et al., 1994), and luteal functions (Tsafriri and Adashi, 1994). Deleterious effects on reproductive integrity of female rats (Rivier et al., 1981) due to neutralization of endogenous GnRH by the administration of a potent GnRH antagonist confirmed the functional importance of this endogenous decapeptide on ovarian physiology and reproduction.

GnRHR (mRNA and protein) expressed by the ovarian granulosa cells in rats (Bauer-Dantoin and Jameson, 1995; Whitelaw et al., 1995) was reported to increase along with follicular development, exhibiting maximum expression in the mature and atretic follicles (Kogo et al., 1995, 1999a,b). High affinity binding of ovarian GnRH receptors (identical to anterior pituitary GnRHR) to GnRH analogs (Clayton et al., 1979; Jones et al., 1980) in rats, led to the hypothesis that ovarian GnRHRs are activated by an endogenous GnRH like substance produced in the ovary, as hypothalamic peptide concentration in the peripheral circulation is not sufficient to activate the ovarian receptors for GnRH (Hsueh and Jones, 1981; Billig et al., 1994). Exogenous GnRH or its agonist through a direct ovarian site was reported to induce a dose dependent inhibition of ovarian functions (Jones and Hsueh, 1980) in rats. Thus, collectively, the existing information in the literature suggests the presence of an endogenous physiologically active ovarian GnRH system (ligand receptor complex) during the estrous cycle in this rodent model which may play a diverse role in the regulation of the ovarian physiology.

There exists a paucity of information regarding the synthesis, localization, and functional significance of endogenous ovarian GnRH/GnRHR system during pregnancy of mammals in general and rodents in particular. Exogenous GnRH/LHRH as a contraceptive vaccine targeting gamete production and a therapeutic agent in treatment of clinical situations such as uterine fibroids, polycystic ovarian syndrome, precocious puberty, etc. (Naz et al., 2005) in human has been widely accepted. GnRH analogs like desorelin have been known to elicit reversible long-term reproductive control in mammals (Trigg et al., 2001, 2006). An earlier

study from our laboratory involving localization of immunoreactive GnRH and its mRNA in the oviduct during pregnancy in rats (Sengupta et al., 2007) indicated the existence of a positive correlation between the expression pattern of oviductal GnRH and ovarian progesterone production during pregnancy. This study provides an indication that endogenous nonneuronal GnRH produced by the reproductive tissues in rats may have a role to play during pregnancy. The present study thus aims at quantification and immunolocalization of ovarian GnRH and study the expression of its mRNA during different time points of pregnancy (Days 7, 9, 16, 20) in this animal model. Further, to explore the possibility of involvement of this endogenous ovarian decapeptide in a receptor mediated local function during pregnancy, we attempted to quantify and immunolocalize its receptor peptide (GnRHR) accompanied by the study of the expression pattern of its mRNA in the rat ovary during each of the chosen time points. The selected time points during pregnancy correspond to changes in the serum profiles of progesterone critical for induction and maintenance of pregnancy in rats related to circulating levels of luteinizing hormone and prolactin (PRL) (Morishige et al., 1973).

MATERIALS AND METHODS

All experimental protocols in this study were approved by the Institutional Animal Care and Use committee (IACUC) and were in compliance with the animal care regulations of Morehouse School of Medicine. Accepted standards of NIH guide for humane care and use of experimental animals were followed in maintaining the animals. Timed-pregnant Sprague-Dawley rats (8 weeks old) used for the present study were obtained from Charles River laboratories (Wilmington, MA) and housed in the Institutional Animal Care Facility for a week under controlled daily light (14 hr light:10 hr darkness) and temperature (23–25°C) conditions. Purina rat chow and tap water were accessible ad libitum to the animals during this period. Considering the day of insemination (identified by the appearance of the sperm plug) as Day 1 of pregnancy, animals (n = 4) were sacrificed subsequently on each of the 7th, 9th, 16th, and 20th day of pregnancy by exposing them to a chamber of carbon dioxide for 5–10 min.

Tissue Collection

Implantation within the uterus was checked for each animal during each of the considered time points to ensure induction of pregnancy prior to collection of the tissues. The paired ovaries along with the hypothalamus, pituitary, and the liver of each animal (n = 3–4) were dissected out on different days of pregnancy. One ovary of each pair, a part of the hypothalamus and pituitary were fixed in neutral formalin solution overnight, embedded in paraffin, cut into 10 μm sections and mounted on 5% gelatin coated slides for immunostaining. The other ovary along with the remaining part of the hypothalamus, pituitary and liver were snap frozen in liquid nitrogen and stored at −80°C for extraction of RNA (for real-time PCR) and protein (for radioimmunoassay and Western blot analysis).

Radioimmunoassay of GnRH

Tissue extraction for GnRH.—The tissues were extracted based on standard published protocols (Pati and Habib, 1998; Schalburg et al., 1999; Yahalom et al., 1999; Montaner et

al., 2002) with modifications. Standardization of the extraction method was done using hypothalamus (positive control), liver (negative control), and ovary (tissue of interest) with or without externally added native GnRH (20 pg; human hypothalamic GnRH, Sigma, St. Louis, MO). Pre-weighed frozen tissues were homogenized in ice cold 1 N HCl: acetone mixture (3:100, v/v; 5 ml extraction medium/g tissue) in a Teflon homogenizer and incubated for 3 hr on ice, homogenizing for 1 min after every hour. The extraction mixture was centrifuged at 1,000*g* for 5 min at 4°C and supernatant (S_1) was separated. The pellet was homogenized and re-extracted in ice cold 0.01 N HCl: acetone (20:80, v/v; 40% of the original extraction fluid) for 5 min. The extraction mixture was centrifuged again at 1,000*g* at 4°Cfor 5 min and the supernatant (S_2) was pooled with (S_1). This process of re-extraction was repeated with the resultant pellets at least five times to ensure completion of the extraction process. Each time the supernatants were pooled together to make a final volume of 1 ml. The combined supernatant was centrifuged at 15,000*g* for 15 min at 4°C and the final suspension thus obtained was subjected to vacuum drying in a speed vacuum (Savant Speed Vac, Global Medical Instrumentation Inc., Ramsey, MN, SPD111V) to remove acetone. The dry pellet containing peptides was suspended in ice-cold RIA buffer (pH 7.4, provided in the RIA kit obtained from Peninsula Laboratories, Inc., Santa Carlos, CA) and used for radioimmunoassay.

Radioimmunoassay (RIA).—RIA was performed by using the rabbit antiserum (polyclonal) raised against the synthetic GnRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) and I^{125} GnRH tracer supplied in LHRHRIA kit [S-2153(RIK 7201)] from Peninsula Lab., Inc. (Division of Bachem) following manufacturer's instructions. Final dilution of the primary antibody used was 1:42,000 and the cross reactivity of this antiserum was mentioned as 100% to mammalian GnRH and 0–0.1% to other proteins. A standard curve was prepared during each assay by using synthetic GnRH, provided in the kit, with a range of 1–128 pg/tube and RIA efficiency was determined in control samples (hypothalamus and liver) with and without externally added synthetic GnRH (32 pg/tube). The volume of the extracted suspension in RIA reaction was pre-normalized by diluting with RIA buffer so that the RIA value remains closer to 50% maximum binding (B/B_0). After the completion of reaction, the radioactivity in the pellet fraction was measured in a gamma counter (ISO-DATA, Model 110, Series 100 operation manual, USA). Intra-assay variations were reduced by analyzing each sample in duplicate while inter-assay variations were minimized by construction of a standard curve during each assay with an internal control (hypothalamus or ovary).

Isolation of Total RNA

Frozen ovaries along with portions of the hypothalamus and pituitary were weighed and homogenized in 1 ml of lysis buffer containing 10 μl of ß-mercaptoethanol provided in the RNeasy midi kit (Qiagen, Inc., Valentia, CA). The homogenate was centrifuged at 3,200*g* for 10 min at room temperature. The supernatant was loaded on an RNeasy midi column (Qiagen, Inc.) and the entire process of RNA isolation and purification was carried out according to the detailed manufacturer's instruction provided in the kit. The RNA samples were eluted in 25 μl nuclease free water and quantified in an Eppendorf AG 2231 Biophotometer (Hamburg, Germany). 260:280 nM ratios (which were in the range of 1.8

and 2.1) helped to assess the purity of the extracted RNA. RNA integrity was assessed by examining the 28S and 18S rRNA bands of representative samples loaded on an Agilent 2100 Bioanalizer using RNA 6000 nano assay kit (Agilent Technologies, Santa Clara, CA).

Reverse Transcription

Two micrograms of RNA samples from each tissue type (ovary, hypothalamus, liver, and pituitary) were subjected to reverse transcription at 48°C for 37 min, using Murine Leukemia Virus reverse transcriptase enzyme and random hexamers (Taqman Reverse Transcription Reagent kit, Applied Biosystems, Foster City, CA) for conversion to cDNA according to manufacturer's instructions. The cDNA samples were stored at −20°C for real-time PCR studies.

Real-Time PCR

Sequence of *Rattus norvegicus* GnRH and GnRHR mRNA (accession numbers; NM_012767 and NM_031038, respectively) was obtained from the gene bank database of the National Center for Biotechnology Information of NIH (http://www.ncbi.nlm.nih.gov/cgi-bin/genbank). Using OligoPerfect™ Designer software (Invitrogen Corporation, Carlsbad, CA), primer sequences were selected to optimally hybridize and amplify target cDNA sequences for real-time PCR assay. To avoid amplification of contaminating genomic DNA, primers specific for GnRH, GnRHR, and 18S rRNA (housekeeping gene) were designed across the exon/intron boundaries (descriptions given in Table 1). The annealing temperatures of the specific primers for GnRH/GnRHR were determined from the temperature curve. Construction of a melt curve determined the absence of primer dimer formation and nonspecific fluorescent signals. The final annealing temperature of the sense and antisense GnRH/GnRHR and 18S rRNA primers were determined to be 54°C (temperature at which the cycle threshold CT value was minimum and fluorescence value was maximum).

The quantitative PCR assay was performed with the help of a MyiQ iCycler (Bio-Rad Laboratories, Inc., Hercules, CA) real-time PCR detection system. Seven hundred and fifty nanograms of cDNA for each reaction was used in case of GnRH and 650 ng of cDNA in case of GnRHR (as determined from the standard curve of rat GnRH/GnRHR constructed using various dilutions of ovarian cDNA). Each gene expression was assessed in a separate PCR reaction. Each reaction mixture contained 2 μl of sense and antisense primers (0.35 nM/μl concentration) for GnRH/GnRHR and 18S rRNA, respectively, 10 μl of 2× quantiTech SYBERGreen PCR Master Mix (containing HotstarTaq® DNA polymerase enzyme, quantiTech SYBERGreen PCR buffer, dNTP mix including dUTP, fluorescent dyes like SYBERGreen 1, and ROX and 5 mM $MgCl_2$) obtained from Qiagen, Inc., and DNAse RNAse free water to make a total volume of 20 μl. Samples were amplified with a pre-cycling hold of 95°C for 15 min, 50 cycles of denaturation at 94°C for 15 sec, annealing at 54°C for 30 sec, and extension at 72°C for 30 sec. The reactions were terminated at 72°C for 5 min and cooled to 4°C. The number of amplification cycles for the fluorescence to reach a determined threshold value (CT) was recorded for every unknown sample and for the internal standard curves for GnRH/GnRHR. CT values for the unknown samples were used to extrapolate the amount of RNA equivalents from the internal standard curves. The RNA

equivalent values were then divided by complimentary 18S RNA equivalents also derived from the same internal standard curve (Young et al., 2002) and the ratio of GnRH/GnRHR to 18S RNA equivalent values were plotted graphically. The rat hypothalamus and liver tissues (with maximum and minimum GnRH gene expression, respectively) were used as positive and negative controls, respectively, for real-time PCR analysis of GnRH mRNA while rat pituitary and liver served as positive and negative controls, respectively, for analysis of the GnRHR mRNA expression in the rat ovary.

Immunohistochemistry of GnRH and GnRHR

Ovarian sections were deparaffinized in xylene, rehydrated in graded ethanol, and rinsed in a running water bath. For immunohistochemical procedures, the tissue sections treated with citrate buffer (10 mM, pH 6.0*)* for antigen retrieval and 0.3% hydrogen peroxide solution in methanol (for quenching of endogenous peroxide activity), were equilibrated in 50 mM Tris Buffered Saline (TBS, pH 7.6). Nonspecific binding was reduced by incubation in 1% normal rabbit serum (provided in the Vector ABC kit, Vector Laboratories, Burlingame, CA) for 1 hr at 37°C. Mouse monoclonal anti-GnRH antibody (100% immunoreactivity with fully processed bioactive form of LHRH, sequence: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2; catalog number MAB5456; Chemicon International, Temecula, CA) diluted to 1:200 in 1× phosphate buffered saline (PBS, Gibco, Invitrogen Corporation) was used for immunolocalization of GnRH. For localization of GnRHR, goat polyclonal anti-GnRHR primary antibody (GnRHR C-18: sc-8681; raised against a peptide mapping at the C-terminus of GnRHR of human origin; Santa Cruz Biotechnology, Inc., Santa Cruz, CA; specificity for GnRHR shown in other studies, Keller et al., 2005) in a dilution of 1:200 in PBS buffer was used. Incubation with primary antibodies was done for 1 hr at 37°C temperature followed by overnight incubation at 4°C. Negative controls included sections in which the primary antibodies were omitted or were pre-absorbed with a synthetic GnRH/GnRHR peptides (Peninsula Laboratories, Inc.; Santa Cruz Biotechnologies, Inc.) respectively. After washing off the unbound primary antibody, the sections were incubated at 37°C for 30 min with biotinylated universal secondary antibody (for GnRH) and rabbit antigoat secondary antibody (for GnRHR) provided in separate Vectastain ABC kits (Vector Laboratories) in dilutions of 1:50 in PBS buffer. Incubation of sections in ABC complex (avidin and biotinylated horseradish peroxidase macromolecular complex, Vectastain ABC kit, Vector Laboratories) at 37°C for 30 min was followed by treatment of the tissue sections with DAB (0.03% of 3,3′-diaminobenzidine in PBS containing 0.01% hydrogen peroxide) for 5 min in darkness for visualization of peroxidase activity at the antigen sites. The sections were then counterstained with hematoxylin (QS, H3404, Vector Laboratories), dehydrated in graded alcohols and xylene and mounted with Vectasheild mounting medium (Vector Laboratories) for light microscopy (preparation of photomicrographs using Axioshop 2 plus software with Zeiss microscope, Carl Zeiss Microimaging, Inc., Thornwood, NY).

Western Blot Analysis for GnRHR

Tissue (rat pituitary and ovary) was homogenized in 1-ml lysis buffer containing 0.15 M NaCl, 2 mM EDTA, 0.15% Triton X-100 and protease inhibitors (protease inhibitor cocktail, catalogue number P8340; Sigma–Aldrich Inc., St. Louis, MO, USA) for extraction of total

protein. The lysate was centrifuged at 18,000*g* for 20 min at 4°C, the supernatant was collected and protein content was estimated by Lowry's method (Lowry et al., 1951) using BSA as standard. The supernatant was diluted with equal volume of lamelli sample buffer (catalogue number 161–0737; Bio-Rad Laboratories, Inc.) and boiled in a boiling water bath for 10 min for linearization of the proteins. The linearized protein samples were then cooled on ice and centrifuged at 11,000*g* for 10 min at 4°C. The supernatant collected was used for Western blot analysis. Total protein from each sample (35 μg) were electrophoresed on 15% Tris–HCl pre-cast mini gels (catalogue number 161–1157; Bio-Rad Laboratories, Inc.) for 45 min under reducing conditions. The proteins were electro-transferred on nitrocellulose paper (pore size 0.2 μm, Bio-Rad Laboratories, Inc.) for 1 hr at 100 V. Nonspecific binding was reduced by incubating the membrane at room temperature in 2.5% nonfat dry milk solution in TBS containing 0.1% Tween 20. The membrane was then incubated overnight at 4°C with anti-GnRHR mouse monoclonal primary antibody (Lab Vision Corporation, Fremont, CA; catalogue number MS-1139-P; Clone designation GNRH03 same as FIG4; raised against a synthetic peptide 1–29 aa long MANSASPEQNQNHCSAINNSIPLMQGNLPY form the N terminal end of Human GnRH receptor) diluted to 1:100 in the same buffer solution containing nonfat dry milk described above. After washing the membrane extensively in washing buffer (TBS—0.1% Tween 20) the membrane was incubated for 1.5 hr at room temperature with horseradish peroxidase-conjugated antimouse IgG (Immun-Star™ GAM-HRP conjugate catalogue number:170–5047; Bio-Rad Laboratories, Inc.) in a final dilution of 1:2,000 in TBS–0.1% Tween 20 solution. The membrane was washed several times in the washing buffer to remove unbound secondary antibody and the signal was detected by enhanced chemiluminescence (ECL; Bio-Rad Laboratories, Inc.). The films were analyzed and the signals were quantified using Image J software (version 1.33U, a program inspired by NIH image; http://rsb.info.nih.gov/ij/docs/index.html). The results were expressed according to the intensity of the signals in arbitrary densitometric units (ADU; Shao et al., 2007) after normalization by alpha tubulin as an internal standard.

Statistical Analysis

All data are presented as mean ± SEM. Statistical evaluation of the mean differences in data amongst the chosen time points of pregnancy were performed by one-way ANOVA with significance levels set at $P \leq 0.05$. If the analysis of variables exhibited significant differences between groups, Student's–Newman–Keuls Multiple Comparisons Test was used for pairwise multiple comparisons.

RESULTS

Quantification of GnRH by Radioimmunoassay

The data reveal the presence of GnRH in the rat ovary throughout pregnancy with a significant increase in the content of ovarian GnRH by Day 20 when compared to Days 7, 9, and 16 of pregnancy (Fig. 1A). Hypothalamic content of GnRH exhibited no significant difference between considered time points of pregnancy. However, the content of hypothalamic GnRH was found to be significantly higher than the content of ovarian GnRH (Fig. 1A, insert).

Expression of GnRH mRNA in the Rat Ovary by Real-Time PCR

18S mRNA expression remained constant (data not shown) in all tissues throughout pregnancy during real-time PCR studies for both GnRH and GnRHR mRNA (discussed later in this section) which is reflective of the integrity of the RNA samples used for the study and the validity of the real-time PCR method. The expression of GnRH mRNA in the rat hypothalamus (n = 5) was significantly higher when compared to the average expression in ovaries (n = 12) (Fig. 1B: insert). Lack of GnRH mRNA expression was noted in the rat liver (data not shown). Pair wise comparison between the chosen time points of pregnancy revealed a significantly higher expression of GnRH mRNA in the rat ovary during Day 7 when compared to Days 9, 16, and 20. The expression of GnRH mRNA from Day 9 through Day 20 remained unchanged (Fig. 1B).

Immunolocalization of GnRH in the Corpus Luteum

Strong immunoreactivity for GnRH was noted in the corpus luteum (CL) within the rat ovary throughout pregnancy (Fig. 2). The luteal cell types (granulosa and thecal luteal cells) in the rat CL are found to be scattered within the CL with no distinct morphological separation. Strong immunoreactivity for GnRH was exhibited in the cytoplasm of both the cell types. The round shaped nuclei of such steriodogenic cells remained immunonegative. The intermediate fibroblast and stromal cells within the CL identified by their elongated nuclei were immunonegative. The size of each immunopositive luteal cells was much smaller toward early pregnancy (Days 7, 9). By Day 16 there was notable increase in the cell size and volume of immunostained cytoplasm which attained its maximum by Day 20 (a marker of luteal regression).

GnRH Immunopositivity in the Follicles

The rat ovary exhibited the presence of a large population of nonatretic and atretic antral follicles during post-implantation period of pregnancy with only a negligible population of developing follicles. The nonatretic antral follicles having a fully grown oocyte, several layers of granulosa cells, well differentiated theca and a fluid filled antrum were present predominantly in the ovary during Days 7, 9, and 16 and were negligible in number during Day 20 of pregnancy (Fig. 3). The granulosa or the thecal cells within the few developing follicles found within the ovary did not exhibit immunostaining for GnRH during any of the time points of pregnancy. Within the nonatretic antral follicles immunostaining for GnRH in the granulosa cells (G) were much less intense when compared to the immunostaining in the thecal cells (T). Intensity of immunostaining for GnRH was highest in the thecal cells of such follicles during Day 20. The oocytes (O) within the nonatretic antral follicles during Days 7, 9 were immunopositive for GnRH although such structures were not visible in the representative follicles during the later stages of pregnancy (Days 16, 20). The atretic antral follicles which dominated the population of the ovarian follicles during pregnancy in rats were identified by the presence of a larger follicular antrum containing cellular debris, lack of basement membrane integrity, and hypertrophy of the thecal layer. The thecal layer of such follicles exhibited intense immunostaining for GnRH during Days 7, 9 when compared to Days 16, 20 while the granulosa cell layer exhibited strong immunopositivity for GnRH during Day 20 when compared to Days 7, 9, and 16.

Western Blot Analysis of GnRHR in the Rat Ovary

Western blot analysis for GnRH receptor in rat ovaries demonstrated expression of the GnRHR protein through signals corresponding to 62 kDa (approx) during each selected time point of pregnancy. Figure 4A depicts the representative GnRHR expression in a single animal. Relative levels of GnRHR protein expressed in the pituitary and ovary of this animal has been shown in ADU (a ratio of the signal intensities of GnRHR to alpha tubulin) during each time point of pregnancy (Fig. 4A) as a representative data. A trend of decrease in ADU of ovarian GnRHR is noted by Day 20 when compared to Day 7 (Fig. 4A). The expression of GnRHR in the pituitary and ovary (Fig. 4A) and the relative levels of the signal intensity measured in ADU were comparable during pregnancy in rats. This experiment was done using a sample size of n = 3 but a representative data from a single animal are presented as the significance of the observation is being masked by the individual variations in the GnRHR expression between animals when the average GnRHR expression from n = 3 was used. But for the convenience of the readers, individual values of the ADU of the other two animals killed during each time point has been documented (n = 1: D7 = 83, D9 = 78, D16 = 66, D20 = 12; n = 2: D7 = 63, D9 = 83, D16 = 73, D20 = 19).

Expression of Ovarian GnRHR mRNA by Real-Time PCR

Expression of GnRHR mRNA in the rat ovary is as much as the expression in pituitary if not more (Fig. 4B, insert). Negligible expression of GnRHR mRNA was noted in the rat liver (data not shown). Expression of GnRHR mRNA in the rat ovary during early pregnancy (D7) was significantly higher when compared to its expression during late pregnancy (D20, Fig. 4B). The decrease in the mRNA expression of GnRHR between Days 7, 9, and 16 is statistically insignificant (Fig. 4B).

GnRHR Expression in the CL

The distribution of GnRHR immunopositive cells in the rat ovary was dependent on the stage of pregnancy. Strong GnRHR immunoreactivity was localized within the cytoplasm of the all luteal cells (the granulosa and thecal luteal cells cannot be differentiated in rat CL as mentioned earlier) within the CL during Days 7, 9, and 16 of pregnancy (Fig. 5). The rounded nuclei of such cells along with the stromal and fibroblast cells within the CL exhibited lack of immunoreactivity during each day of pregnancy. GnRHR immunopositive cells were less abundant and scattered within the CL toward late pregnancy (Day 20) and size of the luteal cells were found to increase considerably from Day 16 through Day 20 when compared to those cells on Days 7 and 9.

GnRHR Expression in the Ovarian Follicles

As mentioned earlier (section of IHC for GnRH), the follicular population within the ovary of rats during pregnancy is predominantly atretic antral follicles, with fewer number of nonatretic antral follicles. Stages of developing follicles are sparse in the rat ovary during pregnancy. The identifying features of atretic and nonatretic follicles have been described previously. The nonatretic antral follicles exhibited intense staining for GnRHR in the oocyte (Fig. 6) with faint expression of this receptor in the granulosa (G) and thecal (T) cells. As such follicles were very few in number in the rat ovary during Day 20 of pregnancy

we could not find many of them. Atretic antral follicles exhibited intense immunopositive staining for GnRHR in the thecal layers (Fig. 6) of Days 7, 9 and 16 which decreased significantly by Day 20. Expression of this receptor protein in the granulosa cell layer during Days 7 and 9 was notable when compared to Days 16 and 20 (Fig. 6).

DISCUSSION

The present study, for the first time, provides evidences for the presence of GnRH-GnRHR system in the rat ovary through a period corresponding between post-implantation to pre-parturition period of pregnancy. The presence of the message of GnRH and GnRHR in the rat ovary during each of the time points tested, demonstrates the local endogenous synthesis of the ligand receptor complex in the rat ovary during pregnancy. The endogenous ovarian GnRH content measured by RIA was found to be significantly lower than the hypothalamic GnRH content. When compared between the specified time points of pregnancy, while the endogenous GnRH content was found to increase significantly by Day 20 when compared to Days 7, 9, and 16, GnRHR mRNA content was found to decrease by Day 20 of pregnancy. There was a decreasing trend in the ovarian GnRHR peptide by Day 20 compared to Day 7. Inverse expression pattern of GnRH/GnRHR in the rat ovary may be indicative toward a physiological role of the endogenous ovarian GnRH system during pregnancy in rats. Immunolocalization of GnRH/GnRHR helped to identify the luteal cell expression of this ligand receptor complex throughout pregnancy while the atretic and nonatretic antral follicles were found to differentially express GnRH and GnRHR depending upon the stage of pregnancy in rats.

The working hypothesis of our laboratory postulates that an endogenous GnRH system present in the ovary/CL has a physiological role to play in regulation, maintenance and/or termination of pregnancy in rats (Sridaran et al., 1999a). Based on this hypothesis as well as established reports regarding the impact of hormones like PRL, lutenizing hormone (LH), rat placental lactogen (rPL), and progesterone on the maintenance of rat pregnancy (Morishige et al., 1973), we selected Days 7, 9, 16, and 20 as the specific time points of pregnancy for this study. The significant increase in the content of ovarian GnRH accompanied by trend of decrease in the signal intensity of GnRHR in the ovary by Day 20 (pre-parturition) of pregnancy supports the contention that ovarian GnRH though a receptor mediated autocrine/paracrine mechanism may result in parturition and thus termination of pregnancy. However expression or presence of endogenous GnRH/GnRHR system in early and mid pregnancy may probably play a role in the regulation or maintenance of pregnancy (Sridaran et al., 1999a,b) in rats. An earlier report in this rodent model (Rivier et al., 1981) describes the functional importance of endogenous GnRH, on events like embryonic development and pregnancy. Collectively, ovarian GnRH may have a receptor mediated local stimulatory/inhibitory role to play on ovarian functions in rats (as is reported in human by Vaananen et al., 1997) depending upon the stage of pregnancy.

The present data exhibit an inverse expression of GnRH mRNA and protein in the rat ovary during post-implantation period of pregnancy. High concentration of GnRH as one of the factors among others like estrogen, FSH/hCG results in down-regulation of its mRNA expression in human ovary exist (Kang et al., 2003; Leung et al., 2003; Harrison et al., 2004;

Choi et al., 2006). Such an interaction between peptide and GnRH mRNA content may result in the inverse expression pattern of the concerned mRNA and the peptide in the rat ovary during the considered time points of pregnancy. But this statement requires further in-depth investigation. The mRNA expression of GnRH in the rat ovary when compared to the hypothalamic GnRH mRNA expression was significantly lower which validates the real-time PCR studies and is in agreement with previous documented literature (Oikawa et al., 1990). Conversely ovarian expression of GnRHR mRNA was as high as in the rat pituitary which is the first report of its kind highlighting the role of endogenous ovarian GnRH/GnRHR system during pregnancy in this rodent model. Relatively higher expression of GnRHR mRNA toward early pregnancy (Day 7) followed by a gradual decrease by Day 20 is interesting. This decrease in the expression of GnRHR mRNA supports the observed trend of decrease in the expression of GnRHR protein (Western blot studies) by Day 20 when compared to Day 7. But confirmation of the observed decrease in the translation of GnRHR peptide toward the tail end of pregnancy in rats requires additional studies, as such a finding may highlight high ligand induced downregulation of the GnRH receptor expression in the rat ovary, as has been reported in the pituitary (Zilberstein and Zakut, 1983; Naik et al., 1984; Garcia-Palencia et al., 2007) and ovarian granulosa cells (Ranta et al., 1982).

It is noteworthy that the immunostaining for GnRH and its receptor were prominently localized in the cytoplasm of the luteal cells (sites of steriodogenesis) within the mature as well as developing CL. The fibroblast and endothelial cells intervening the luteal cells were immunonegative. Despite GnRHR being a G protein coupled transmembrane receptor; its cytoplasmic expression is believed to represent neo-synthesized or internalized receptors (Hopkins and Gregory, 1977; Choi et al., 2006). In the rat ovary, based on the stringent stereospecificity of GnRH action in the ovarian cells, local production of GnRH in the ovarian component is speculated to contribute in events like ovulation, luteinization, luteolysis (Jones and Hsueh, 1981), regulation of steroidogenesis (Clayton et al., 1979), and apoptosis (Sridaran et al., 1998; Sridaran et al., 1999b; Zhao et al., 2000). Some of these functions like lutenization may be modulated indirectly through regulation of progesterone receptor expression, a critical factor for lutenization (Natraj and Richards, 1993; Rivier et al., 1981) while others like luteolysis through induction of matrix metalloproteinases which degrades collagens result in involution of CL in rats (Goto et al., 1999). In the light of these observations we can hypothesize that expression of GnRH and GnRHR ligand receptor system in the luteal cells of rat ovary during the chosen time points of pregnancy provides an indication toward involvement of endogenous ovarian GnRH in events like lutenization during early pregnancy and luteolysis during late pregnancy through a receptor mediated mechanism. Given that a number of studies have implicated a role of GnRH in the regulation of progesterone production in both rat (Clayton et al., 1979) and human (Peng et al., 1994) ovary, it is tempting to speculate that ovarian GnRH depending upon its content, through its local receptors stimulate or inhibit progesterone secretion thus influencing processes like lutenization and luteolysis within the ovary. But further studies will be required for validation of this prediction in terms of identification of the exact molecular mechanism through which GnRH may induce these events during early or late pregnancy.

In rats, the regulatory peptide GnRH and its agonist have been shown to exert direct inhibitory effects on follicular differentiation by acting through specific ovarian receptors on

granulosa and thecal cells of the follicles (Jones et al., 1980). Furthermore endogenous GnRH in the rodent ovary is reported to play a potential role as an atretogenic factor through induction of ovarian apoptotic DNA fragmentation (Billig et al., 1994; Hsueh et al., 1994). Down regulation of FSH stimulated LH receptor expression by GnRH in rat granulosa cells (Harwood et al., 1980; Seguin et al., 1982) leads to loss of follicular integrity and appearance of atretic characteristics like reduction of the granulosa cells layer and appearance of pyknotic nucleus (Whitelaw et al., 1995). During late antral development persistent expression of GnRH receptor in the absence of LH receptor expression appears to be indicative of follicular atresia (Whitelaw et al., 1995). In the present study, differential expression of GnRH and GnRHR is restricted to the thecal cells of the antral follicles undergoing atresia during Days 7, 9, 16, and 20 of pregnancy with comparatively lesser expression in the granulosa cells. The expression of the ligand receptor complex in such cell layers within the nonatretic antral follicles is also notable. This study taken together with the previous reports (Bauer-Dantoin and Jameson, 1995), provides support for a role of autocrine/paracrine receptor mediated role of ovarian GnRH in the induction and progression of follicular atresia under certain physiological conditions like pregnancy in rats when hypothalamic GnRH concentrations in the peripheral circulations may not be sufficient to activate the ovarian GnRH receptors (Hsueh and Jones, 1981). Strong immunoreactivity for both GnRH and GnRHR in the oocytes of nonatretic antral follicles was noted throughout pregnancy. This is consistent with the studies which have suggested that GnRH may play a role in the induction of ovulation through enhancement of meiotic maturation of oocyte and stimulation of germinal vesicle breakdown (Hillensjo and LeMaire, 1980). The expression of the ligand receptor complex in the granulosa and thecal cell layers of nonatretic antral follicles cannot very well be explained at this stage of the present study as a detailed study on follicular maturation and the role of GnRH and GnRHR if any on such mechanisms in rats/mammals is required in future.

In conclusion, our study for the first time quantifies ovarian GnRH/GnRHR by RIA and Western blot analysis, respectively, during pregnancy in rats accompanied by the cell specific localization of decapeptide and its receptor in the ovary during each chosen time point of pregnancy. Quantitative expressions of GnRH and GnRHR mRNA by real-time PCR in the ovarian extracts highlights the local synthesis of this hormone receptor system in the ovarian cells during pregnancy in this rodent model. High content of GnRH and by the tail end of pregnancy (Day 20) indicates the possible functional involvement of this ovarian decapeptide in processes like parturition. Although a trend of decrease in the expression of GnRHR peptide by Day 20 of pregnancy when compared to Day 7 is supported by a subsequent significant decrease in the transcription of GnRHR mRNA toward tail end of pregnancy (Day 20), this study requires further confirmation through future in-depth investigation. Differential expression of the ligand and receptor in the luteal cells within the CL and the thecal/granulosa cells of the atretic antral follicles within the ovary throughout pregnancy provides an indication toward a possible concentration dependent involvement of ovarian GnRH/GnRHR in promoting processes like lutenization/luteolysis in the CL and follicular atresia in the atretic antral follicles in an autocrine/paracrine mechanism during pregnancy in rats. The expression of the ligand receptor system in the oocyte or thecal/granulosa layers in the nonatretic antral follicles requires further in-depth investigation. The

current data thus provide groundwork for further research into the exact functional significance of ovarian GnRH/GnRHR system during pregnancy in rats.

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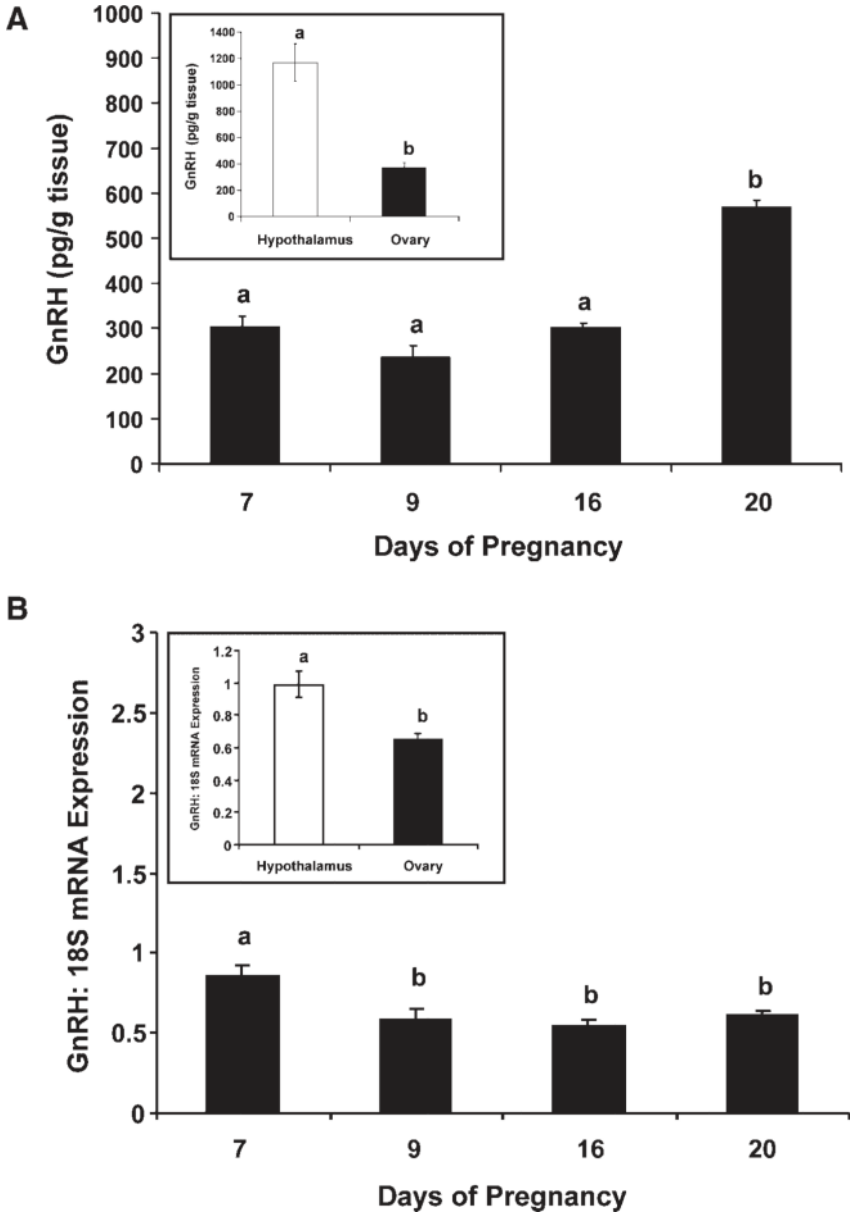

Fig. 1.

A: Ovarian GnRH content (mean ± SEM) during chosen time points of pregnancy in rats (n = 3/day). Insert represents significant difference in the mean hypothalamic (n = 13) and ovarian (n = 13) GnRH content during pregnancy. **B**: GnRH mRNA expression in the rat ovary (mean ± SEM). Values were standardized to respective 18S control values. Group means with different superscript letters differ significantly ($P \leq 0.05$). Insert represents comparison between the mean GnRH mRNA expression in the hypothalamus (n = 5) and ovary (n = 12).

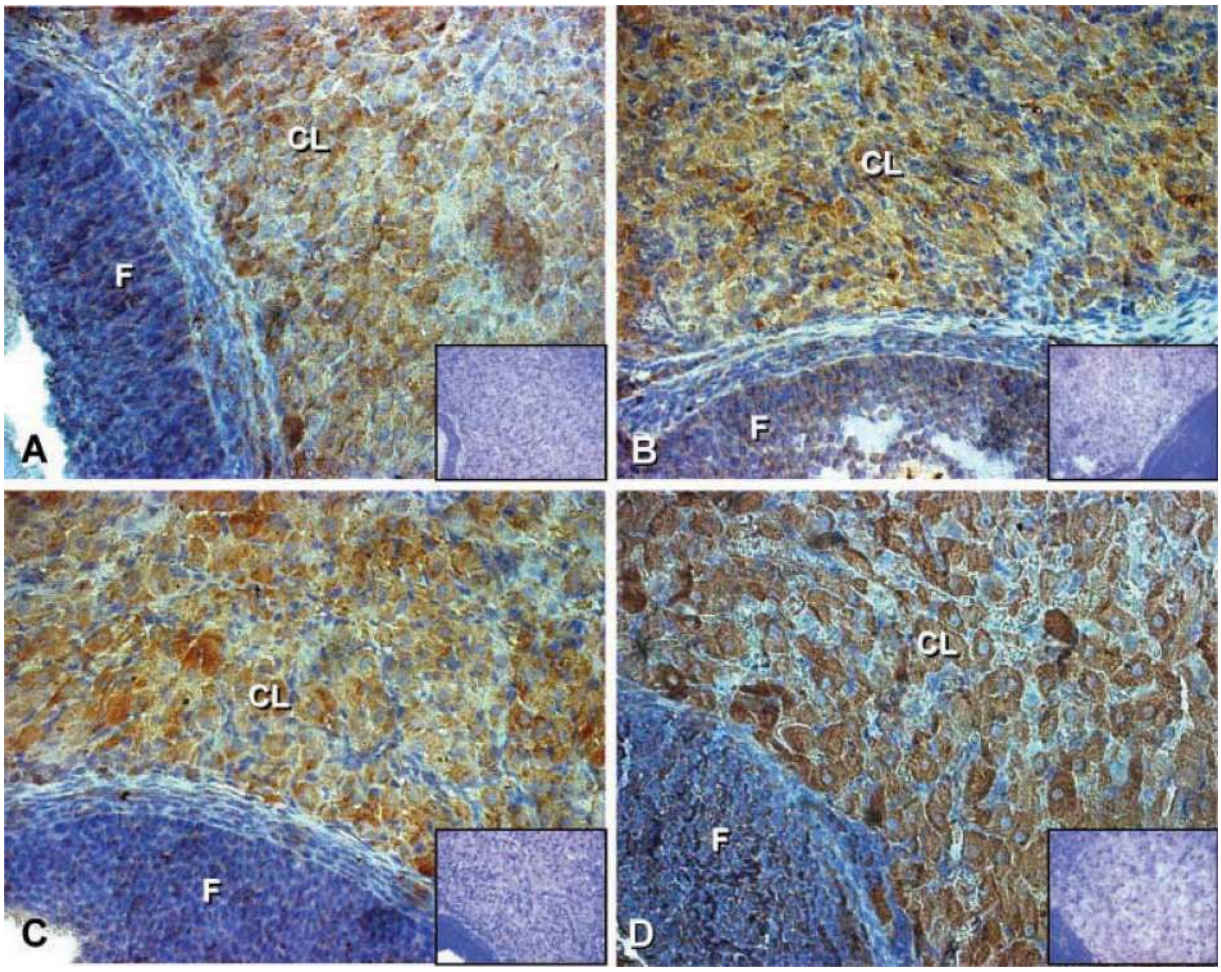

Fig. 2.

Photomicrographs of ovarian sections (magnification, 400×) showing immunohistochemical localization of GnRH in the corpus luteum (CL) during days 7 (A), 9 (B), 16 (C), and 20 (D) of pregnancy in rats. F denotes the ovarian follicles. Immunopositivity for GnRH is restricted to the cytoplasm of the luteal cells during each time point of pregnancy. The nucleus of the luteal cells and the intervening stromal and fibroblast cells are immunonegative. Inserts on each photomicrograph represent the negative control (without primary antibody) sections of the corresponding day of pregnancy.

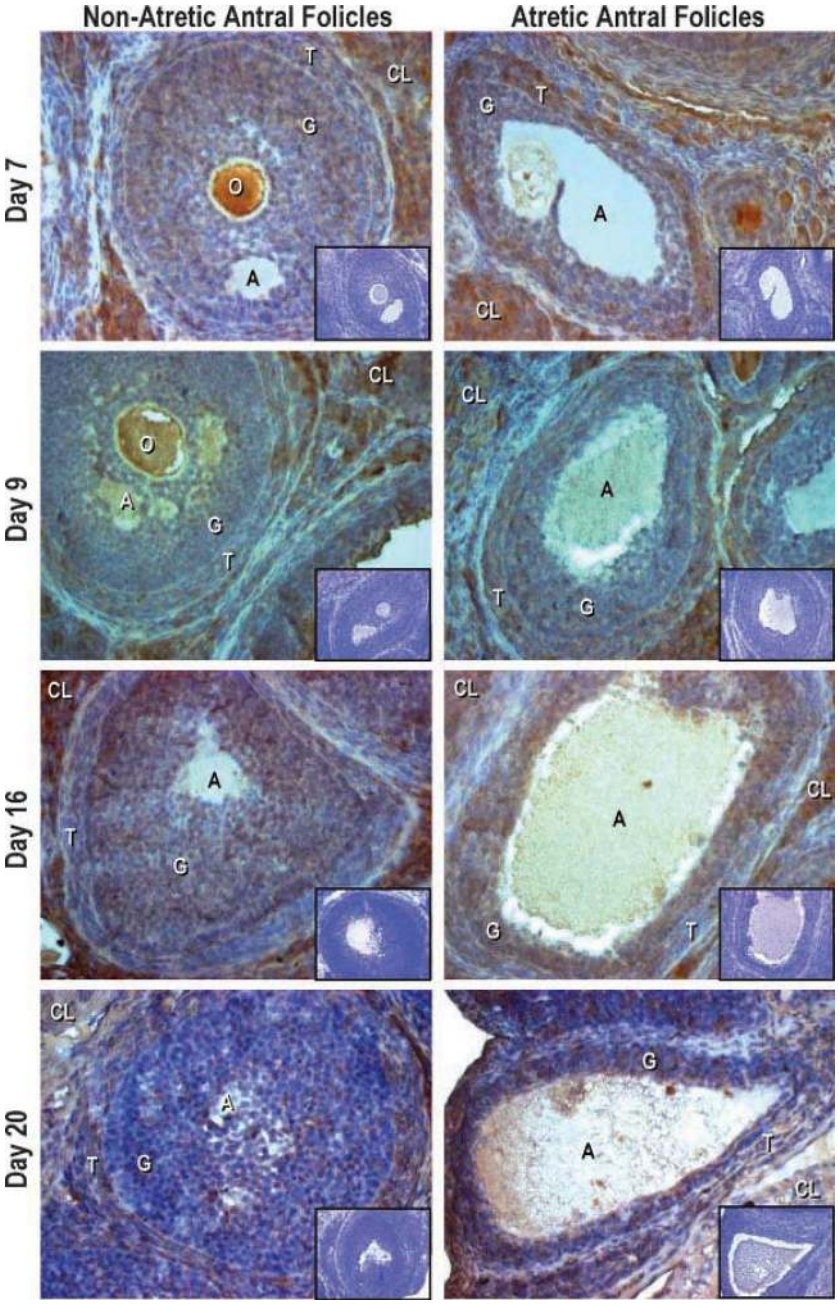

Fig. 3.

Photomicrographs (magnification, 400×) of ovarian sections exhibiting immunohistochemical localization of GnRH in the oocytes (O), granulosa (G), and thecal (T) cell layers of the atretic and nonatretic antral follicles within the rat ovary during days 7, 9, 16, and 20 of pregnancy. The large antrum (A) with cellular debris within the atretic follicles as well as the developing antrum (A) within the nonatretic antral follicles is immunonegative. The negative control (without primary antibody) sections are represented by the inserts on the photomicrograph of each day of pregnancy.

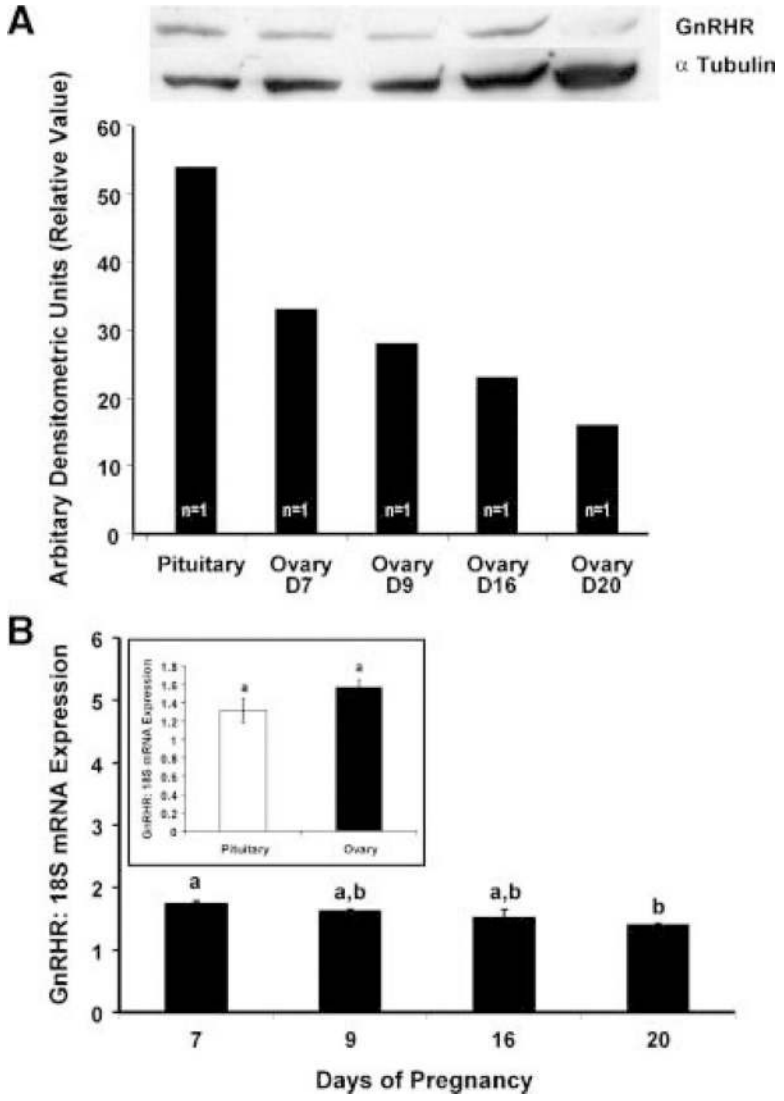

Fig. 4.

A: Representative Western blot of ovarian protein for expression of GnRHR and relative levels of the protein in the rat ovary expressed as a ratio of the densitometric values of GnRHR to alpha tubulin during days 7, 9, 16, and 20 of pregnancy in rats. Data represent values from a single animal. Expression of GnRHR in the rat pituitary is used as positive control for validation of the experiment. Alpha tubulin (50 kDA) used as an internal standard protein is expressed in all samples. **B**: GnRHR mRNA expression (mean ± SEM) in the rat ovary during chosen time points of pregnancy. Values were standardized to respective 18S control values. Comparison between the mean GnRHR mRNA expression in the rat pituitary (n = 4) and ovary (n = 12) during pregnancy is represented in the insert. Different superscript letters over histograms representing group means indicate significant difference ($P \leq 0.05$).

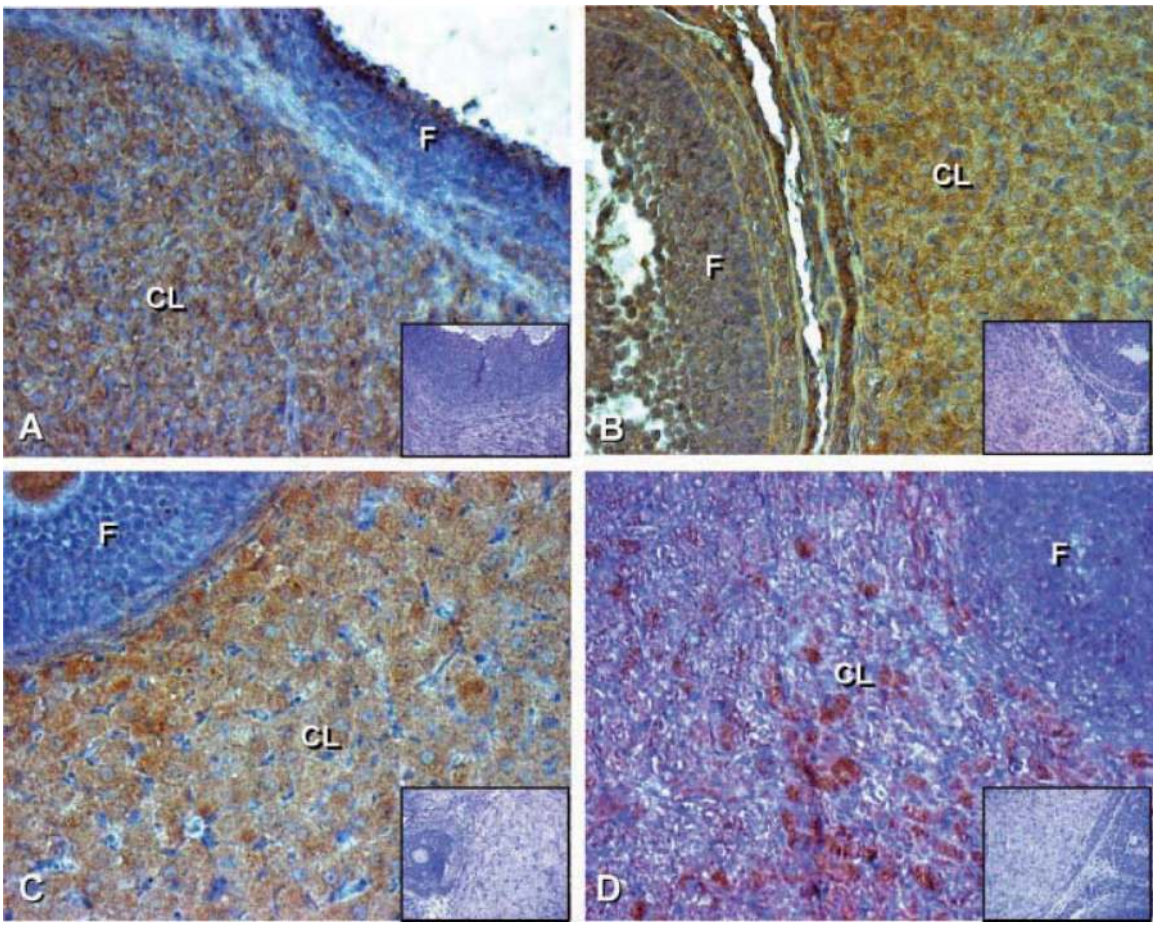

Fig. 5.

Immunohistochemical staining for GnRHR in rat CL during days 7(A), 9(B), 16(C), and 20(D) of pregnancy. F denotes the ovarian follicles. Cytoplasm of the luteal cells exhibits strong immunopositivity for GnRHR during each time point of pregnancy. Absence of immunostaining for GnRHR is noted in the nucleus of the luteal cells, the intervening fibroblast and the stromal cells within the CL and the negative control sections (inserts on photomicrographs of Days 7, 9, 16, and 20).

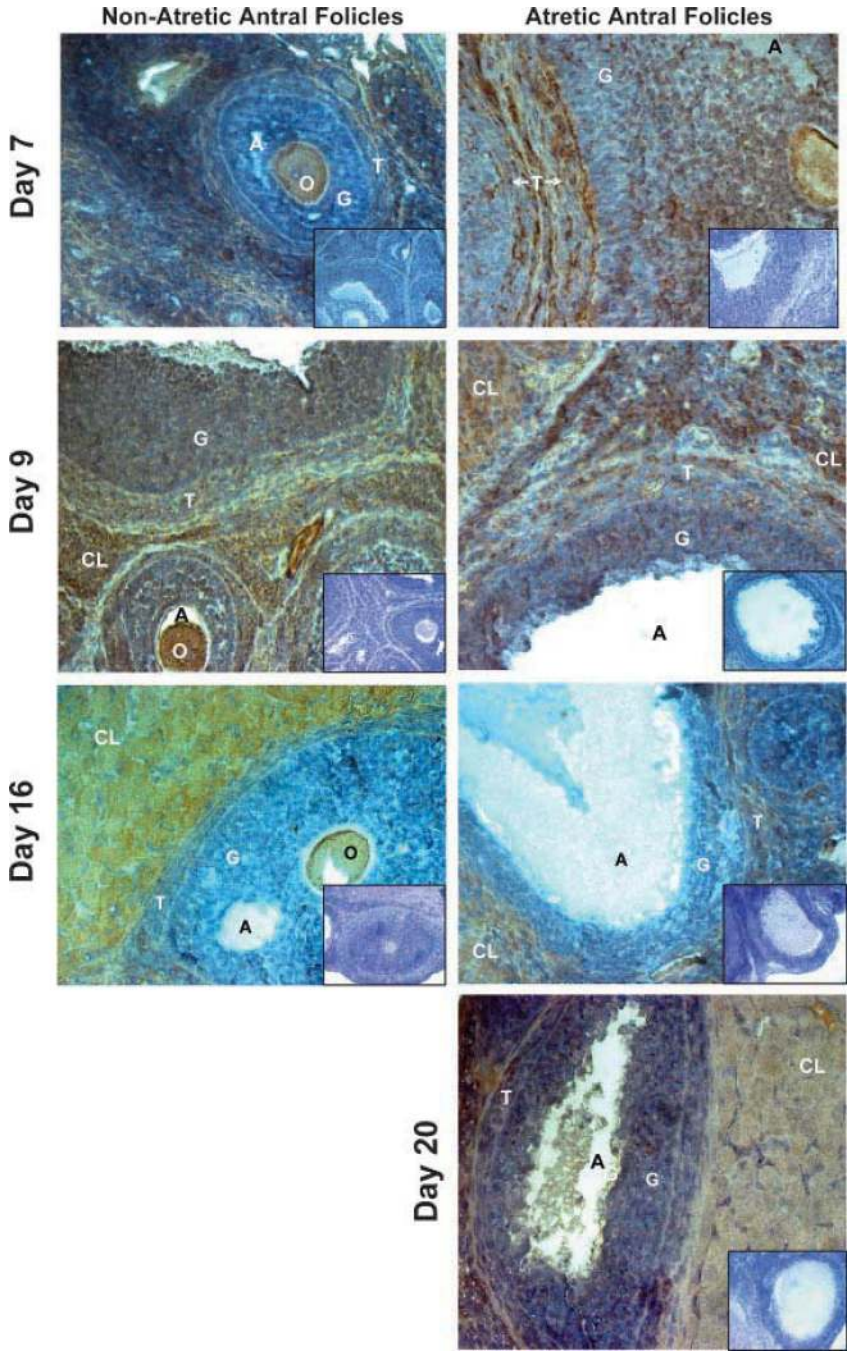

Fig. 6.

Photomicrographs (magnification, 400×) showing immunolocalization of GnRHR in the thecal (T), granulosa cells (G) of the nonatretic and atretic antral follicles during pregnancy. The oocyte (O) within the nonatretic antral follicles also exhibits strong immunopositive reactive reaction during Days 7, 9, and 16 of pregnancy. Inserts on each micrograph represents the negative control (without primary antibody) sections showing lack of immunostaining for GnRHR.

TABLE 1.

Description of the Sense and Antisense Pairs of Primers Used for Amplification of GnRH, GnRHR, and 18S mRNAs by Real-Time Polymerase Chain Reaction

Gene name	Sequence (5′ –3′)	Product length	Primer temperature (°C)
GnRH	Sense: AGGAGGATCAAATGGCAGAACC Antisense: TCTTCAATCAGACGTTCCAGAGC	103	54
GnRHR	Sense: CTAACAATGCGTCTCTTGA Antisense: TCCAGATAAGGTTAGAGTCG	101	54
18S rRNA	Sense: AATTCCGATAACGAACGAGA Antisense: ATCTAAGGGCATCACAGACC	141	54

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