Regulation of Rat Ovarian Gonadotropin Releasing Hormone Receptor mRNA Levels

by

Céline Claire Magali Väänänen

Maîtrise de Biologie Cellulaire,
Université Jussieu-PARIS VII, Paris, France, 1991

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ABSTRACT

The studies undertaken herewithin sought to characterize the pattern of regulation of the gonadotrophin-releasing hormone receptor (GnRH-R) mRNA levels in the rat ovary.

The demonstration of gonadotrophin-releasing hormone (GnRH) transcripts in steroid-producing and steroid-dependent tissues suggests that GnRH may be implicated in the modulation of steroid action in those target tissues. Expression of GnRH-R mRNA increases in gonadal tissues and the pituitary with age, supporting the concept of GnRH as a local regulator in the developing rat as well as in the adult.

During the peri-ovulatory period, stimulation of the ovarian luteinizing hormone receptor (LH-R) by an ovulatory dose of human chorionic gonadotrophin (hCG) is capable of inducing a transient and pronounced decrease in GnRH-R mRNA levels, thus bringing more evidence to the role of GnRH in the regulation of ovarian function during ovulation.

The gene encoding GnRH-R was found to be expressed in granulosa cells. In a follicle stimulating hormone (FSH) pre-treated granulosa cell model, GnRH-R gene transcript levels were negatively influenced by LH, but not FSH, in a time- and concentration-dependent manner. Auto-stimulation of GnRH-R by GnRH was also seen. In a second granulosa cell model obtained from pregnant mare serum gonadotrophin (PMSG) synchronized immature rats, the levels of GnRH-R transcripts were found to be positively regulated by hCG and GnRH, while prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) inhibited GnRH-R mRNA levels in a bell curve-like fashion. However, combinations of these treatments canceled each other's effects. In this model, GnRH stimulated progesterone (P_4) production and only slightly inhibited hCG-stimulated progesterone production. This modulation in the responses to GnRH and in the levels of GnRH-R mRNA suggest that GnRH may have different actions at different times of follicular development.

In conclusion, the findings of the present study demonstrate wide spread expression of the GnRH-R gene in steroidogenic or steroid-dependent tissues. Gonadotrophin-releasing hormone receptor mRNA levels were shown to be regulated by gonadotrophins, $PGF_{2\alpha}$ and GnRH in the whole ovary and in granulosa cells, strongly supporting the postulated role of GnRH-R in the local modulations of ovarian function mediated by GnRH.

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LIST OF ABBREVIATIONS

Standard Abbreviations

AA	Arachidonic acid
ATP	Adenosine triphosphate
bp	Base pairs
C	Celcius
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CAT	Chloramphenicol Acetyl Transferase
cDNA	Complementary DNA
Ci	Currie
CRE	cAMP response element
DI	Diestrus day I
DII	Diestrus day II
ddH_2O	Double distilled water
dCTP	Deoxycytosine-triphosphate
dNTPs	Deoxynucleotide-triphosphate(s)
DAG	Diacyl glycerol
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified eagles medium
DNA	Deoxyribonucleic acid
DTT	Dithiothrietol
e (as in eLH)	equine
E	Estrus
E_2	Estradiol

EDTA	Ethylenediaminetetraaceticacid
Estradiol	17ß-estradiol
ERE	Estradiol response element
FBS	Foetal bovine serum
FSH	Follicle stimulating hormone
FSH-R	Follicle stimulating hormone receptor
g	Grams
G3PDH	Glucose-3 phosphate dehydrogenase
GLB	Gel loading buffer
GnRH	Gonadotrophin-releasing hormone
GnRH-R	Gonadotrophin-releasing hormone receptor
GTP	Guanosine triphosphate
G-protein	GTP binding protein
GRB	Gel running buffer
GTC	Guanosine thiocyanate homogenizing buffer
h	Hours
h (as in hCG)	Human
hCG	Human chorionic gonadotrophin
HRE	Hormone response element
IP_3	Inositol triphosphate or inositol trisphosphate

IU	International unit
IVF	*In vitro* fertilization
Kd	Equilibrium dissociation constant
KDa	kilodaltons
l	Litre
L	Lactating
LH	Luteinizing hormone
LH-R	Luteinizing hormone receptor
m (as in mGnRH-R)	Mouse
ml	Milliliters
min	Minutes
mRNA	messenger RNA
m.w.	Molecular weight
M	Moles/Liter
n (as in nM)	Nano (10^{-9})
P	Pregnant
P_4	Progesterone
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Proestrus
PG	Prostaglandin
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
$PGF_{2\alpha}$-R	Prostaglandin $F_{2\alpha}$ receptor
PGI_2	Prostaglandin I_2
PI	Phosphatidiyl inositide or phosphoinositide

PKA	Protein kinase A
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
pM	Picomolar
pmole	Pico moles
PMSG	Pregnant mare serum gonadotrophin
r (as in rGnRH)	Rat
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RT	Reverse transcription
s	Seconds
SDS	Sodium dodecyl sulfate
SSC	Sodium chloride and Sodium citrate buffer
TBE	Tris borate EDTA
TRIS	Tris(hydroxymethyl)aminomethane
Tx	Thromboxanes
TxA_2	Thromboxane A2
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume
x	Times (or multiplied by)
x g	Times gravity

Abbreviations Starting with Greek Characters

α	Alpha
ß	Beta
γ	Gamma
λ_{260}	wave length at 260 mm
μ (as in μg)	Micro (10^{-6})

List of suppliers

Amersham	Amersham Ltd., Oakville, ON
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Bio/Can	Bio/Can Scientific, Mississauga, ON
Bio-Rad	Bio-Rad, Mississauga, ON
Boehringer Mannheim	Boehringer Mannheim, Laval, QU
BRL	Gibco BRL, Gaithersburg, MD
Canlab	VWR/CANLAB, Toronto, ON
Pharmacia	Pharmacia, Upsala, Sweden
Sigma	Sigma Chemical Company, St. Louis, MO

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BACKGROUND

I. The hypothalamo-pituitary-gonadal axis

In the human, the term menstrual cycle describes the monthly cycle of physiological events which prepare the female for pregnancy. In non-primate mammals, the cycles, varying in duration and frequency from one species to the other, are called the estrous cycle. The endocrinology of the menstrual and estrous cycle are comprised of three levels, known as the hypothalamo-pituitary-ovarian axis (H-P-O axis). Hormones are produced at each level, and controlled by positive and negative feedback mechanisms from the pituitary and the ovary (Figure 1).

A. Hypothalamic GnRH

Gonadotrophin-Releasing Hormone is a decapeptide produced primarily by the hypothalamus. It is secreted in rapid pulses of varied amplitude and frequency during the cycle [Smith et al., 1985]. GnRH exerts its actions on the gonadotrophs of the anterior pituitary. The half-life for GnRH is only a few minutes, and in that time, it must travel to the pituitary via the hypothalamo-pituitary portal vein, and bind to GnRH-receptors present on the surface of the gonadotrophs [Eidne et al., 1992; Fan et al., 1994; Crumeyrolle et al., 1994], and exert its actions.

GnRH is responsible for increasing synthesis, storage, activation and secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH), from the gonadotropes [Joshi et al., 1993, Baldwin et al., 1984; Rommler et al., 1979]. GnRH secretion is under the influence of cathecolaminergic neurons, such as noradrenaline, or dopamine [Sawyer, 1975; Rotsztejn et al., 1976; Yen et al., 1977; Advis et al., 1978; Balthazart et al., 1982; Dufour et al., 1988; Laatikainen, 1991; Donham; 1993]. Its activity is modulated, partly by auto-stimulation of its own receptor, and partly by short feedback loops from the pituitary, but mainly the ovarian steroids, progesterone: P_4 and estradiol: E_2 [Gregg et al, 1990; Ortmann et al, 1995].

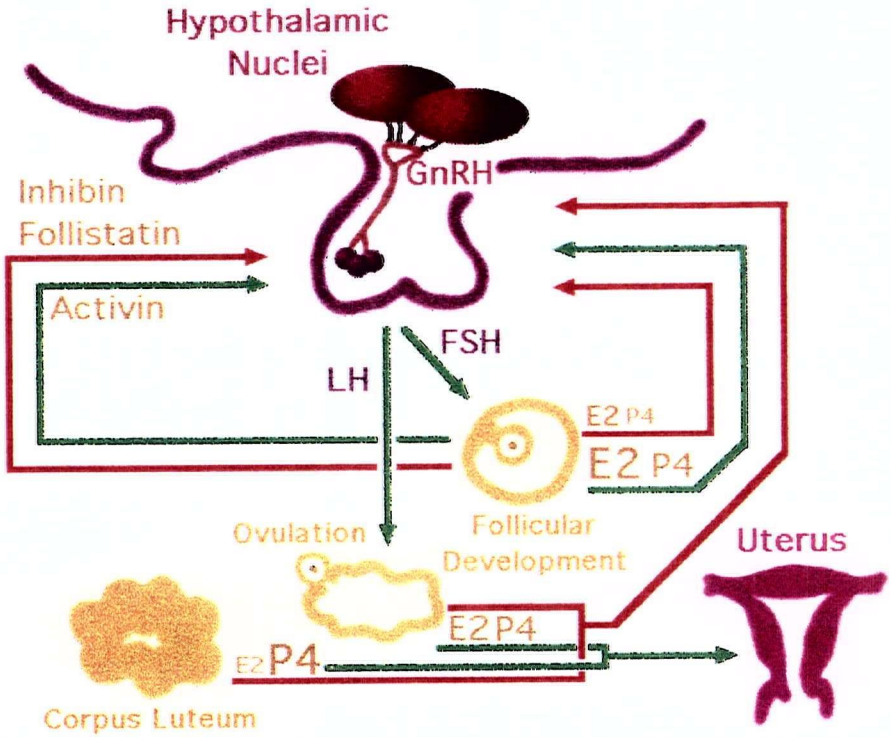


Figure 1. Hypothalamo-pituitary-ovary axis. (Green arrows represent stimulatory actions, while red arrows indicate inhibitory effects). The pulsatile release of GnRH stimulates the production of the gonadotrophins (LH and FSH) by the anterior pituitary. Acting on the ovary, FSH stimulates steroidogenesis (P_4 and E_2) and follicular development. The rising levels of E_2 first feedback negatively on the pituitary productions, but when E_2 reaches a threshold, E2 stimulates an LH surge that provokes ovulation. The subsequent steroidogenesis also targets the uterus, preparing it for an eventual implantation. Thus, depending on the stage and the levels of steroids, P_4 and E_2 can either have stimulatory or inhibitory effects.

B. Pituitary LH and FSH

LH and FSH are secreted by the gonadotrophic cells of the anterior pituitary in response to GnRH stimuli. In turn, LH and FSH act on the ovary to stimulate steroidogenesis [Hillier et al., 1995]. LH and FSH pulsatile secretion is modulated by GnRH secretion and ovarian steroids [Hsueh et al., 1980; Fink, 1988].

During the first half of the cycle (known as the follicular phase) E_2 exerts a negative feedback on the hypothalamo-pituitary unit by inhibiting FSH secretion [McNeilly, 1988]. At midcycle, high levels of E_2 induce the LH surge responsible for ovulation [Suzuki et al., 1974; Wu et al., 1974; Yen et al., 1977; Haour et al., 1978; Channing et al., 1980; Turgeon et al., 1980; Leung et al., 1992]. P_4 enhances the midcycle LH surge [Lee et al., 1990], and is mainly responsible for the FSH surge. During the second phase of the cycle (luteal phase), high levels of P_4 act with E_2 to inhibit gonadotrophin secretion.

C. Ovarian function

The ovarian cycle is divided into three phases: Follicular development, ovulation, and luteal phase. Under appropriate gonadotrophic stimulation, primordial follicles are recruited to undergo further maturation. At the beginning of a cycle, rising levels of FSH stimulate follicular development and E_2 synthesis [Hillier et al., 1995]. Negative feedback from E_2 causes FSH levels to decline, withdrawing support to the less developed follicles, while the dominant follicle(s) still develops due to the greater number of FSH receptors they carry. At mid cycle, high levels of E_2, positively feeding back on the pituitary, cause the midcycle surge of LH and FSH [Suzuki et al., 1974; Wu et al., 1974; Yen, 1977; Haour et al., 1978; Channing et al., 1980; Turgeon, 1980; Leung et al., 1992]. The gonadotrophin surge is responsible for the final follicular maturation and ovulation. Ovulated follicles become corpora lutea, secreting both E_2 and P_4. A feedback loop from E_2 and P_4 inhibits gonadotrophins, which causes them to decline, allowing the rise of FSH of the next cycle.

C.1. The follicular phase

C.1.a. *The dominant follicle*

FSH which is responsible for follicular recruitment, rapidly diminishes under E_2 negative feedback. Because of the decreasing FSH stimulation, only the dominant follicle, which, having more granulosa cells, carries more FSH receptors, continues to be stimulated. Progesterone production by the dominant follicle increases in response to LH due to the newly acquired LH receptors.

C.1.b. *Morphology of the pre-ovulatory follicle*

During follicular maturation, accumulation of follicular fluid within the proliferative granulosa cell mass forms the antrum. Maturation is accompanied by enlargement of the oocyte, and formation of the zona pellucida, a mucopolysaccharide layer surrounding the oocyte. The cumulus oophorus is composed of a dense layer of granulosa cells surrounding the zona pellucida. The stroma in contact with the granulosa cells differentiates into the theca layer, with a well vascularized internal layer and a fibrous external layer (Figure 2).

FSH receptors are carried by the granulosa cells only, while the theca interna contains LH receptors. Granulosa cells also express LH receptors in response to FSH stimulation. LH stimulation causes theca cells to produce androstenedione (an androgen) which is a substrate for the aromatase enzyme produced under FSH stimulation by the granulosa cells. The aromatase is a key enzyme for E_2 production (Figure 3).

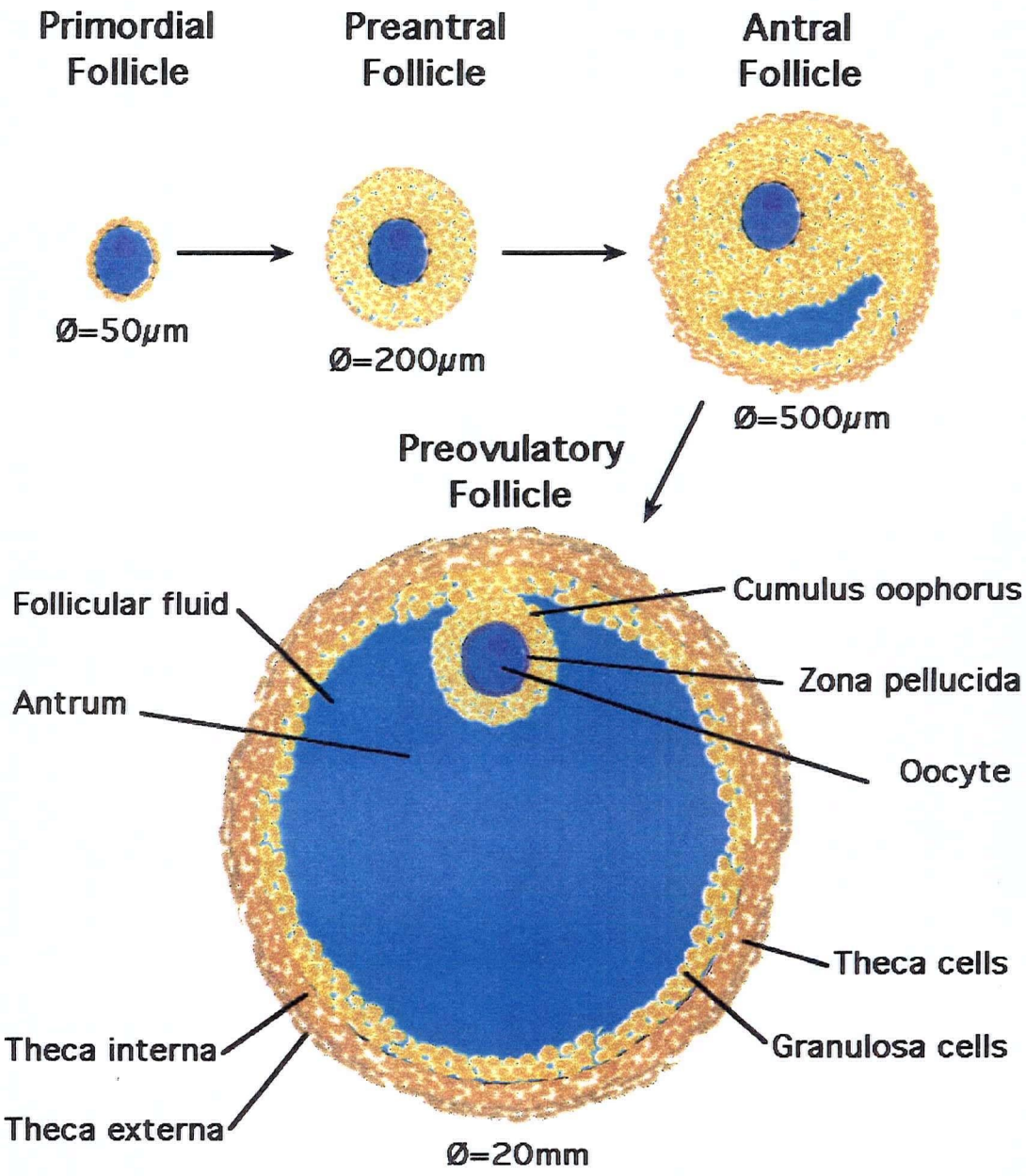


Figure 2. Follicular maturation process. The main morphological stages of the human follicle are depicted here. The diameters of the follicles at different stages of maturity are indicated. Under FSH stimulation, primordial follicles are recruited to undergo maturation. Only the dominant follicle will reach the pre-ovulatory stage, and complete the cycle.

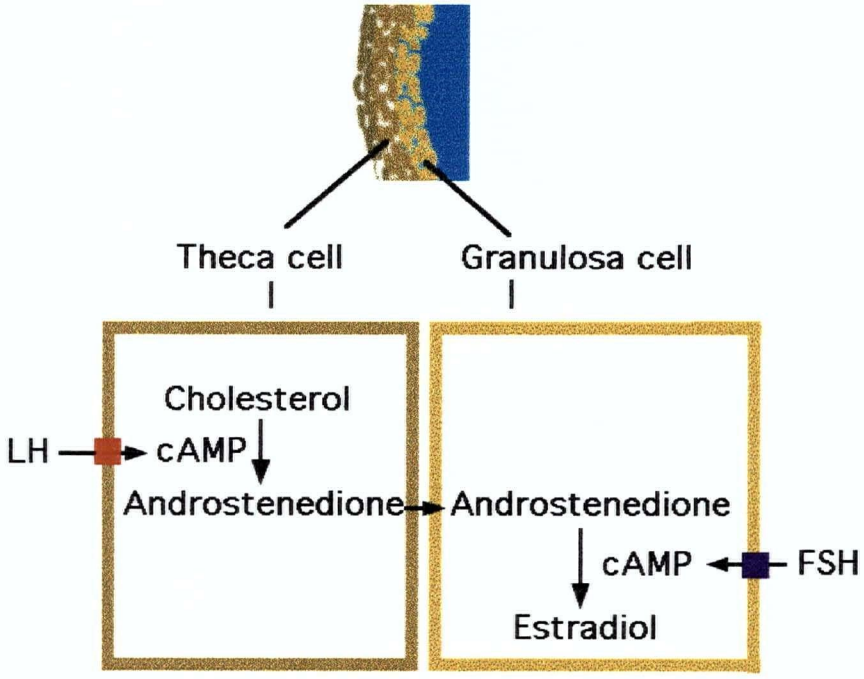


Figure 3. Compartmentalization of steroidogenesis. Also known as the two cells theory, steroidogenesis is assured by the two cell types of the follicle, the theca cells and the granulosa cells. The theca cell responds to LH by producing androstenedione. Androstenedione is transported to the granulosa cell where, following FSH activation of the aromatase enzyme, it can be transformed into estradiol.

C.2. The process of ovulation

The LH surge is triggered by the high, plateauing levels of E_2, and synergistic action of increasing levels of P_4. After initiation of the surge, E_2 levels drop dramatically, due to down-regulation of LH receptors on the theca cells by LH, depriving the granulosa cells of androgen substrate. Further granulosa cell proliferation is also inhibited by P_4 [Channing et al., 1978].

In response to the LH surge, synthesis of proteolytic enzymes, involved in the lysis of the follicular wall, increases. $PGF_{2\alpha}$ levels in the follicular fluid also increase. E_2 levels drop while P_4 production by the granulosa cells increase. Further nuclear maturation occurs, as the first meiotic division resumes, only to be arrested again at the metaphase of the second meiotic division [Channing et al., 1978].

The loss of positive feedback from E_2, combined with the increasing negative feedback from P_4, leads to down-regulation of GnRH receptors in the pituitary. These events, combined with the exhaustion of LH stores, terminate the LH surge.

$PGF_{2\alpha}$ increases intra-follicular pressure by increasing ovarian contractions and activates the proteolytic enzymes. Rupture of the follicular wall and expulsion of the egg constitute the ovulation *per se* [Dennefors et al., 1983].

C.3. Luteinization

Luteinization starts in response to the LH surge by a significant rise in P_4 prior to ovulation. After ovulation, the follicle becomes the corpus luteum. The granulosa cells enlarge and become luteal cells. Vascularization invades the granulosa layer, bringing cholesterol, carried by LDL (low density lipoproteins), as a substrate for P_4 synthesis in response to gonadotrophin stimulation. Although circulating levels of FSH are low, significant quantities still reach the granulosa-luteal cells through the increased vascularization, and E_2 is still synthesized.

The peak activity of the corpus luteum coincides with peak vascularization and P_4 and E_2 levels in the luteal phase. Unless pregnancy rescues the corpus luteum [Stouffer et al., 1989], luteal regression is inevitable, resulting in P_4 decline and death of the corpus luteum.

II The uterine cycle

A. Uterus, and other targets of ovarian steroids

The cycling hormonal patterns, determined by the ovarian cycle, fulfill different functions throughout the body, with the purpose of preparing the uterus for pregnancy [Yen, 1978; McCarty, 1983]. The functions of the ovarian steroids include:

-Increased ciliation and secretory activity by the fallopian tube in response to E_2, while P_4 has opposite effects.

-Increased mitotic activity and keratinazation of the epithelium of the vagina under E_2 influence, and opposite effects of P_4.

-Breast enlargement prior to menstruation.

-Increase in skin pigmentation, and sometimes, acne, due to P_4.

-Small rise in temperature due to P_4 effects on thyroid function leading to a slight increase in metabolic rate.

-Symptomatic changes including bloatedness, breast tenderness, and what is generally described as PMS (pre-menstrual syndrome).

B. Endometrial changes

The uterus is affected at several levels. The myometrium, the uterine blood flow, the endometrium and the cervix are all influenced by P_4 and E_2 [Jaffe, 1974]. The endometrial changes can be divided into three phases:

B.1. The proliferative phase

The proliferative phase coincides with the follicular phase. Under E_2 stimulation, the endometrium grows and rich vascularization invades the thickening tissues. The effects of E_2 are enhanced by up-regulation of its own receptor. Priming with P_4 receptors is also under the control of E_2.

B.2. The secretory phase

After ovulation, P_4 becomes the dominant hormone. P_4 causes endometrial growth to cease, and prepares the tissues for implantation by stimulating secretion. The secretions are composed of glycogen, sugar, amino-acids, mucus and enzymes.

If implantation occurs, the secretory activity is maintained, and the superficial stroma cells differentiate in a strong compact layer. However, if implantation does not occur, the absence of gonadotrophic support causes a reduction in tissue weight. Prostaglandins (PGE_2, $PGF_{2\alpha}$, PGI_2) and trombaxane (TXA_2), vasodilatation and vasoconstriction effects cause blood to leak into the interstitial space, eventually leading to haemorrhage, known as menstruation.

B.3. Menstruation

The non-viable tissues formed as a result of cell and vascular necrosis is extruded into the endometrial cavity and contributes to the menstrual flow. Menstrual flow is limited, and eventually stops. Secretion of E_2 by the developing follicles resumes, inducing healing and new tissue growth.

III. Gonadal control of the gonadal functions

A. Paracrine/autocrine factors

An intricate interplay takes place between endocrine and other cell types within the ovary. Paracrine/autocrine factors may act on neighboring cells as intercellular modulators [Tsafriri, 1988]. Paracrine control mechanisms involve local diffusion of hormones to their neighboring cells without entering the circulatory system. Autocrine regulation occurs when the target of a hormone is the producing cell itself.

Local steroids, eicosanoids, and peptide hormones are suspected to play a role in the regulation of ovarian function, affecting steroidogenesis, oocyte maturation and ovulation through paracrine and autocrine mechanisms. These regulators include P_4 and E_2, prostaglandins ($PGF_{2\alpha}$,

PGE_2, PGI_2...), gonadotrophins, GnRH, inhibin and activin, growth factors, insulin and insulin-like growth factors, angiogenic factors, angiotensin, neurotransmitters, OMI (oocyte maturation inhibitor) [Ling et al., 1990; Leung et al., 1992; Chun et al., 1994].

B. GnRH as an ovarian modulator

GnRH is also thought to be an important paracrine/autocrine regulator in the gonads [Hsueh et al., 1981; Tsafriri, 1988; Leung et al., 1992]. GnRH and its receptor mRNA have recently been characterized in the human granulosa cell [Peng et al., 1994].

In the ovary, GnRH is considered to act differentially during follicular maturation, and has been shown to be both steroidogenic and antisteroidogenic [Clayton et al., 1979; Leung, 1985]. It is shown to stimulate basal steroidogenesis, but attenuate gonadotrophin induced cAMP and P_4 production. There is a multitude array of reports demonstrating the effects of this peptide on ovarian steroidogenesis [Hsueh et al., 1984; Leung et al., 1989; Sridaran et al., 1988; Clayton, 1988]. Acute stimulatory effects on P_4 production are seen in both cultured rat and human granulosa and luteal cells [Massicotte et al., 1984; Srivastava et al., 1994]. However, an inhibitory effect of LH-stimulated P_4 production has also been demonstrated [Smith et al., 1991; Srivastava et al., 1994]. Furthermore, GnRH has been shown to be luteolytic under *in vivo* and *in vitro* conditions [Massicotte et al., 1984].

The importance of GnRH is further illustrated by the GnRH-induced appearance of P_4 receptor mRNA and protein [Natraj et al., 1993] in rat granulosa cells. Furthermore, GnRH has the capability to induce oocyte release [Ekholm et al., 1981; Koos et al., 1985], oocyte maturation [Hillensjö et al., 1980] and luteinization [Morris et al., 1993].

IV. The hormones of the endocrine reproductive system

A. The gonadotrophins

A.1. LH and FSH

Luteinizing hormone (LH) and follicle stimulating hormone (FSH) are both glycoproteins composed of two non-covalently bound subunits [Ryan et al., 1987; Combarnous, 1988; Gray, 1988; Wierman et al., 1988]. While the 96-amino acid-long α-subunit is common to the two hormones, they have different β-subunits. The carbohydrate moieties of those glycoproteins contribute to 15% of their molecular weight and are contributing to the conformation of the molecule, forming the epitope that will bind to the receptor [Ryan et al., 1987; Combarnous et al., 1988; Gray et al., 1988; Wierman et al., 1988].

LH and FSH releases are pulsatile. The periods between peaks of LH during the menstrual cycle is 1 to 7 hours, and the amplitude of FSH release is lower than LH [Filicori et al., 1979]. Both are under GnRH control, although FSH is less sensitive to GnRH and also regulated by E_2 [Hall et al., 1992].

A.2. hCG

Human Chorionic Gonadotrophin is a glycoprotein composed of an α-subunit identical to the hLH/hFSH α-subunit. The beta-subunits of hCG and hLH share 85% sequence identity in their first 114 amino acids but differ in the carboxy-terminal peptide because hCG beta contains a 31-amino acid extension (beta-CTP) [Yoshimura et al., 1995]. Human CG is the hormone of pregnancy of chorionic origin in the human female. It binds to the LH receptor [Ziecik et al., 1992] and its effects are very similar [Yoshimura et al., 1995]. It is used clinically, in place of LH, for triggering ovulation in patients undergoing *in vitro* fertilization [Tarin et al., 1992], and in animal reproduction [Shelton et al., 1990].

A.3. PMSG

Pregnant Mare Serum Gonadotrophin (PMSG) is a glycoprotein hormone similar in structure to LH, FSH and hCG. Like the other gonadotrophins, it is composed of two non-covalently bound subunits (α and β). The alpha-subunit contains 96 amino acids and is identical to the α-subunit of the equine pituitary hormones eLH, eFSH and eTSH. The beta-subunit is composed of 149 amino acids. Its primary structure is identical to that of beta-eLH, and it shares similarities with beta-hCG, as both possess a C-terminal extension. PMSG is, in fact, of chorionic origin. Thus, it should be more rightfully named equine Chorionic Gonadotrophin (eCG) [Hoppen, 1994].

PMSG, together with other progestagens, are widely used in animal reproductive technology with the goal of increasing productivity of farm animals through enhanced control of reproductive function [Shelton, 1990]. It is also used on laboratory animals for synchronization of the estrous cycle and the study of reproductive function. PMSG is predominantly follicle-stimulating: Its effect closely resemble those of the follicle-stimulating hormone (FSH) of the anterior pituitary.

B. GnRH and its receptor

B.1. GnRH

Gonadotrophin-releasing hormone (GnRH) is a decapeptide derived from a larger preprohormone [Nillius et al., 1974; Hsueh et al., 1983]. It is found in the hypothalamus of all mammalian species studied so far [Illing et al., 1993], and is also present in non-mammalian species [Battisti et al., 1994; Grober et al., 1995]. Active transcription of the gene encoding GnRH has been confirmed in the hypothalamus of mammalian species, (Figure 4), and also the rat ovaries [Oikawa et al., 1990; Clayton et al., 1992; Goubau et al., 1992], the rat testis and the human placenta [Sherwood et al., 1993] (Figure 5). The gene encoding GnRH is composed of four exons and three introns. The detailed sequence with the position of the exonic and intronic portions is

shown in Figure 4. In the hypothalamus, the mature transcript is spliced of all three intronic sequences. The resulting 0.6 Kb mRNA is composed of a 3' and a 5' untranslated region; the start codon is located at the beginning of the second exon, and translation begins with the signal peptide. GnRH follows, within the second exon, and is followed by GAP (GnRH associated peptide) which has its stop codon in the fourth exon (Figure 5).

The gene encoding for GnRH has multiple transcription initiation start sites that are differentially used in various tissues. The mRNA species found in other tissues, although derived from the same gene, are also much longer as they retain some intronic regions. In the ovary, the main transcript is a 3.3 Kb mRNA with a transcription start located in the first intron and with conserved intronic sequences 2 and 3. The placental mRNA is 1.47 Kb: Transcription starts upstream of the first exon and the first intron is not spliced. The mRNA species found in the testis is 1.4 Kb, has an identical transcription start as the hypothalamic transcript, and, as the placental transcript, retains the first intron.

Although the non-hypothalamic transcripts have different transcription start sites, the presence of immunoreactive GnRH in those tissues suggest that the non-hypothalamic transcripts also have an open reading frame encoding for this peptide.

B.2. GnRH-receptor

There exists extensive evidence that all hormones and other local regulators relay their information through receptors. Some are membrane bound, expressed on the cell surface or the nucleus. Others are soluble receptors present within the cytosol or the nucleus. The receptors for GnRH are membrane bound.

GnRH-R immunoreactivity and mRNA have been found in the brain, [Jennes et al., 1994] the anterior pituitary, [Reinhart et al., 1992], ovary, [Moumni et al., 1994; Whitelaw et al., 1995] and Leydig cells [Reinhart et al., 1992].

In the ovary, another important piece of evidence for GnRH action as a paracrine factor is the finding of high affinity binding sites (Kd = 0.5 nM) for GnRH in ovarian cells [Huckle et al., 1988] and the demonstration of the expression of specific mRNA encoding the receptor for GnRH

in the different ovarian cells during ovulation and luteolysis [Olofsson et al., 1994; Peng et al., 1994; Latouche et al., 1989]. This evidence lends strong support to the concept of GnRH as a significant intra-ovarian hormone.

The first report of the cloning of a cDNA-sequence for the murine receptor for GnRH [Reinhart et al., 1992] revealed that the transcript encodes for a 327 amino acids protein, organized in seven trans-membrane domains characteristic of G-Protein coupled receptors [Probst et al., 1992] (Figure 6). Since then, several groups have characterized cDNA sequences encoding functional GnRH-R in the mouse [Tsutsumi et al., 1992], rat [Eidne et al., 1992; Kaiser et al., 1992; Kakar et al., 1994; Perrin et al., 1993], sheep [Brooks et al., 1993] and human [Kakar et al., 1992; Chi et al., 1993].

The binding of GnRH to a G-Protein coupled receptor results in the initiation of two possible transduction pathways -PKA, PKC [Leung et al., 1992]. In the pituitary, the cellular response initiated by the binding of GnRH on its membrane receptor results in activation of adenylate cyclase by the α-subunit of a Gs protein followed by PKA activation by cAMP. Upon dissociation of PKA into the regulatory and the catalytic unit, the catalytic unit is phosphorylated by ATP and, in turn, phosphorylated membrane proteins allowing calcium to enter the cell [Kiesel et al., 1986]. Ca^{2+} movement leads to the excretion of LH and FSH granules [Huckle et al., 1987]. Binding of GnRH on its receptor also activates PLC which hydrolyses phosphatidyinositol to generate IP3. IP3 binds on the endoplasmic reticulum and opens calcium channels. The other product of PLC hydrolytic activity is DAG. DAG remains in the membrane and, with calcium, activates PKC.

In the ovary, GnRH transduction involves three pathways: PLA_2, PLC, and PLD [Leung et al., 1992]. The stimulation of PLC activity results in the hydrolysis of polyphosphoinositides, generating IP_3 and DAG. IP_3 is responsible for the release of Ca^{2+} from intracellular stores, while DAG stimulates PKC. The stimulation of PLD also contributes to PKC activation and Ca^{2+} regulation (from extracellular stores). The stimulation of PLA_2 results in the generation of AA, which is a substrate for lipoxygenase metabolites, also mediating GnRH actions.

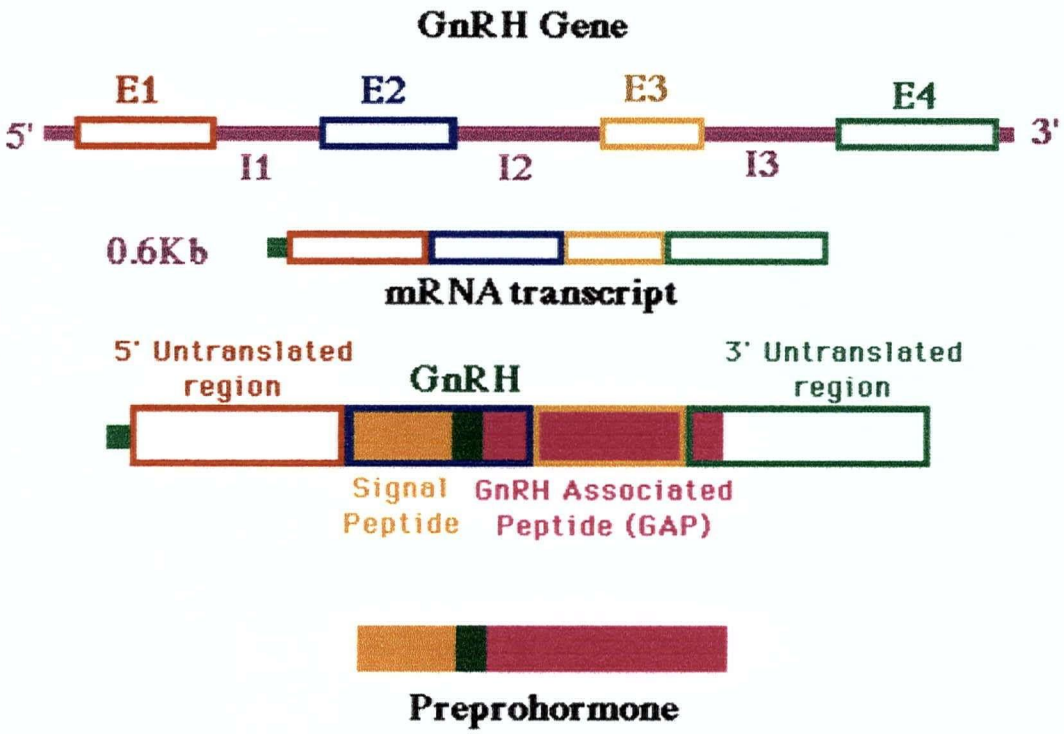


Figure 4. Schematic representation of GnRH gene structure. (not to scale). The gene encoding for GnRH in the hypothalamus is composed of 4 exons and three introns. During the process of transcription, the introns are spliced from the hypothalamic transcript, leaving a 0.6 Kb mRNA transcript. The translated region starts at the second exon and ends in the fourth exon. The decapeptide GnRH is encoded in the second exon, preceded by the signal peptide and followed by GnRH associated peptide (GAP).

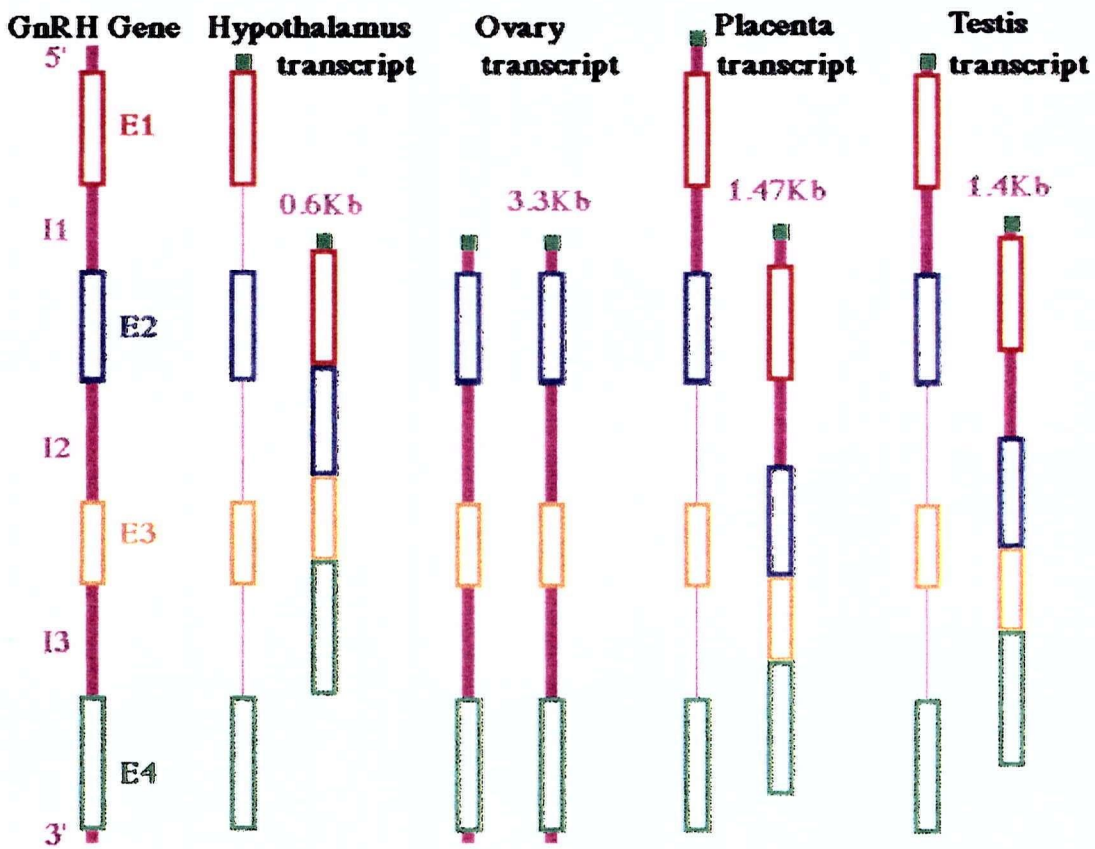


Figure 5. Differential GnRH expression. GnRH is expressed in the hypothalamus, the ovary, placenta and testis of the rat . For each tissue, the left transcript is the precursor mRNA and the right transcript is the spliced mature mRNA. Boxes (E1, E2, E3 and E4) are the exonic sequences of the gene, lines (I1,I2and I3) are the intronic sequences. Thin lines are the intronic sequences spliced in the final mRNA, while thick lines are the conserved intronic regions.The transcripts expressed in these different tissues have different transcription starts and ends, and different splicing patterns. Thus, while the hypothalamic transcript is spliced of all intronic regions, the main ovarian transcript starts in the first intron and retains introns 2 and 3. The placenta transcript starts upstream of the first exon and retains the first intron. The testicular transcript is similar to the placenta transcript, but starts closer to the first exon.

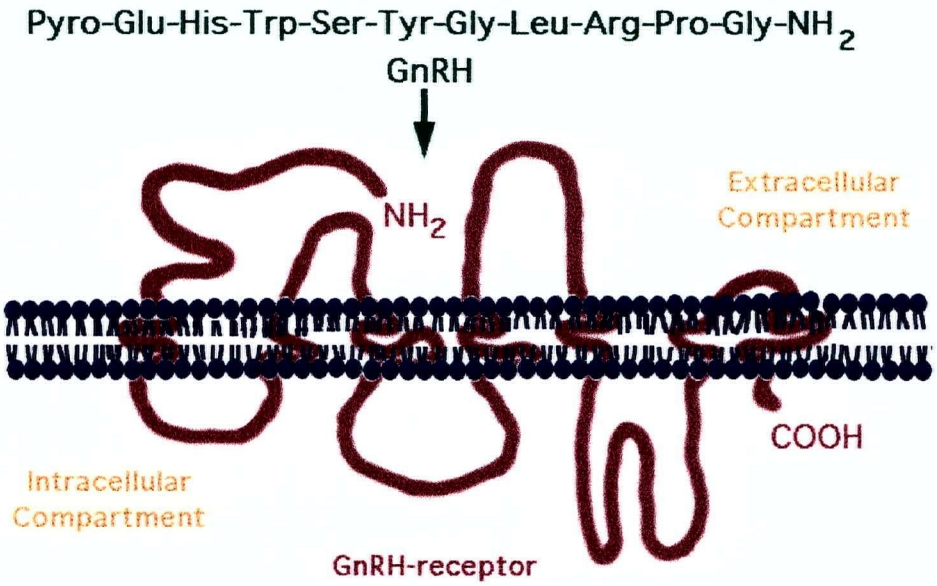


Figure 6. GnRH and GnRH-receptor structure. GnRH is primarily a hypothalamic decapeptide with an aminated C-terminus and a cyclized N-terminus. The hypothalamic form of GnRH has a short half-life of 2-4 minutes only. It reaches its receptor, a seven trans-membrane serpentine-G protein coupled receptor, via the portal vein of the pituitary. GnRH-like peptides are also produced locally in the ovary, testis, and placenta of mammalian species. There, they also find a locally expressed, similar GnRH-R.

C. Prostaglandin $F_{2\alpha}$ and its receptor

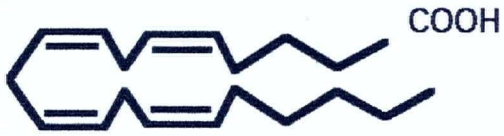
C.1. Prostaglandin $F_{2\alpha}$

Prostaglandin F_2 alpha ($PGF_{2\alpha}$) is an eicosanoid derived from arachidonic acid (Figure 7). Prostaglandins and their receptors are present in practically all tissues where they exert various local effects. The reproductive tissues where $PGF_{2\alpha}$ has been detected include the human decidua, amnion, pregnant myometrium and ovary [Aksel et al., 1977; Satoh et al., 1981]. $PGF_{2\alpha}$ has been localized to the follicle, theca-, granulosa- and luteal-cell of the human ovary [Plunkett et al., 1975; Aksel et al., 1977; Patwardhan et al., 1981].

Prostaglandin F_2 alpha is believed to regulate the life span of the corpus luteum. Various studies suggest that prostaglandin F2 alpha ($PGF_{2\alpha}$) is a luteolytic factor in the mammalian ovary [Korda et al., 1975; Grinwich et al., 1976; Richardson et al., 1980; Hanzen et al., 1984; Moon et al., 1986; Jalkanen et al., 1987; Michael et al., 1991] and the rat corpus luteum [Hall et al., 1979; Luborsky et al., 1984; Bjurulf et al., 1994]. It is most abundant in corpora lutea of pseudopregnant rats, [Olofsson et al., 1992] where it exerts auto/paracrine modulation of luteal steroidogenesis [Olofsson et al., 1994]. Prostaglandin synthesis is stimulated by LH or hCG in pre-ovulatory follicles [Larson et al., 1991; Tsafriri, 1995]. In turn, $PGF_{2\alpha}$ inhibits LH-, hCG- and PGE_2-stimulated P_4 production, through inhibition of LH/hCG receptor mRNA levels and the alpha -mRNA subunit of inhibin [Luborsky et al., 1984; Bjurulf et al., 1994; Fraser et al., 1995] and/or reduction in adenylate cyclase activation [Dorflinger et al., 1984], increased P_4 catabolism [Moon et al., 1986], and increased cAMP phosphodiesterase activity through PKC [Lahav et al., 1989].

$PGF_{2\alpha}$ also appears to be luteotrophic under certain conditions depending on the time and concentration. During the mid-luteal phase and pregnancy, $PGF_{2\alpha}$ was found to exhibit strong luteotrophic effects [Suginami et al., 1976; Khan et al., 1989; Webley et al., 1989; Michael et al., 1993].

arachidonic acid



Prostaglandin $F_{2\alpha}$

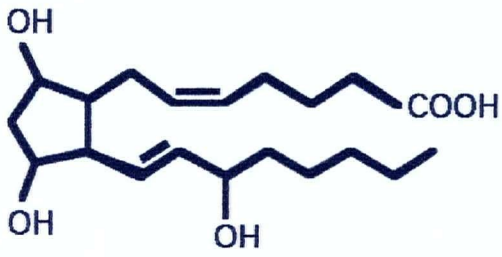


Figure 7. $PGF_{2\alpha}$ molecular structure. $PGF_{2\alpha}$ (bottom) is a cyclo-oxygenase product derived from arachidonic-acid (top). It is synthesized in most tissues, including the ovary, uterus, placenta, testis and other tissues where it exerts local actions. The receptor for $PGF_{2\alpha}$ is a seven transmembrane serpentine receptor of the G-coupled receptor family.

C.2. $PGF_{2\alpha}$ -receptor

Numerous prostanoid receptors have been identified in mammals. Amoung these is the $PGF_{2\alpha}$-R, recently cloned [Lake et al., 1994; Abramovitz et al., 1994]. Sequence analysis of this receptor suggests that it belongs to the family of G-protein coupled receptor. Transduction is linked to intracellular calcium and cAMP production [Leung, 1985; Rodway et al., 1991 et al., 1992; Currie et al., 1992; Steele et al., 1992; Lopez et al., 1995].

In non-pregnant rats, corpora lutea, thecal cells, primary and secondary interstitial cells were found to contain immunoreactive $PGF_{2\alpha}$-R. During luteolysis, cells undergoing apoptosis stained for the presence of $PGF_{2\alpha}$-R [Orlicky et al., 1992]. The physiologically induced changes occurring during the life span of the corpus luteum are believed to be regulated by $PGF_{2\alpha}$ could be related to the binding properties of $PGF_{2\alpha}$.

D. Steroid hormones

The two main steroids involved in the regulation of the female reproductive function are progesterone (P_4) and 17ß-estradiol, usually referred to as estradiol (E_2). Steroids are derived from cholesterol (Figure 8). They are insoluble in water, and are transported into the circulation by proteins. The functions of P_4 and E_2 have been described in previous paragraphs. They mediate their actions via cytoplasmic receptors migrating directly to the nucleus and binding to regulatory elements of the targeted genes.

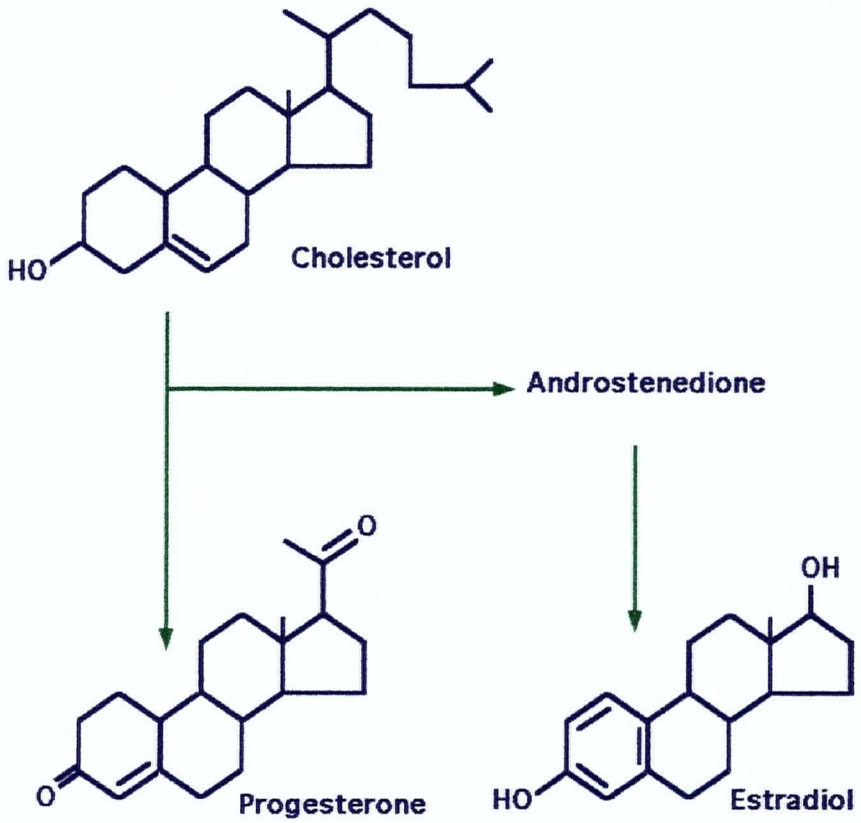


Figure 8. Progesterone and estradiol structure. Progesterone and estradiol (17β-estradiol) are sex steroid hormones derived from cholesterol.

V. The rat model

The in vivo and in vitro models studied here were derived from the Sprague Dawley rat. General information on characteristics and biology of reproduction of the rat model are developed here. [Baker, 1979 et al., 1990; CCAC, 1984; Fox et al., 1984].

A. General characteristics

A.1. Origin and development

The wild brown rat (*Rattus norvegicus*), originating from the borders of the Caspian sea, spread with the movements of modern civilization over the Old World during the 18th century, but did not reach North America until 1775. Laboratory rat breeding experiments were first reported from Germany (Circa 1880), and soon laboratory bred rats were brought to North America where they were first established at a Chicago laboratory for neurological studies. In 1906, some of this stock was transferred to the Wistar Institute in Philadelphia, giving rise to the present strain of Wistar rats.

Nowadays, next to the laboratory mouse, the rat is the most commonly used laboratory animal, accounting for 20% of the total number of mammals used for scientific purpose. Like mice, several varieties of rats are available. The two most common are Wistar and Sprague-Dawley, both originating from US colonies and spreading world wide over the past half century. The model used in these studies was the Sprague-Dawley rat.

Upon arrival, the rats are normally given one to four days for recovery, as transit, especially transport by air, is very stressful to them. [CCAC, 1984].

A.2. Characteristics

Laboratory rats, unlike wild ones, are year round breeders. Pups weigh around 5 g at birth, adult males weigh 400 to 500 g, and females 100 g less, but size and weight will vary between strains. Healthy rats will live for 2.5-3 years, depending on strains. For the purpose of these studies, Sprague-Dawley rats will be used. Their characteristics lay on the lower end of the range (Table I).

B. Biology of reproduction

B.1. Maturation and sexing

Laboratory rats breed year round, without appreciable seasonality. Sexing pups is easily achieved by comparing the ano-genital distances between litter mates. This distance in the male is about twice that of the female. Litters are weaned at three weeks and should be segregated by sex by about seven weeks to avoid precocious breeding.

Sexual maturity occurs between 6 and 8 weeks for both sexes, although the onset of the first estrus in females occurs at about 5 weeks while the vagina opens between 34 and 109 days. In the male testes descend between 15 and 51 days; however, they remain fully retractable in the adult [CCAC, 1984; Laboratory Animal Medicine, 1984].

B.2. Estrous cycle

Rats are polyestrous, and will accept the male and ovulate every four or five days for a 12-14 hour period. Because vaginal changes in the rat are closely related to the estrous cycle, examination of vaginal fluid and cells provides a valuable method for determining its stages. Table II summarizes the criteria used to classify the different stages of the estrous cycle.

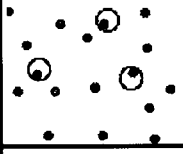
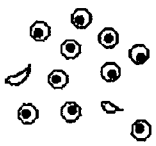
During the estrous cycle, as for the human menstrual cycle, the rat estrous cycle is coordinated by hormonal fluctuations within the classical hypothalamo-pituitary-gonadal axis (Figure 9). However, the luteal phase of the rat is shortened unless (pseudo-)pregnancy occurs.

Table I. Rat characteristics.

Adult Weight	
Male	300-400 gm
Female	250-300 gm
Life span	2.5-3 years
Body temperature	37.5 C
Chromosome number	42 (diploid)
Puberty	50 +/- 10 days
Gestation	21-23 days
Litter size	8-14 pups
Birth weight	5-6 gm
Weaning	21 days
Blood volume	6 ml/100 gm body weight

[adapted from: Laboratory Animal Medicine, 1984]

Table II . Histological changes associated with the estrous cycle.

Stage	Vaginal Fluids		Uterus	Ovary
DI	cornified cells and leukocytes		regeneration of epithelium	egg in oviduct
DII	epithelial cells and leukocytes		lumen to 25mm diameter	formation of corpora lutea
PE	small, round nucleated cells and up to 25% cornified cells		distention by fluid and vascilar engorgement	follicle enlargement
E	cornified, denucleated cells		epithelial degeneration	egg maturation; ovulation

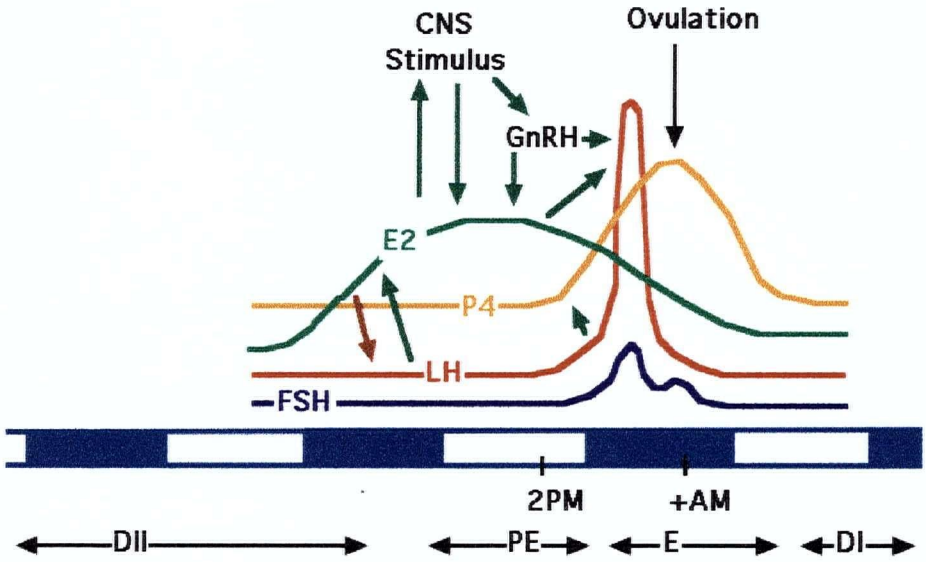


Figure 9. Hormonal levels in the female rat. During the estrous cycle, the hormonal patterns vary with the different stages of the cycle: Stimuli from the central nervous system (CNS) cause hypothalamic GnRH pulses to release LH and FSH from the anterior pituitary. Starting at the end of diestrus II, gonadotrophins induce a progressive increase in E_2 levels which, in turn, down regulate the gonadotrophins. The elevation of E_2 eventually provokes an LH peak, and the elevated LH stimulate further P_4 production. Ovulation and receptivity are coincident with the peak of P4. If mating does not occur, a new cycle starts on the morning of estrus.

B.3. Pregnancy

Fertility in both sexes is considered maximal between 100-300 days of age. Polygamous mating is commonly used and may involve from two to six females caged with one male. Males will mount estrous females numerous times in a 15 to 20 minute period, and the ejaculated semen from one to two ejaculations forms a copulatory plug that remains in the distal vagina for a few hours. Gestation length varies with strain, age, litter size (6 to 14), and ranges from 19 to 23 days (21 days on average for Sprague-Dawley) [Laboratory Animal Medicine, 1984].

B.4. Persistent estrus

Persistent estrus describes the end of the cycling in an old female rat. Anovulation begins around 450-540 days of age, when efficient breeding life already ceased long before the onset of persistent estrus.

HYPOTHESIS

Gonadotrophin-releasing hormone (GnRH) and its receptor (GnRH-R) are expressed in the rat ovary, where the local actions of GnRH are modulated, in part, by regulation of GnRH-R mRNA levels.

SPECIFIC OBJECTIVES

1. To characterize GnRH-R mRNA expression in respect to tissue localization.

2. To define the onset of GnRH-R mRNA expression during ontogeny in the rat.

3. To reveal any variation in GnRH-R mRNA levels, in the ovary of cycling adult female.

4. To detect potential ovarian GnRH-R mRNA responses to PMSG/hCG priming treatment of immature rats.

5. To examine the *in vitro* effects of LH, FSH, hCG, GnRH and $PGF_{2\alpha}$ on the levels of GnRH-R mRNA expression in granulosa cells.

RATIONALE

Gonadotrophin-Releasing Hormone (GnRH) is primarily known as a hypothalamic hormone controlling the synthesis and release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary. Receptors for GnRH are expressed on the surface of anterior pituitary gonadotrophs. Although not totally elucidated, regulation of hypothalamic GnRH and its pituitary receptor is already well defined. Short feedback loops from the pituitary gonadotrophins, as well as auto-regulation of GnRH-receptor (GnRH-R) by GnRH, and most importantly, positive and negative feedback by ovarian steroids, regulate GnRH/GnRH-R pattern of expression and activity.

With the sequencing of the mouse and human pituitary GnRH-R cDNA, and, later, the rat GnRH-R cDNA, it became possible to study the regulation of the rat GnRH-R mRNA, thus adding support to GnRH-R binding studies already performed on the pituitary receptor. However, these studies focused on the regulation of the ovarian GnRH-R mRNA.

GnRH is also expressed locally in the ovary of some (but not all, e,g not in cow ovary, [Kakar et al, 1993]) mammalian species, including human and rat. The more recent concept of GnRH as an autocrine regulator of the ovarian function, and the less explored field of investigation offered by the ovarian system seemed to be able to reveal new insight in the complex mechanisms regulating the reproductive function. It is now accepted that GnRH is a local regulator of ovarian function [Massicotte et al., 1984; Clayton et al., 1988; Tsatriri et al., 1988; Sridaran et al., 1988; Leung et al., 1989; Smith et al., 1991; Srivastava et al., 1994], and is itself subjected to control from within the ovary and from the pituitary gonadotrophins. GnRH-R and its mRNA were found to be expressed locally in the ovary of the human and the rat [Huckle et al., 1988; Goubau et al., 1992; Crumeyrolle-Arias et al., 1994; Moumni et al., 1994; Kogo et al., 1995; Whitelaw et al., 1995], thus supporting the role of GnRH as a paracrine factor.

Whatever levels of GnRH may be present in the ovary, a hormone requires a receptor to exert its actions. As GnRH and its receptor are coupled, the regulation of one can affect the activity of the other. Therefore, it is of great interest to study the regulation of GnRH-R expression, to

more comprehensively understand the regulatory processes of regulation of the reproductive endocrine system. Thus, these studies aimed to examine the regulation of GnRH-R mRNA in the ovary of the rat, chosen for its similarity with the human ovary concerning the presence of GnRH/GnRH-R and its ease of experimentation.

Tissue localization and ontogeny

Although there have been numerous studies showing GnRH binding in various reproductive tissues, there is little information on the exact distribution of GnRH-R mRNA. Therefore, it is of great interest to study the synthesis, occurrence and cell-specific distribution of this receptor molecule. In these studies, GnRH-R mRNA expression was localized to various tissues, and ontogeny of this mRNA was established in the rat pituitary and gonads.

Cycling rat

In the ovary, local production of GnRH is considered to act differentially during follicular maturation, stimulating basal steroidogenesis but inhibiting gonadotrophin-induced P_4 production. Moreover, GnRH induces the expression of P_4 receptors in rat granulosa cells [Natraj et al., 1993] and is capable of inducing oocyte release [Ekholm et al., 1981], oocyte maturation [Hillensjö et al., 1980] and luteinization [Massicotte et al., 1984]. GnRH is primarily known to exerts its effects through a specific receptor, present in the pituitary [Eidne et al., 1992], where GnRH of hypothalamic origin exerts its primary effects. However, receptors are also present in the ovary [Latouche et al., 1989] where their expression, as it is the case in the pituitary, might be affected and regulated by different factors such as E_2 levels [Gregg et al., 1989] or its own ligand, GnRH. Since it is not known whether GnRH-R mRNA levels are actually similarly regulated at different stages of the estrous cycle, it is important to demonstrate any differential regulation during the adult female rat reproductive cycle.

Peri-ovulatory PMSG/hCG induced ovulation

In several species including the rat, the number of pituitary GnRH binding sites have been observed to increase during the proestrous period immediately prior to the ovulatory surge [Bauer-Dantoin et al., 1993]. Since it is not known whether GnRH-R mRNA levels are similarly regulated prior to, during or following ovulation in the rat ovary, the study of *in vivo* expression of the ovarian GnRH-R was undertaken. Although the normal adult female is physiologically unaltered, detection of variations in GnRH-R mRNA levels in the whole ovary at a particular stage of follicular development may be masked by the presence of other follicles at different developmental stages. Thus, a synchronized model for follicular development, ovulation and luteinization (the PMSG/hCG immature rat model) is more likely to reveal alterations in mRNA levels.

Granulosa cell models

Based on the previous findings that GnRH-R mRNA is actually dramatically and transiently down-regulated after an ovulatory dose of hCG in PMSG primed immature rats, *in vitro* studies were designed to determine the influence of potential regulators of GnRH-R gene expression in pre-ovulatory rat granulosa cells. In a first series of experiments, FSH/androstenedione pre-treatment was applied to granulosa cell culture with the belief that it would promote further cellular development *in vitro* and still maintain the cells in a pre-ovulatory state. In a second series of experiments, the granulosa cell cultures did not receive FSH/androstenedione pre-treatment and were obtained 56 hours after PMSG treatment instead of 48 hours. This model is less manipulated than the first one and may be more representative of an ovulatory state.

MATERIALS AND METHODS

I. Steroid assays

A. Progesterone RIA

Serum steroids were measured using a ^{125}I-RIA kit. (Diagnostic Systems Laboratory), while media from cultured granulosa cells were assayed by a specific [^{3}H]RIA as described below.

Progesterone-RIA was performed as previously outlined [Leung et al., 1979]. Briefly, the assay used rabbit P_4 antisera (P_4-2; Kindly provided by D.T. Armstrong, University of Western Ontario) raised against 4-pregnen-6ß-ol-3, 20-dione hemisuccinate: bovine serum albumin conjugate (Steraloids, Wilton, NH). The final antisera concentration was 50 μg/ml. A standard competition method was employed, utilizing P_4 (Sigma) standards, and ^{3}H-P_4 at 10,000 cpm/tube (Amersham). The range of the assay standards was from 0.5 to 128 ng/ml. A 0.04 M phosphate buffer (pH 7.4) was used for diluting samples and controls with a final assay volume of 600 μl/tube. Phosphate buffer with dextran (0.025% w/v) and charcoal (0.25% w/v) was used to separate free P_4 from bound. Free P_4 in the supernatant was diluted in 3.0 ml of scintiverse scintillation cocktail (Canlab), and counted for 60 sec on a Wallac 1217 Rack beta-counter. Concentrations were a function of the counts and deduced from the equation generated by the standard curve (Figure 10). Samples were assayed in duplicate. Intra- and inter-assay variation was less than 10 %.

B. Estradiol RIA

Estradiol-RIA used specific rabbit antisera (Kindly provided by D.T. Armstrong) raised against estratriene-3, 17ß-diol-6-carboxymethyl-oxime:BSA conjugate (Steraloids). The final antiserum dilution was 1:200,000 w/v in phosphate buffer. As with the P4-RIA, a standard competition method was employed, utilizing E_2 (Sigma) standards, and ^{3}H-E_2 (Amersham) at 10,000 cpm/tube. The E_2-RIA was performed as described above for the P4-RIA. Furthermore, the range of concentrations and intra- inter-assay variation were similar to the P4-RIA.

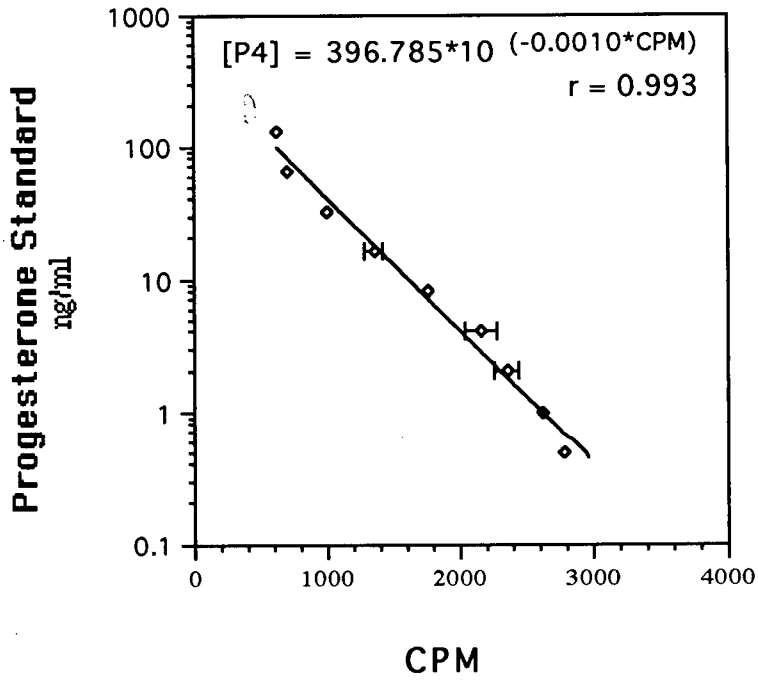


Figure 10. Progesterone standard curve. For each P_4 and E_2 [^{3}H]RIA assay, the standards were plotted against their respective counts and a curve was fitted. The equation of the best fitting curve (usually either exponential or logarithmic) was deduced and used for calculating the concentration of the samples. The correlation factor (r) was always high (minimum around 0.85).

II. Molecular biology tools

A. RNA extraction

Tissue and cell preparations were extracted for RNA using two different methods, depending on the amount of tissue or cells available and the expected yield. When a tissue was abundant (total mature ovary and all the tissues tested for tissue-localization), the cesium chloride gradient extraction method was used, as it was less expensive (but time consuming and yielding less product). When a tissue was in limited quantity, such as ovaries and neonate rats pituitaries and cultured granulosa cells, RNA was prepared using a RNA extraction kit (Bio 101).

A.1. Extraction by cesium chloride / chloroform:butanol protocol

Tissue preparations were lysed in 1 to 8 ml of homogenizing buffer (4 M guanidine-thiocynate, 5mM sodium citrate, 0.5% sarcosyl, 0.7% β-mercaptoethanol). RNA was separated on a cesium chloride gradient for 12 hours at 40.000 rpm, in a SW60 rotor and further purified by chloroform:butanol extraction and ethanol precipitation. The extracted RNA was solubilized in 50 to 200 μl of sterile DEPC treated water, and stored at -70 C until further processing [Glisin et al., 1974].

A.2. Extraction with RNaid kit

Cell preparations were lysed in homogenizing buffer (300 μl per pooled sample). Then, total RNA was extracted using a RNA extraction kit (Bio 101) according to specifications. Samples of extracted RNA were reconstituted in 10 to 20 μl of sterile DEPC treated water. RNA concentration was determined by UV-spectrophotometry (DU-64 spectrophotometer, Beckman) at a wave length of 260 mm (λ_{260}). When RNA yields were abundant, RNA integrity was checked on a 1% denaturing agarose gel stained with ethidium bromide. Gels were visualized under UV light. The rest of the RNA was stored at -70°C until further examination.

B. Reverse transcription/polymerase chain reaction

B.1. RT reaction

For all samples from each experiment, cDNA was synthesized from 0.5-5 μg of total RNA (depending on the experiment) using a first strand cDNA synthesis kit (Pharmacia) and 0.5 μg oligo(dT_{12-18}). The thermocycler (normally intended for PCR) was programmed for the reverse transcription protocol specification. After incubation at 37 C for 90 minutes, the reaction was quenched by heating to 100 C for 10 minutes and rapidly cooled down to 4 C, after which samples were spun down (to pellet the condensed sample) and stored at -20 C.

B.2. PCR reactions

B.2.a. *Cold PCR*

The polymerase chain reaction is an automated series of heating and cooling steps utilized to exponentially amplify DNA segments that are in very scarce quantity. In these experiments, double stranded cDNA was synthesized via reverse transcription for subsequent amplification. A PCR sample contains 1μl of cDNA obtained by reverse transcription, 1μl of each primer (sense and antisense at 100 pM each), 0.04 μl (0.4nmol) of each dNTP (Boehringer Mannheim), and 0.2 μl (0.2 U) of Taq polymerase (BRL) in PCR buffer (10 mM TRIS-HCl, pH 8.3, 50 nM KCl, 1.5 mM $MgCl_2$, 0.01% Gelatin).

Primers for amplifying specific portions of the genes of interest were designed from published cDNA sequences of those genes (Table III). The complete rat GnRH gene sequence as determined by Bond (1989) was used to design GnRH primers (Table IV). Primers for GnRH-R (Table IV) were originally generated from the murine cDNA sequence [Reinhart et al., 1992] and later corrected to match the complete GnRH-R cDNA sequence of the rat [Kakar et al., 1994]. The $PGF_{2\alpha}$-R primers (Table IV) were designed from the published rat $PGF_{2\alpha}$-R cDNA sequence [Lake et al., 1994]. Control RT/PCR reactions utilized the human cDNA derived primers for β-

actin (Table IV). The primers for the other control gene, G3PDH (glucose 3-phosphate dehydrogenase), an enzyme involved in glucose metabolism and used as a housekeeping gene, were commercial rat primers designed to be used specifically as controls (Table IV).

The primers used for the PCR reactions are described in Table IV and Figure 11. In short, they consist of 5 pairs of primers: A pair of GnRH-R primers, yielding a 703 bp product; a pair of GnRH primers amplifying a 195 and a 1785 bp product; a pair of $PGF_{2\alpha}$-R primers, generating a 730 bp product; a pair of β-actin primers, amplifying a 506 bp product; and a pair of G3PDH primers, targeting a 983 bp product. For each reaction, 1.0 μl aliquots of the reverse transcribed RNA was amplified by PCR on a thermocycler (Perkin-Elmer Cetus) for 21 to 36 cycles, with a 30 sec. denaturation step at 96 C, 30 sec annealing step at 50 or 55 C, 1 min 30 sec extension step at 72 C, and, at the end of the final cycle, a 7 min extension step at 72 C. PCR products were visualized under UV light on agarose gels stained with ethidium bromide (as described page 43).

Table III. GENBANK sequences for GnRH gene, GnRH-R and $PGF_{2\alpha}$-R.

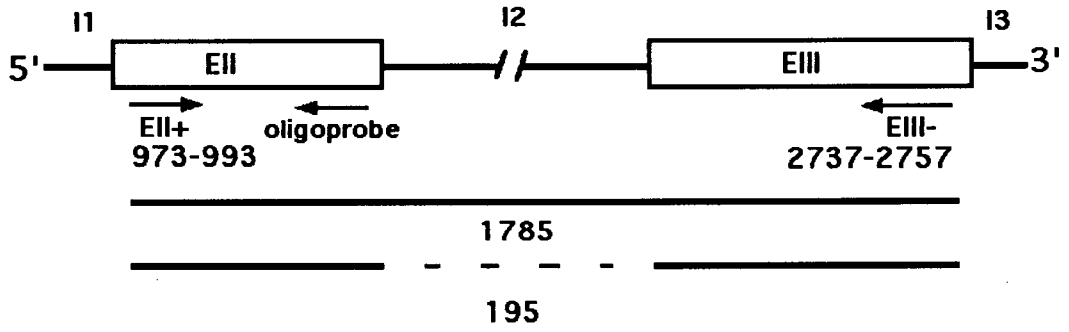
GENE	SPECIES	GENBANK ACCESSION	FROM	LENGTH	REFERENCE
GnRH	Rat	M31670	DNA (gene)	5873 bp	Bond (1989)
GnRH-R	Rat	U00935	cDNA (mRMA)	2891 bp	Kakar (1994)
$PGF_{2\alpha}$-R	Rat	S74898	cDNA (mRNA)	1101 bp	Lake (1994)

Table IV. Primers designed for PCR.

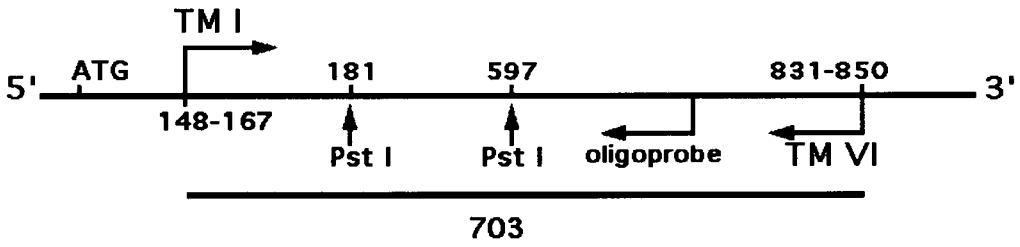
PRIMER	NAME	Sense/ Antisense	SIZE (bp)	MW	SEQUENCE (5'-3')
GnRH	**GEII+**	sense	20	6056	ATGGAAACGATCCCCAAACT
	***GEII-**	antisense	20	6077	ATCAACCAAGTGTTCAGTAT
	GEIII-	antisense	20	6079	CTCGCAGATCCCTAAGAGGT
GnRH-R	**TMI+**	sense	20	5970	CTGCCTTCAATGCCTCTTTC
	TMVI-	antisense	20	6191	ACGTAGTAGGGAGTCCAGCA
$PGF_{2\alpha}$-R	**PGFR+**	sense	20		CTCATGAAGGCATATCAGAG
	PGFR-	antisense	19		GTTGCCATTCGGAGAGCAA
	***PR+**	sense	26		GCTTCTGATAAAGACTGGATCCGCTT
β-ACTIN	**AC2**	sense	20	6117	TGATCCACATCTGCTGGAAG
	AC3	antisense	20	6037	GACCTGACTGACTACCTCAT
G3PDH	**G3+**	sense	24	7284	CATGTAGGCCATGAGGTCCACCAC
	G3-	antisense	26	8035	TGAAGGTCGGTGTCAACGGATTTGGC

(The primers preceded by * were used as oligoprobes for Southern blotting).

GnRH: GEII+/GEIII- Combination: 195 bp spliced product and 1785 bp unspliced product



GnRH-R: TMI+/TMVI- Combination: 703 bp.



$PGF_{2\alpha}$-R: PGFR+/PGFR- Combination: 730 bp

β-actin: AC2/AC3 Combination: 506 bp.

G3PDH: G3+/G3- Combination: 983 bp.

Figure 11. Size of expected PCR products. The different combinations of primers anneal to their cDNA targets to generate products determined by the position of the primers within the sequence amplified. The primers used to amplify a portion of the GnRH cDNA yield a 1785 and a 195 base pair, depending on the splicing of the second intron; GnRH-R fragment is 703 bp long; $PGF_{2\alpha}$ product is 730 bp; β-actin generates a 506 bp product, and G3PDH fragment is 983 bp.

B.2.b. *Incorporation of ^{32}P-dCTP during PCR amplification*

When hot PCR was performed, 4 nCi of α-^{32}P dCTP (Amersham) were added to each 25 µl sample. PCR products were run on a 1% agarose gel, and post-stained with ethidium bromide. Eventually, a Polaroid photograph was taken before further processing of the gel. The gel was then dried and autoradiographed and/or the dried bands were cut out and counted (Wallac 1217 Rack beta-counter). The bands of the gel could also be cut and counted directly.

B.3. RT/PCR Combination Experiments

B.3.a. *RT experiment/hot PCR*

Different amounts of total ovarian RNA (2, 1, 0.5 µg) were submitted to reverse transcription, and 1µl of the reaction was subjected to a hot amplification of GnRH-R using a standard PCR program (denaturation: 96 C, 30 sec; annealing: 50 C, 1min 30sec; extention: 72 C, 1min 30sec; 30 cycles). The PCR products were processed as described above in order to generate an autoradiogram and β-counting.

B.3.b. *PCR cycle experiments*

Once the ideal quantity of RNA to be reverse-transcribed was defined, the ideal number of cycles for PCR was determined. For each cDNA and each gene to be amplified with specific primer combinations, a PCR cycle experiment was performed to determine the optimum number of cycles for the reaction, with the optimum temperature calculated for each pair of primers. This was done in order to detect a gene of interest and visualize its different levels of expression in different samples.

The experiment consisted of running aliquots of the same PCR sample for various number of cycles, ranging here from 20 to 48, in increments of 4 cycles. After the PCR was completed,

samples were run on a 1% agarose gel containing ethidium bromide, and a negative picture of the gel was scanned with the densitometer.

C. Cloning and sequencing of a portion of the rat GnRH-receptor cDNA

Primers derived from the murine GnRH-R sequence were used to amplify a segment of the rat ovarian GnRH-R. The generated PCR product was analyzed on a 5% polyacrylamide gel electrophoresis, and amplified fragments were recovered from the gel by electroelution and purified using Gene-Clean II kit (Pharmacia). DNA was cloned into *Sma*I-digested pUC19 vector. A competent *Escherichia coli* strain was transformed with the constructed plasmid and successful transformation was verified and detected by α-complementation with X-gal. Double-stranded DNA sequencing was performed using the dideoxy chain termination method with α-^{35}S-dATP (Amersham). Nucleotide sequence comparison was performed with the program from the Genetics Computer Group of the University of Wisconsin-Madison, (Madison, WI).

D. Northern and Southern blot analysis

D.1. Northern and Southern blotting

For Northern analysis, 20μg of denatured total RNA was prepared on 1% denaturing agarose gels. Transfer to a nylon membrane and hybridization were perfofmed as the Southern blots.

Southern blot hybridization was utilized for the verification of PCR products by hybridizing a probe designed to bind to the internal portion of the predicted PCR product. The procedure used was as follows: The 1.5% agarose gels containing the expected PCR products were denatured by immersion and agitation (15 minutes at room temperature) in a solution containing NaCl (1.5 M) and NaOH (0.5 M). The reaction was then stopped with a solution of NaCl (3 M) and Tris (0.5 M) at pH 8.0. Three washes with 3xSSC (sodium chloride:sodium citrate buffer at pH 7.0) followed, each for 5 minutes, after which an overnight transfer to a nylon membrane was performed. After transfer, the membrane was washed in 3xSSC, dried, wrapped in

saran wrap, and exposed to UV light for 2 minutes. At this time the membrane was stored at 4 C until hybridization, which was performed with a radiolabeled oligo nucleotide, specific to the inner sequence of the predicted PCR product, or a cDNA probe.

D.2. Generation of an oligonucleoprobe

Radiolabeling of the oligo nucleotide was performed by a kinase reaction as follows: Primer (10 pmol; 1 μl), T_4 kinase buffer (1 μl; 10x), H_2O (2 μl), [γ^{32}P]-ATP (5 μl) and T_4 kinase (10 U; 1μl) were mixed and incubated (1 h; 37 C). The probe was then boiled for 2 minutes , and spun for 1 minute at 10,000 xg.

Just prior to hybridization, the nylon membrane was removed from the refrigerator and pre-incubated in pre-hybridization solution (6x SSC, 1x Denhart's, 0.5% SDS, 100 μg/ml salmon sperm DNA, 0.05% sodium pyrophosphate). The probe was then diluted in hybridization solution (6x SSC, 1x Denhart's, 0.5% SDS, 0.05% sodium pyrophosphate, 4 ng/ml tRNA), and hybridized at 40 C over night. The following day, the membrane was washed repeatedly in a sodium chloride:sodium citrate buffer (Wash 20 minutes, 40 C in 1x SSC, 10 minutes at 50 C, in 0.1x SSC, and 10 minutes, at 50 C in 0.1x SSC).

The washed membrane was blotted, re-wrapped, and an autoradiography was performed at -70 C for a period varying from 20 minutes to several days depending on the strength of the signal. The autoradiograph was then scanned with a video densitometer (Model 620, Bio-Rad) which quantifies the relative strength of each of the bands by their optic density. Data were then treated as below.

D.3. Generation of a cDNA Probe for the rat GnRH-receptor Gene

The rat GnRH-R cDNA obtained with the mouse primers was used as a probe in Northern blot analysis to characterize the occurrence of GnRH-R during the estrous cycle and the peri-ovulatory period in the rat. The same positive colony used for sequencing was amplified in liquid LB broth media (1% bactotryptonem 0.5% bacto-yeast extract, 1% NaCl, pH 7.0-7.5). Thereafter, the plasmids carrying the GnRH-R cDNA insert were extracted, digested, and labeled with α-^{32}P dCTP (Amersham) via random priming reaction (Random Primers DNA Labeling System, BRL).

III Quantification protocols

A. Visualization methods.

A.1. Electrophoresis

A.1.a. *RNA gels*

Electrophoresis of RNA were run on a 1% agarose gel made in GRB (gel running buffer) with 20% formaldehyde. Samples of 2 to 20 μg were made up in a 10 μl volume, and 3 μl of GLB containing 1:10 (vol/vol) ethidium bromide were added to each sample. The chambers were loaded with the samples and a RNA molecular ladder, and 100V was applied to the gel until good separation of the dye bands was obtained.

A.1.b. *DNA gels*

For DNA gels, 1.5% agarose gels were made up in TBE (Tris, Boric Acid, EDTA) and ethidium bromide (2μl/50 ml=200μg/100ml) was added directly in the gel. Samples of cDNA (16μl) were mixed with 5 μl of GLB (Gel Loading Buffer; 50% glycerol, 20% 0.5M EDTA, pH8.0, 0.1% bromophenol blue, 0.1% xylene cyanol) and loaded in the chambers. A DNA molecular ladder (10 μl) was loaded in the first well of each lane. A voltage of 100 to 150 V was applied to the gel until good separation of the dye bands occurred.

A.2. Polaroid photography

Both RNA and DNA gels could be visualized on a UV transilluminator (Foto/Prepl, Bio/Can). Polaroid pictures were taken for records (using positive film, T667) and/or for scanning the bands from a negative (positive/negative film T665).

A.3. measurements of dCTP incorporation in β-counter

When quantification was performed by hot PCR, the hot PCR products were run on a 1.5% agarose gel. The bands of the expected product were cut out, and counted in a β-counter (Wallac 1217 Rack beta-counter).

A.4. Autoradiography

Northern or Southern blots were autoradiographed at -70 C for 20 minutes to several days depending on the strength of the signal. The autoradiographs were developed with a Kodak automatic developer (model 35A, X-OMAT processor).

Hot PCR products were electrophoresed and the gel dried (Bio-Rad, model 583, 80 C for 45 minutes) and exposed.

A.5. Transluminescence video densitometry

Negative Polaroid pictures of gels and autoradiographs of Northern or Southern blots or dried gels were scanned with a transluminescence video densitometer (Model 620, Bio-Rad) which quantifies the relative strength of each of the bands by their optic density.

B. Evaluation of the visualization methods.

B.1. Northern versus Southern

In order to compare the outcome of Northern of RNA and Southern blot hybridization of RT/PCR, both quantification methods were performed for some experiments.

B.2. Quantification by transluminescence densitometry

An experiment was conducted in order to correlate O.D. reading and quantity of product present in a band. β-actin PCR was performed on an ovarian sample, and serial dilutions of the generated product were run on a standard agarose gel. Dilutions were as follows: 2, 4, 8, 16 μl of

sample were made up to a total volume of 16 μl in TBE. To each sample, 5 μl of loading dye (GLB) was added, and samples were run in duplicates. After electrophoresis, the gel was visualized on a UV transilluminator, and positive/negative Polaroid photographs were taken at two different exposures. The negatives were scanned, and the O.D. readings averaged and plotted against the amount loaded.

B.2. Hot versus cold RT/PCR

Initially, two gels of the same products were run simultaneously, and processed as described below:

1) Negative and positive Polaroid pictures of the two gels were taken and the negatives were scanned with the video densitometer.

2) One of the gels was dried, autoradiographed and scanned with the densitometer.

3) The bands corresponding to the GnRH-R product were cut out from either the hydrated or the vacuum-dried gel and counted with a β-counter.

III. Animal protocols

The rats used in these studies were supplied by UBC Animal Care Centre, Vancouver, BC. A 24 hour recovery period after transportation was considered sufficient. These animals were housed under controlled environmental conditions (12 hour day/night cycle) and had free access to standard pellet and filtered water. All experimental protocols were approved by the University of British Columbia Animal Care Committee.

A. Tissue collection

Animals were euthanized by decapitation following light halothane/NO_2 anesthesia. Tissues of interest, depending on the experiment, were extirpated, rinsed in 0.15M sterile saline solution, quickly blotted on sterile gauze, and either kept in DMEM:F12 medium for further processing and cell culture or immediately homogenized and RNA extracted. When serum measurements were planned, trunk blood was collected and serum separated by refrigerated centrifugation to be stored at -20 C until further analysis for content of steroid hormones (Figure 12).

For tissue localization experiments, adult male and female rats were used. At least two samples of each tissue were generated from different animals.

For ontogeny experiments, animals (4 to 6) of the same sex, at the same age and from the same litter were pooled together for each representative sample.

Experiments during the estrous cycle examined animals of the four different stages of the cycle as determined by vaginal smear, sampled on the morning of each day (at 9:00 am).

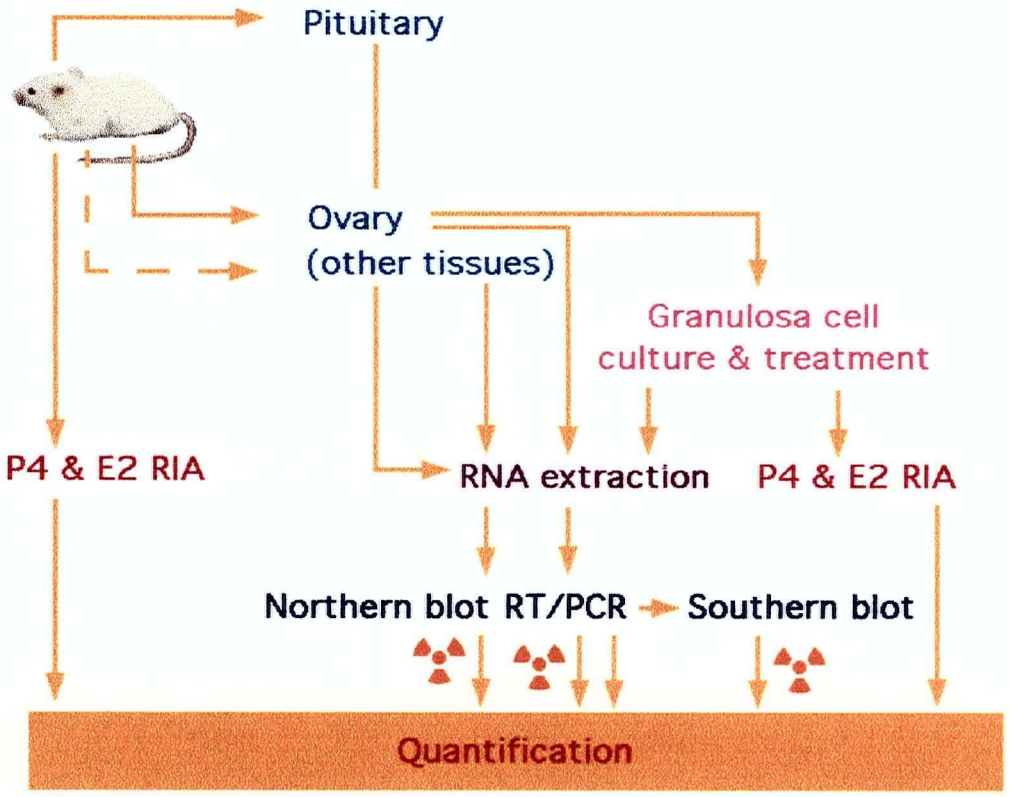


Figure 12. General protocol for *in situ*, *in vivo* and *in vitro* studies. Tissues were obtained from female Sprague Dawley rats of different age groups and following different treatments depending on the experiments (cf. methods for more details). RNA extractions from tissues were done by Cesium Chloride gradient, while extractions from granulosa cells were performed using the RNaid kit from Bio 101.

B. PMSG/hCG synchronized ovaries

EQUINEX (Ayerst Laboratories, Montreal, QU) is the PMSG preparation used for inducing the first ovulation in synchronized immature laboratory animals. It provides a source of gonadotrophin derived from pregnant mares serum (PMSG). Assayed by comparison with the International Standard, its potency is expressed in International Units (IU). One IU is the specific gonadotrophic activity of 0.25 mg of the standard preparation held by the World Health Organization.

At 09:00h on day 28 of age, and at least 24 hours after arrival, 24 rats were injected with 10 IU of PMSG s.c. in a 0.2 ml volume to induce a synchronized growth of pre-ovulatory follicles. An additional 8 rats were injected with 0.2 ml of 0.15 M saline solution, serving as vehicle treated controls. At 09:00h on day 30, 16 animals were injected with 10 IU of hCG s.c. to induce ovulation and follicular rupture, 12-15 h after injection. For each group, 8 animals were euthanized as described in the previous paragraph at 09:00h on day 30 (control group and PMSG pre-ovulatory group), or 21:00h on day 30 (PMSG + hCG, 12h; ovulatory group), or 09:00h on day 32 (PMSG + hCG, 48h; post-ovulatory group). Groups were designed according to the follicular development following PMSG/hCG stimulation [Hillensjö et al., 1974]. Ovaries and pituitaries were collected for immediate RNA extraction and trunk blood was collected and processed as described in the previous paragraph (Figure 13).

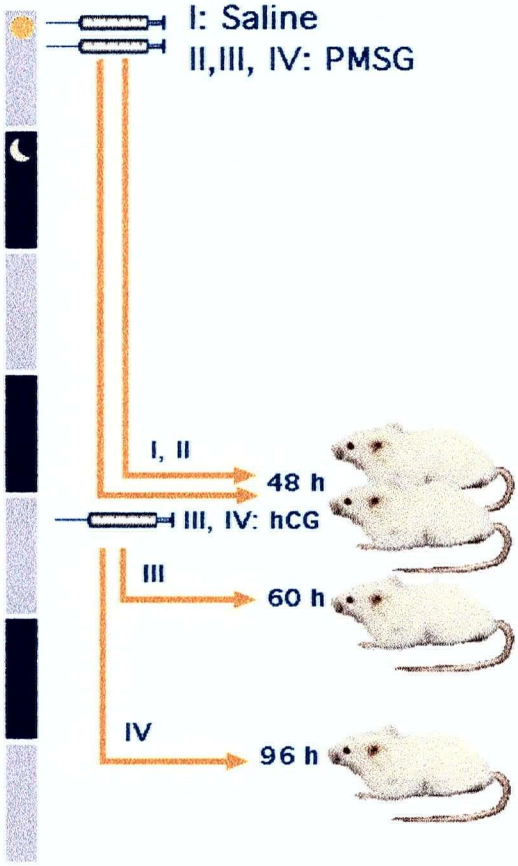


Figure 13. Protocol for peri-ovulatory period experiments. Twenty-eight day old immature Sprague-Dawley female rats were injected with 10 IU PMSG s.c. or 0.2 ml saline (control group I) and sacrificed 48 hours later (pre-ovulatory group II). hCG (10 IU) was injected 48 hours after PMSG treatment and rats were killed after 12 hours (ovulation group III) or after 48 hours, during luteinization (group IV).

C. Granulosa cells model I

At 09:00h on day 28 of age, and at least 24 hours after arrival, animals (2 batches of 5 per experiment) were injected with 10 IU of PMSG s.c. in a 0.2 ml volume to induce a synchronized growth of pre-ovulatory follicles. Animals were euthanized following light anesthesia at 09:00h on day 2 after PMSG injection (i.e., after 48h). Whole ovaries were retrieved and kept on medium for immediate processing. Ovarian follicles, easily visible on the surface of the ovaries, were punctured with a sterile 23 gauge needle under the stereomicroscope. Expressed granulosa cells were collected, spun down at 500 g for 10 minutes, and washed twice in medium. Cells were then counted, and volume adjusted with DMEM/F12 medium containing FBS (1%), penicillin/streptomycin (1% of stock solution), FSH (50 ng/ml) and androstenedione (10^{-7}M), and plated $0.5x10^{6}$ cells/well . On average, 5 rats generated two 24-well culture dishes (16 mm diameter; Falcon). After a 24 hour pre-incubation period, cultured cells were treated following different protocols described in detail in Table V. Treatments, made up in DMEM/F12 + androstenedione (10^{-7}M) were added (in quadruplets) for 24 hours. Released media were then collected for steroid assay, and plates were treated with homogenizing buffer and frozen for further RNA extraction (Figure 14).

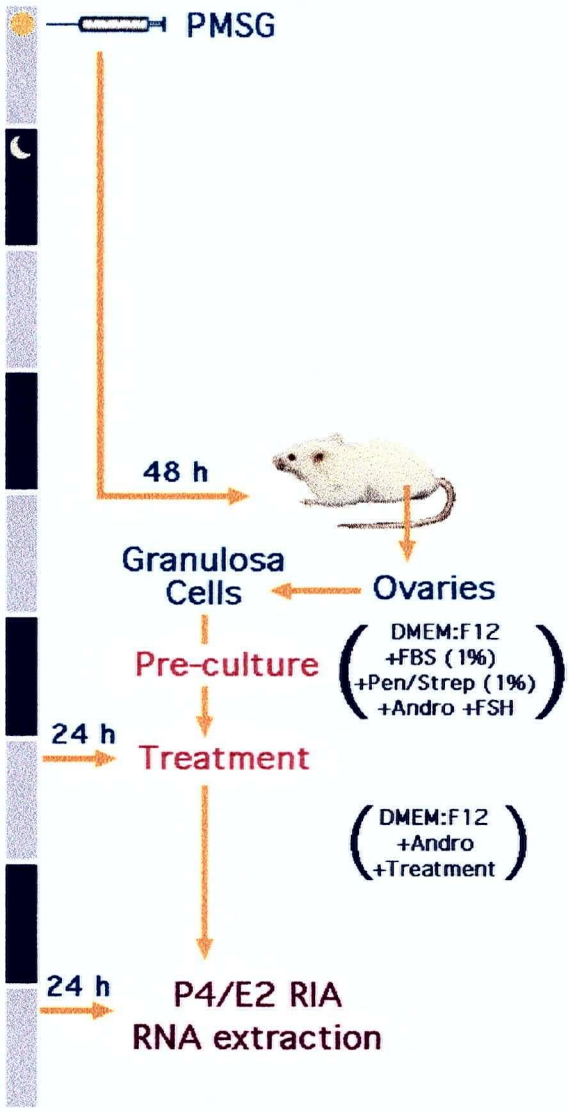


Figure 14. Protocol for granulosa cell experiments - Model I. Twenty eight day old immature rats received 10 IU PMSG s.c. at 09:00h. They were sacrificed 48 hours later, granulosa cells were retrieved from the ovaries and pre-cultured for 24 hours. Treatments were applied for 24 hours, and released media and cells were processed.

Table V. Treatments applied to granulosa cell culture - Model I.

Treatment	**Interactions**	**LH_{tc}** (1000 ng)	
1	Control	LH, 0 hours	
2	LH, 1000 ng	LH, 3 hours	
3	FSH, 1000ng	LH, 5 hours	
4	GnRH, 10^{-6}	LH, 7 hours	
5	LH + GnRH	LH, 9 hours	
6	FSH + GnRH	LH, 12 hours	
7		LH, 24 hours	
8		LH, 48 hours	
Treatment	**LH_{dr}**	**FSH_{dr}**	**GnRHdr**
1	Control	Control	Control
2	LH, 10^{-10} g/ml	FSH, 10^{-10} g/ml	GnRH, 10^{-11} M
3	LH, 10^{-9} g/ml	FSH, 10^{-9} g/ml	GnRH, 10^{-10} M
4	LH, 10^{-8} g/ml	FSH, 10^{-8} g/ml	GnRH, 10^{-9} M
5	LH, 10^{-7} g/ml	FSH, 10^{-7} g/ml	GnRH, 10^{-8} M
6	LH, 10^{-6} g/ml	FSH, 10^{-6} g/ml	GnRH, 10^{-7} M
7			GnRH, 10^{-6} M
8			GnRH, 10^{-5} M

A series of five experiments were performed on this granulosa cell model. An interaction study examined the effects of various treatments, alone or in combination. A time-course (**tc**) study of LH was undertaken, and various dose responses (**dr**) were also tested for their effects on steroid production and alteration of GnRH-R mRNA levels.

D. Granulosa cells model II

At 09:00h on day 25 of age, and at least 24 hours after arrival, animals (6 per experiment) were injected with 10 IU of PMSG s.c. in a 0.2 ml volume. Animals were euthanized as described above. At 15:00h on day 2 after PMSG injection (i.e., after 54h), whole ovaries were retrieved and granulosa cells were collected, spun down and washed twice in medium. Cells were then counted, and volume adjusted with DMEM:F12 medium containing FBS (10%) and penicillin/streptomycin (1%). They were plated at a density of 10^6 cells/well . On average, 6 rats generated four 24-well culture dishes (16 mm diameter; Falcon). After a 24 hour recovery period, cultured cells were treated following different protocols described in detail in Tables VI to VIII. Treatments, made up in DMEM/F12 + androstenedione (10^{-7}M), were added in replicates (r) of two for 18 hours. Experiments were run in triplicate (n). Released media were then collected for steroid assay, and plates were treated with GTC and frozen for further RNA extraction (Figure 15).

Photographs of rat ovaries and uteri were taken with a hand-held Contax 167 MT camera body with a Carl Zeiss (100 mm) Makro lens, and a TLA 360 daylight flash (set at TTL-through the lens- metering). Fuji Provia (100 ASA; daylight) slide film was exposed for 1/125th of a second. Photographs of rat granulosa cells were taken with a Nikon TMS inverted tissue culture microscope, using either a Nikon N2000 or Contax 167 MT body, and film as above.

Slides were scanned with a Nikon Coolscan II on a Power Macintosh, and printed on a Photo-enhanced Colour Stylewriter 2500.

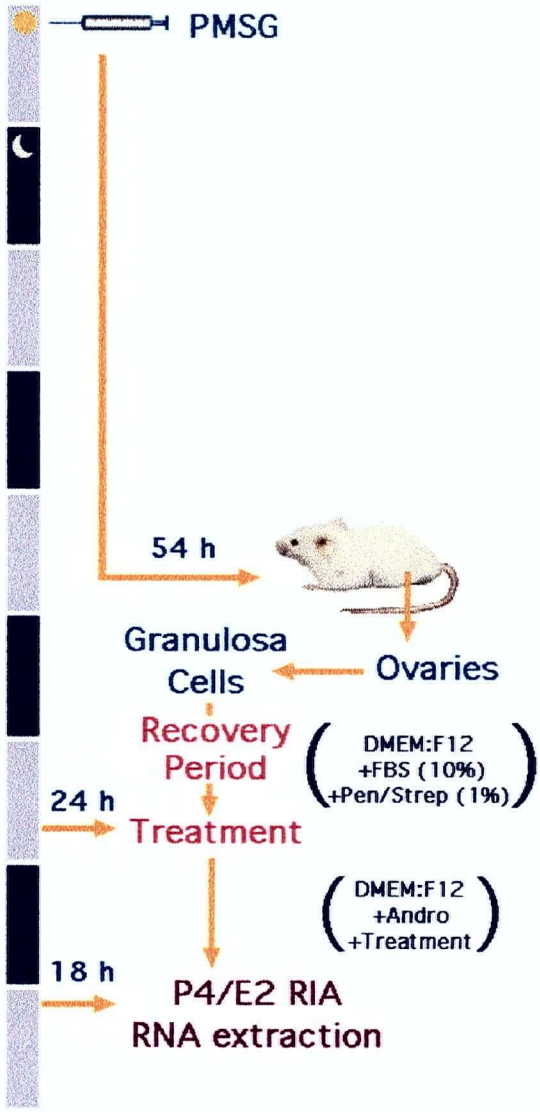


Figure 15. Protocol for granulosa cell experiments - Model II. Twenty-five day old immature Sprague-Dawley female rats were injected with 10 IU PMSG s.c. and sacrificed 54 hours later. Granulosa cells were obtained from the ovaries and pre-cultured for 24 hours. Treatments were applied for 18 hours, and released media and cells were processed.

Table VI. Time-course experiments on granulosa cell culture - Model II.

Treatment	$culture_{tc}$	hCG_{tc}	$GnRH_{tc}$
1	Control	Control	Control
2	hCG, 1 IU/ml	hCG, 0.1 IU/ml	GnRH, 10^{-11}M
3	GnRH, 10^{-9}M	hCG, 1 IU/ml	GnRH, 10^{-9}M
4	hCG + GnRH	hCG, 10 IU/ml	GnRH, 10^{-6}M
	at Day 1	for 3 h	for 3 h
	Day 2	6 h	6 h
	Day 3	18 h	18 h
	Day 4	24 h	24 h

Time-course (**tc**) studies on steroid production and alteration of GnRH-R mRNA levels were undertaken to determine the optimum time for the maximum mRNA response to treatments.

Table VII. hCG, GnRH, $PGF_{2\alpha}$ interactions - Model II.

Treatment	interactions		
1	Control	Control	Control
2		hCG, 1 IU/ml	
3	GnRH, 10^{-9}M	hCG + GnRH, 10^{-9}M	GnRH, 10^{-6}M
4	$PGF_{2\alpha}$, 10^{-9}M	hCG + $PGF_{2\alpha}$, 10^{-9}M	$PGF_{2\alpha}$, 10^{-6}M
5	GnRH + $PGF_{2\alpha}$	hCG + GnRH + $PGF_{2\alpha}$	

Interaction studies on steroid production, alteration of GnRH-R and $PGF_{2\alpha}$-R mRNA levels were examined.

Table VIII. Concentration-response experiments - Model II.

Treatment	hCG_{dr}	$GnRH_{dr}$	$PGF_{2\alpha dr}$
1	Control	Control	Control
2	hCG, 1 mIU/ml	hCG, 1 IU/ml	hCG, 1 IU/ml
3	hCG, 0.01 IU/ml	GnRH, 10^{-11}M	$PGF_{2\alpha}$, 10^{-11}M
4	hCG, 0.1 IU/ml	GnRH, 10^{-10}M	$PGF_{2\alpha}$, 10^{-10}M
5	hCG, 10 IU/ml	GnRH, 10^{-9}M	$PGF_{2\alpha}$, 10^{-9}M
6		GnRH, 10^{-8}M	$PGF_{2\alpha}$, 10^{-8}M
7		GnRH, 10^{-7}M	$PGF_{2\alpha}$, 10^{-7}M
8		GnRH, 10^{-6}M	$PGF_{2\alpha}$, 10^{-6}M

Dose response (**dr**) studies examined the effects of treatments on steroid production, alteration of GnRH, GnRH-R and $PGF_{2\alpha}$-R mRNA levels.

IV. Statistical analysis and graphing

Following scanning of negative film, counts of gel bands or RIA assay replicates were averaged when applicable. The values of each separate experiment (n =3 to 8) were then expressed as percentage of the control. For mRNA quantification, results were expressed either as percentage of control with a separate graph for the housekeeping gene (also expressed in percentage of control), or as a ratio over actin or G3PDH (also expressed as percentage of control). Then, the average and standard error of the mean (SEM) between these experiments were calculated.

Data were then analyzed by ANOVA and a post-hoc test. Treatments differing by a 'p' value less than 0.05 were considered to be significantly different.

RESULTS - CHAPTER I

Preliminary studies

I. Setting up the methodology parameters

A. RNA gel and reverse transcription

Before reverse transcription, integrity of RNA was verified on a denaturing gel. A typical RNA gel, revealing the intact 18 and 28S bands, is shown on Figure 16. Following the reverse transcription experiment, significant differences in the message were obtained and a minimum of 1 μg was chosen as the quantity of total RNA to be routinely reverse transcribed (Figure 17).

B. PCR cycle experiments

The appearance of the different PCR product generated is depicted in the schematic representation of an electrophoresis gel in Figure 18, showing the relative intensity and position of the bands in comparison to the molecular ladder.

To determine the optimum number of cycles for PCR, areas of the bands (O.D. x mm) obtained from density reading of the gels were plotted against the number of cycles, and a curve was mathematically fitted. The number of cycles to be used for each cDNA/gene combination was chosen within the linear portion of a curve, with a high enough number of cycles for the product to be detected, and yet low enough to avoid reaching the plateau part of the curve, where variations between samples would not be detectable any more (Figure 19-21).

Subsequent reverse transcriptions were followed by different PCR conditions depending on the nature of the cDNA source and the portion of gene to be amplified. The annealing temperature was adjusted for the optimal amplification with the minimum number of side products. The conditions used are summarized in the following table (Table IX).

Table IX. PCR conditions for different cDNAs with different primer combinations.

	GnRH-R		GnRH		$PGF_{2\alpha}$-R		β-Actin		G3PDH	
	Cycles	Temp	Cycles	Temp	Cycles	Temp	Cycles	Temp	Cycles	Temp
Whole tissue	27	50 C	27	50 C	N/A	N/A	24	50 C	N/A	N/A
Whole ovary	27	50 C	27	50 C	N/A	N/A	24	50 C	21	50 C
Granulosa cells	32	50 C	32	55 C	32	50 C	30	50 C	24	50 C

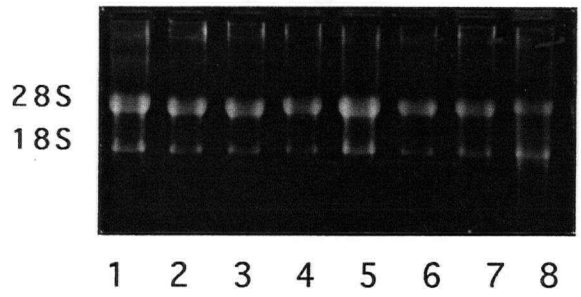


Figure 16. Integrity of total RNA. To check for the integrity of total RNA after extraction, RNA electrophoreses were performed on a 1% agarose gel prepared as described in the methodology section . The gels were visualized on a UV transilluminator and a Polaroid picture was taken. The picture shown here is that of a typical experiment where granulosa cell RNA was extracted from 8 samples (corresponding to 8 treatments) and 2 μg were run .The main ribosomal bands, 28 S and 18 S, highly expressed in all tissues, were visible if the RNA sample was intact. A degraded RNA sample would only show a long smear, or nothing.

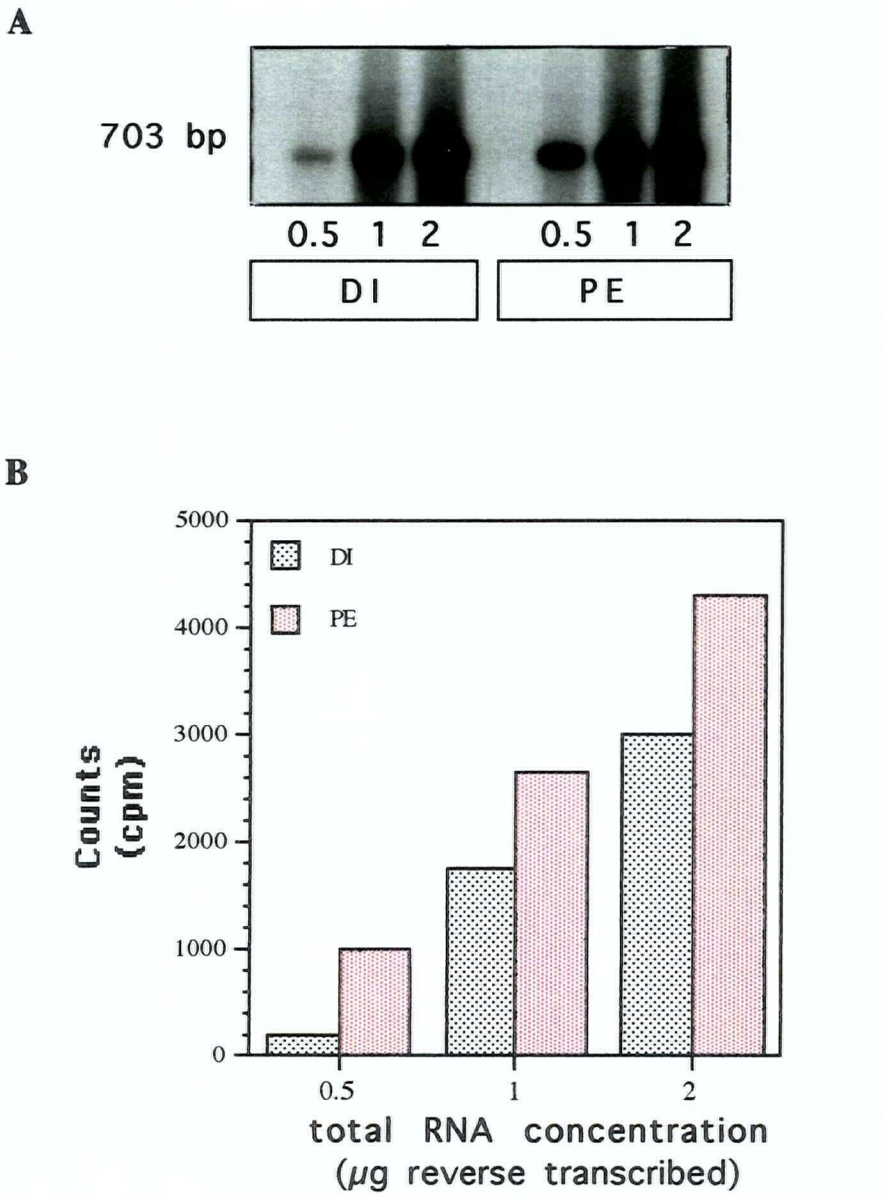


Figure 17. Reverse transcription experiment. Different amounts, i.e., 0.5, 1 or 2 μg of ovarian RNA samples from diestrus I stage (**DI**) and proestrus stage (**PE**), were reverse transcribed and subjected to a standard PCR (50/72/96 C, for 33 cycles) using GnRH-R primers TMI/TMVI and [^{32}P]dCTP. Products were run on a 1.5% agarose gel. The gel was then dried and directly autoradiographed. The autoradiogram shown here (**A**) reveals a GnRH-R product whose intensity is proportional to the amount reverse transcribed, as determined by densitometry reading of the autoradiogram (**B**). For further experiments on ovarian RNA, 1 μg was chosen to be routinely reverse transcribed.

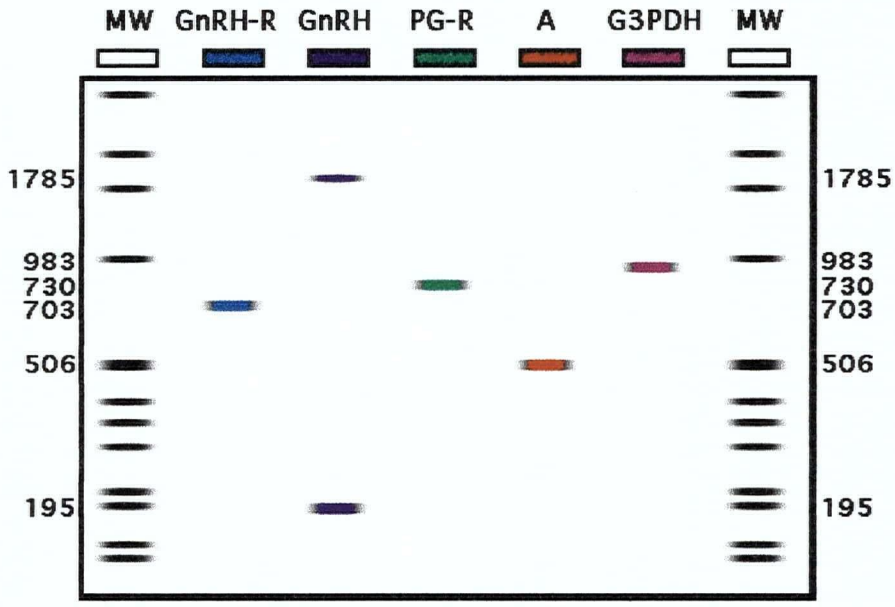


Figure 18. Schematic representation of PCR products showing sizes of amplified segments. When run on an agarose gel, the different PCR products obtained with the primer combinations described earlier should yield bands of the expected sizes migrating, as shown on this diagram, in relation with the molecular ladder. GnRH-R product is a 703 bp segment; GnRH gives two products of 1785 bp and 195 bp respectively; $PGF_{2\alpha}$-R yields a 730 bp product; β-actin band is 506 bp and G3PDH segment is 983 bp.

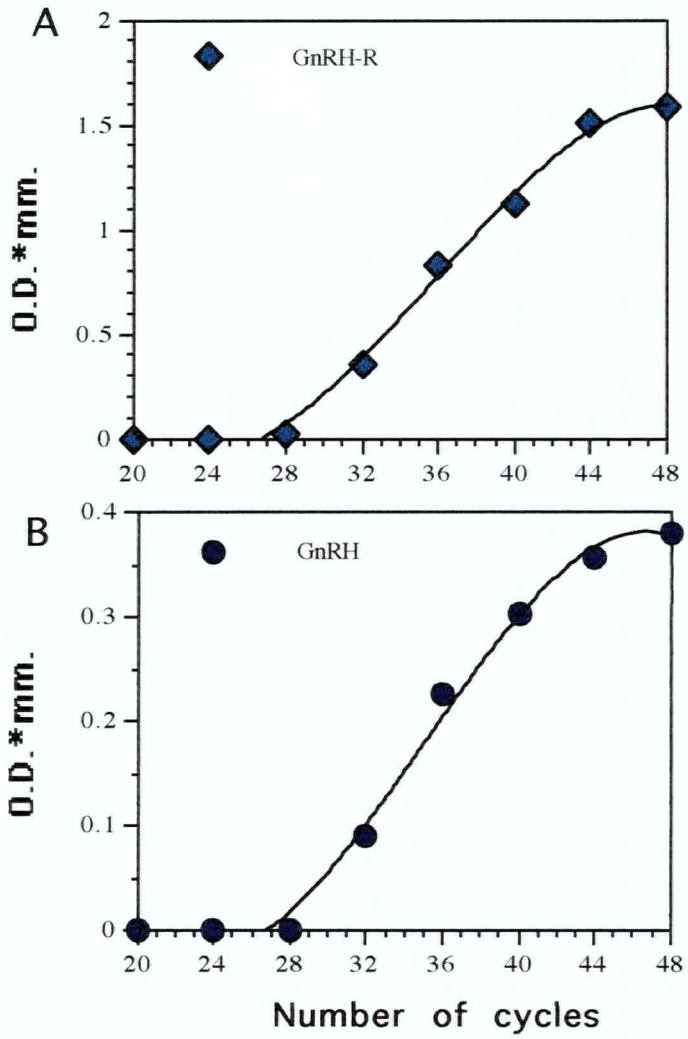


Figure 19. PCR cycle experiment. (GnRH, GnRH-receptor). To determine the optimal PCR conditions, PCR cycle experiments were run for each cDNA type, with every PCR primer combination. Standard PCR conditions (96/50/72 C) were used with a number of cycles varying from 20 to 48, in increments of 4. PCR products were run on a 1.5% agarose gel containing ethidium bromide and photographed with a positive/negative Polaroid film. The negative was scanned and optic density values (area in O.D. x mm) were plotted against the number of cycles. The number of cycles to be routinely used was chosen within the linear part of the fitted curve: 35 cycles for GnRH-R (**A**), GnRH (**B**).

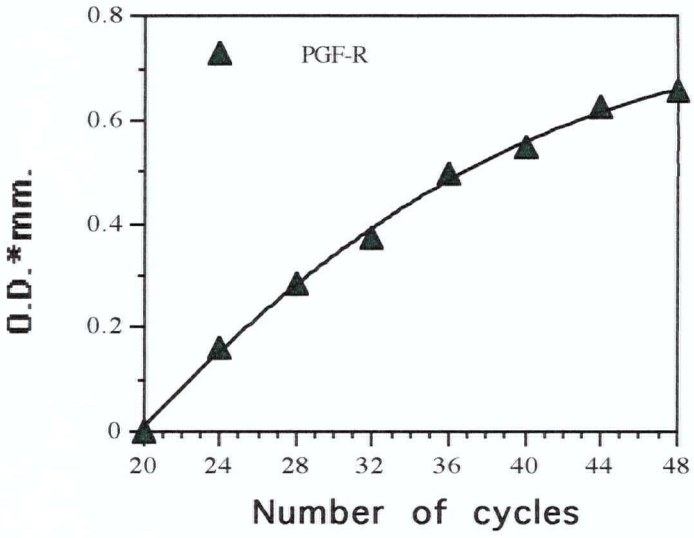


Figure 20. PCR cycle experiments. ($PGF_{2\alpha}$ -receptor). PCR cycle experiments were run as described in Figure 16. For $PGF_{2\alpha}$ -receptor, 35 cycles were chosen to be run routinely.

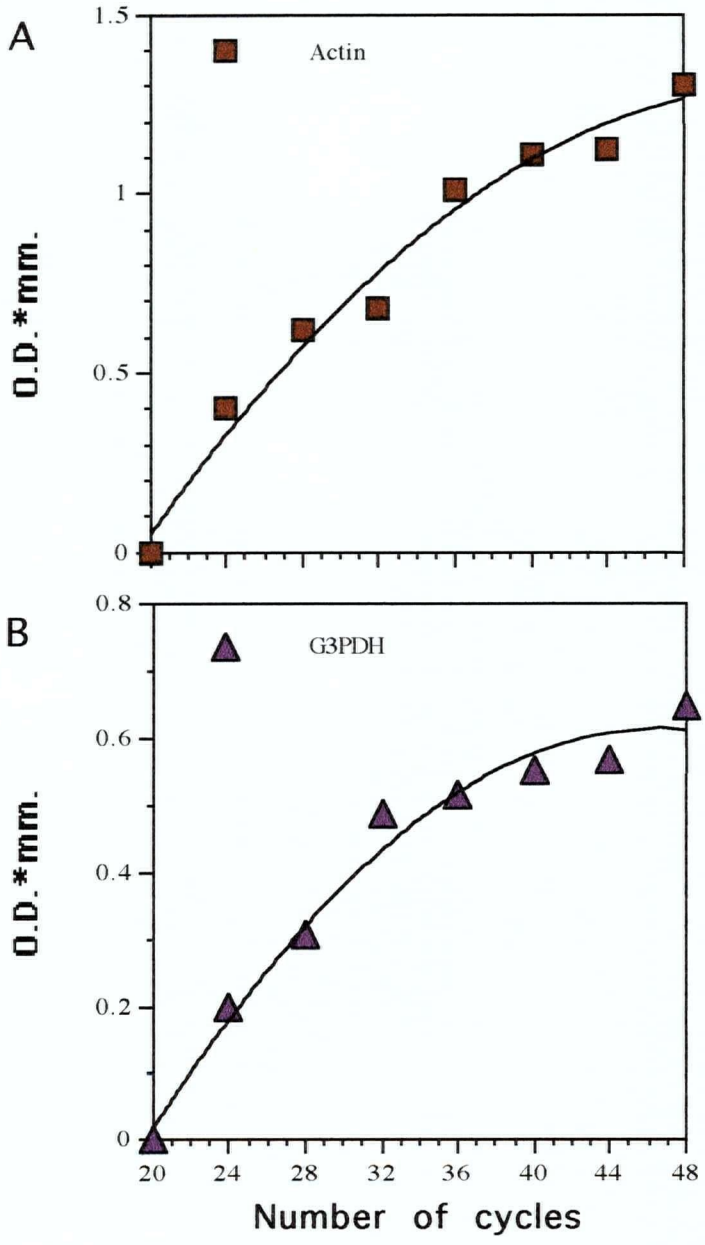


Figure 21. PCR cycle experiments. (Actin, G3PDH). PCR cycle experiments were run as described in Figure 16. For β-actin (**A**), 30 cycles were chosen, and for G3PDH (**B**), 28 cycles.

II Comparison of the different quantification protocols

A. Quantification by transluminescence densitometry

Figure 22.A shows a direct representation of the O.D. function of the volume. The correlation between O.D. and quantity (or volume) is not linear. For example, the O.D. for 4 μl is 1 Unit (O.D.), while the O.D. for 16 μl (i.e., 4 times more) is only 2.5 units (i.e., 2.5 times more). If a user was to read 1 unit for their first sample, and 2.5 for the second sample, he/she could be led to believe that the concentration of product in the second sample is 2.5 times that of the first sample, when it is in fact 4 times. Hence, the densitometer underestimates the variations in the quantity of products present in the bands.

To correct for this large underestimation error, the relative volumes can be plotted on a logarithmic scale as a function of the O.D. reading, and a curve mathematically fitted. A logarithmic curve will fit the data, although in this example, a power function, with a very good r value of 0.995, fits it even better (Figure 22.B). Entering the O.D. reading in this system (curve or equation) gives an accurate estimation of the quantity of product present in the band scanned.

The equation obtained for this particular standardization would be different for each gel, and each exposure of the picture. Hence, it is not possible to establish a standard for each experiment. For smaller variations, the correlation is linear enough for the relative concentrations to be evaluated directly. Thus, when running subsequent experiments and using the O.D directly for expressing the variations in mRNA levels, one must keep in mind that variations may be greater in the biological system studied than they appear to be as visualized by the technique used and that the evaluation as determined by the O.D. readings might be an underestimation of the real differences between samples. Therefore, the quantification methods employed here are semi-quantitative.

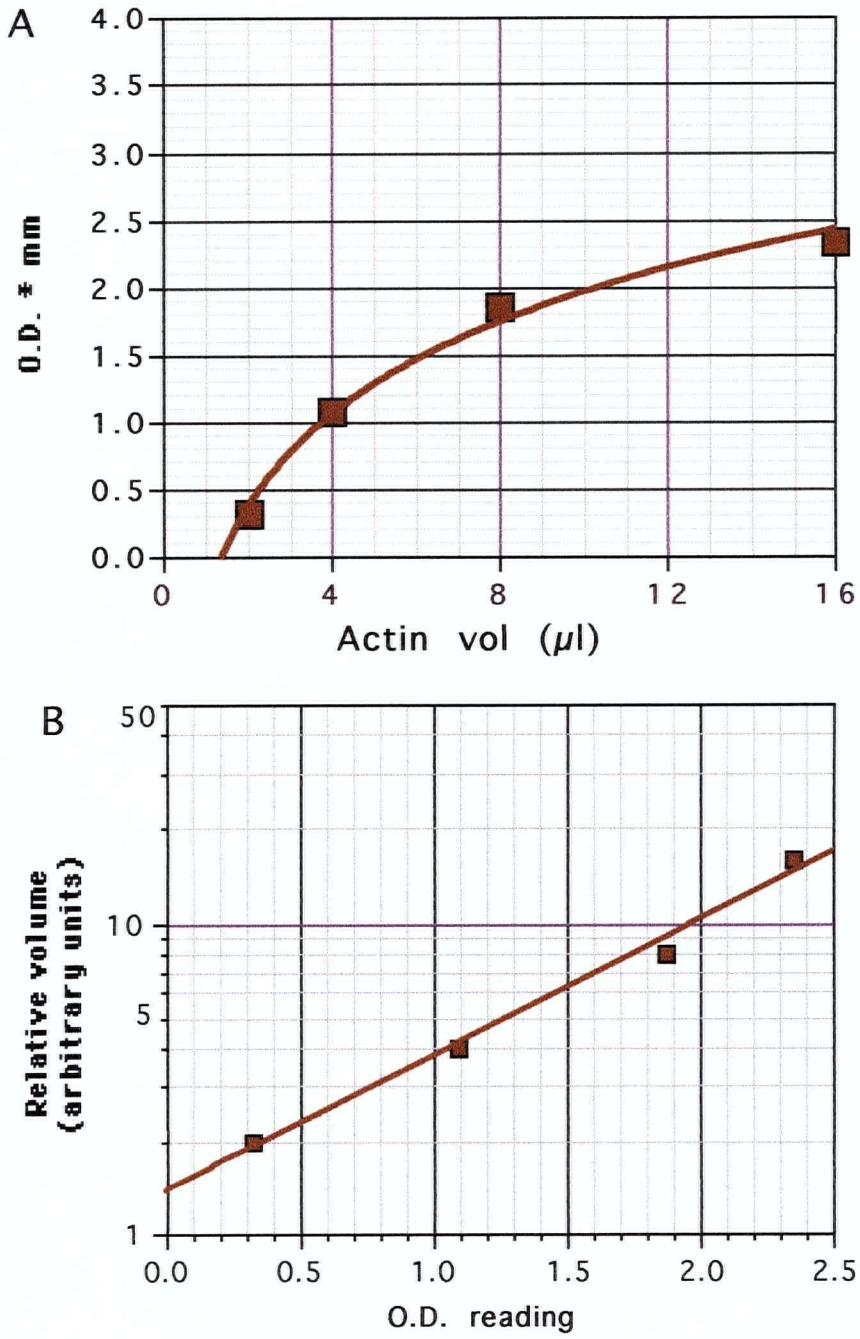


Figure 22. Densitometer standardization. To verify the validity of the scanning technique, different amounts of PCR products (2, 4, 8 or 16 μl of β-actin PCR) were run on a 1.5% agarose gel. A Polaroid photograph was taken and the negative scanned with a densitometer. **A**: The optic density (O.D.) was plotted as a function of the volume. The O.D. was not directly proportional to the volume loaded. **B**: The relative volumes were plotted as a function of the O.D. on a log scale. A curve was fitted and the equation deduced (Relative volume = $1.380 \times 10^{(0.436 \times OD)}$; r=0.995). The correct relationship between the O.D. and the quantity of cDNA present in the bands of the gel is in fact a power function.

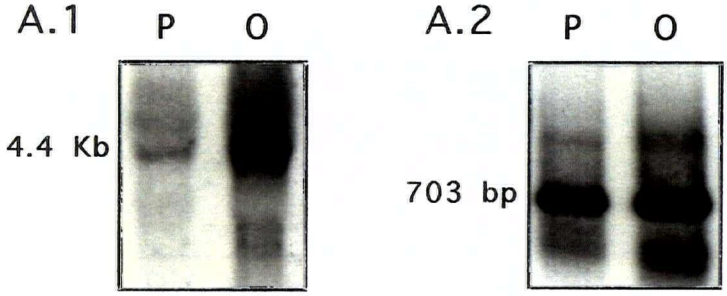
B. Northern versus Southern

Both Northern blot analysis of RNA and Southern blot hybridization of the RT/PCR products were performed for some early experiments. The results obtained by the two techniques were comparable (Figure 23). Southern blots are more time consuming and work intensive than Northern blots. However, when hybridization was required (e.g., for confirmation of the identity of a PCR product), Southern blots were preferred as the total RNA extracted from the different samples was usually too low for Northern blot hybridization.

Northern blots of GnRH-R mRNA showed multiple bands, with a main transcript of 4.4 Kb. The other bands represent alternative transcripts. Southern blots also displayed multiple bands, although they were not always visible on the gel of the PCR products.

C. Hot versus cold PCR

Once plotted in percentage of control, all three methods gave similar results. Thus, a gel of a cold PCR, photographed and scanned directly could be utilized, avoiding the necessity of radiolabeled material.



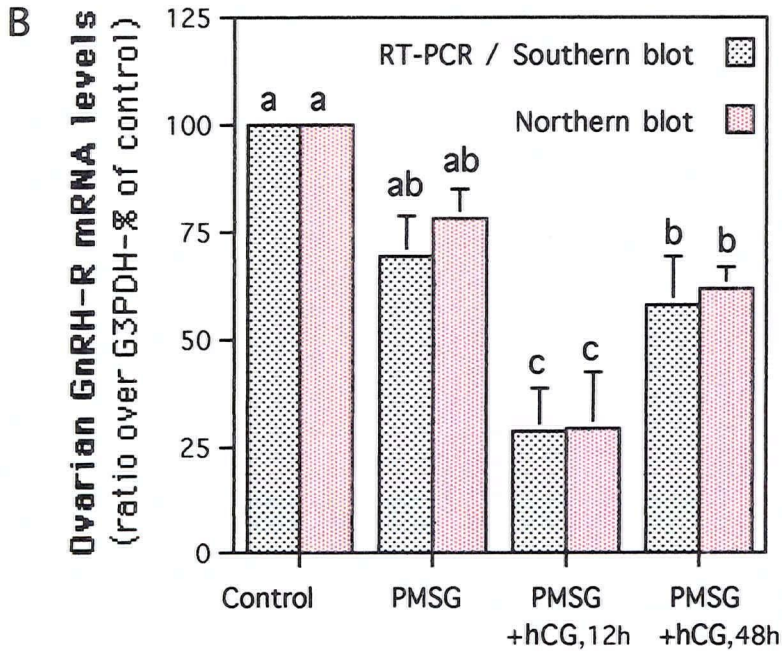


Figure 23. Northern versus Southern. Northern blotting and RT-PCR/Southern hybridization were used to confirm the presence of GnRH-R in the rat ovary and pituitary (**A1.** and **A2.**) and quantify variations between different stages or treatments of a tissue, such as ovary in the present experiment (**B**). Both techniques were equally efficient in detecting GnRH-R transcript or quantifying them, although Northern blotting required higher yields of RNA. Thus, when hybrydization was necessary, Southern blotting was preferred over Northern because RNA yields obtained from the samples studied were often low.

III. Sequencing of a portion of the ovarian GnRH-R

The cDNA sequence for the rat GnRH-R being unknown at the beginning of this study, in 1992, the primers used to amplify a portion of the rat GnRH-R cDNA were derived from the murine cDNA sequence [Reinhart et al., 1992].

TM1+ (5' CTGCCTTCAATGCTTCCTTC 3')
and
TM6- (5' ACATAGTAGGGAGTCCAGCA 3')

The PCR amplification generated a 703 bp product. Sequencing revealed a 663 bp (703-[2x20]) cDNA sharing 93% of homology with the reported sequence from the mouse pituitary GnRH-R (Figure 24). Since then, it was verified to be identical to the published rat pituitary GnRH-R sequence [Kakar et al., 1994].

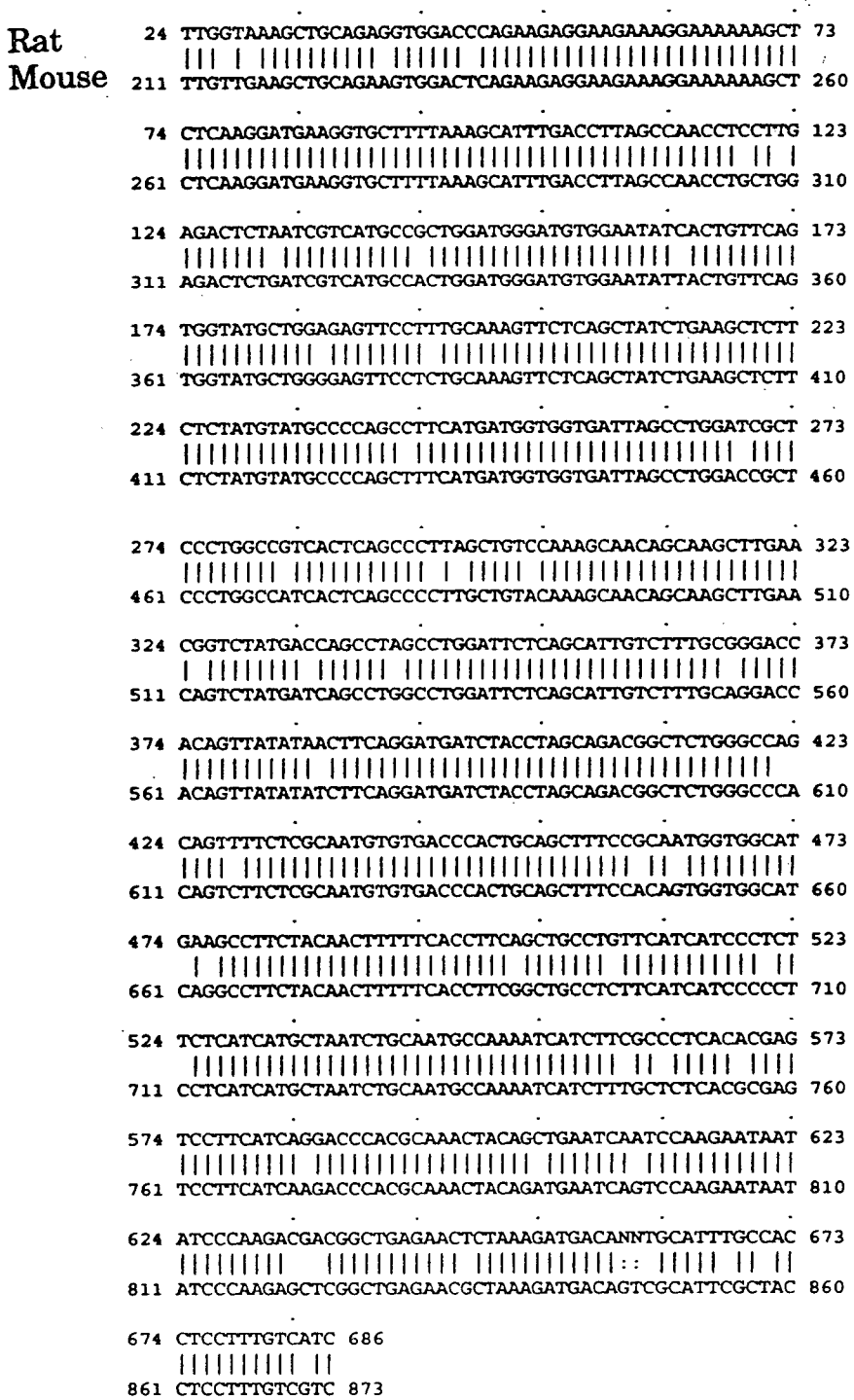


Figure 24. GnRH-receptor partial cDNA sequence: rat versus mouse. The high degree of homology expected between those two species was confirmed after the sequencing of a partial GnRH-R cDNA sequence. The cDNA obtained was used for Southern and Northern blottings.

RESULTS - CHAPTER II

Detection of GnRH-receptor mRNA

I. Tissue localization of GnRH-receptor expression

Amplification of GnRH-R transcripts, from two sets of rat cDNA, generated fragments corresponding to the expected product (703 bp) in the following tissues (Figure 25): Total brain, pituitary of cycling rats, lung, kidney, adrenals, uterus of cycling rats, ovary, corpus luteum, oviduct, testis, ventral prostate, and epidydimis. However, no expression of GnRH-R mRNA was detected, even after a 2 week exposure of the autoradiograms, in the following tissues: cerebellum, whole hypothalamus, pituitary of pregnant animals, heart, iliopsoas (muscle of the leg), mammary gland, duodenum, ileum, colon, spleen, liver, pancreas, placenta, or the uterus (of pregnant or lactating animals). β-actin PCR amplified a product in all samples.

II. Ontogeny of GnRH-R expression

In the rat pituitary (Figure 26-27), the transcript appeared to be present, as early as 8 days old, with a progressive increase in transcripts with age (5-fold increase by adulthood). In the female gonads (Figure 26-27), the GnRH-R transcript was hardly detectable in the early neonatal animal (day 6). In the gonads, a rapid increase in GnRH-R transcripts occurred, with testicular and ovarian transcripts increasing 2- and 20-fold, respectively, by puberty. Both pituitary and gonadal GnRH-R mRNA seem to follow a similar pattern.

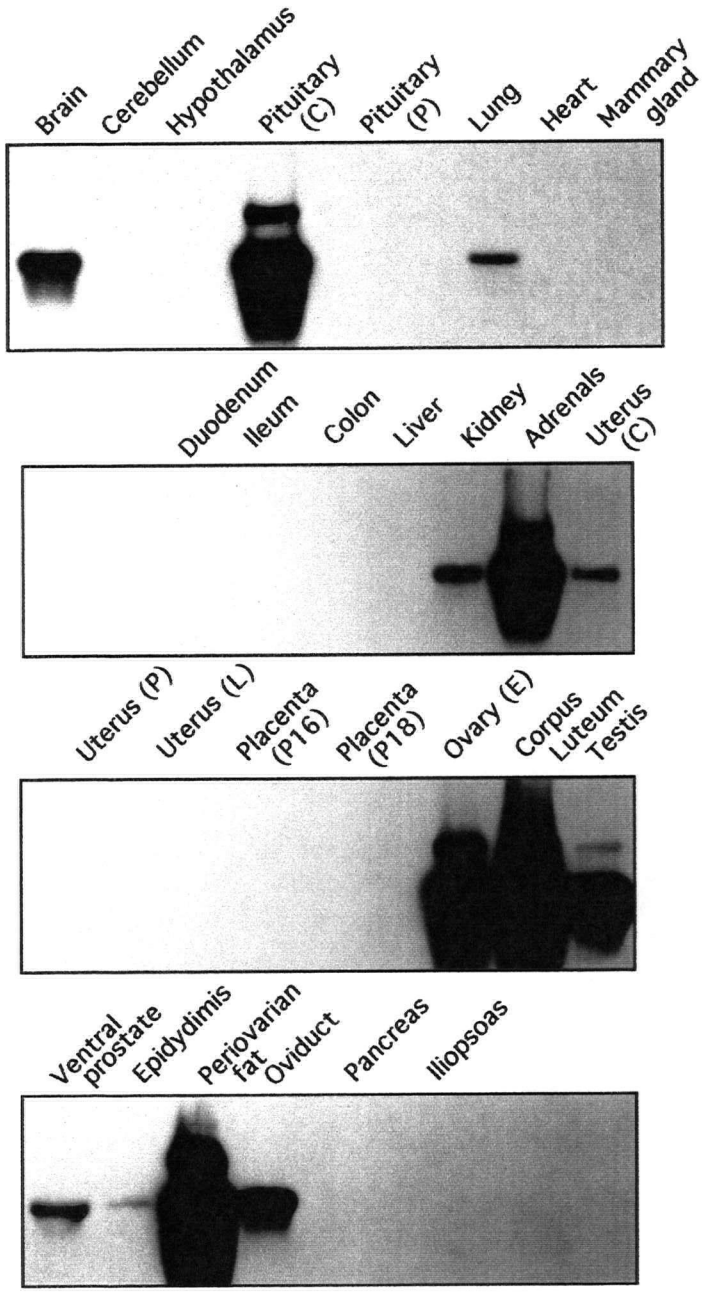


Figure 25. GnRH-receptor tissue localization in the rat. Cycling (C); pregnant (P), days of pregnancy 16 or 18 (P16, P18); lactating (L). GnRH-R was found to be expressed in different reproductive tissues of the male and female rat, as well as other steroidogenic or steroid-sensitive tissues, as revealed by Southern blottings.

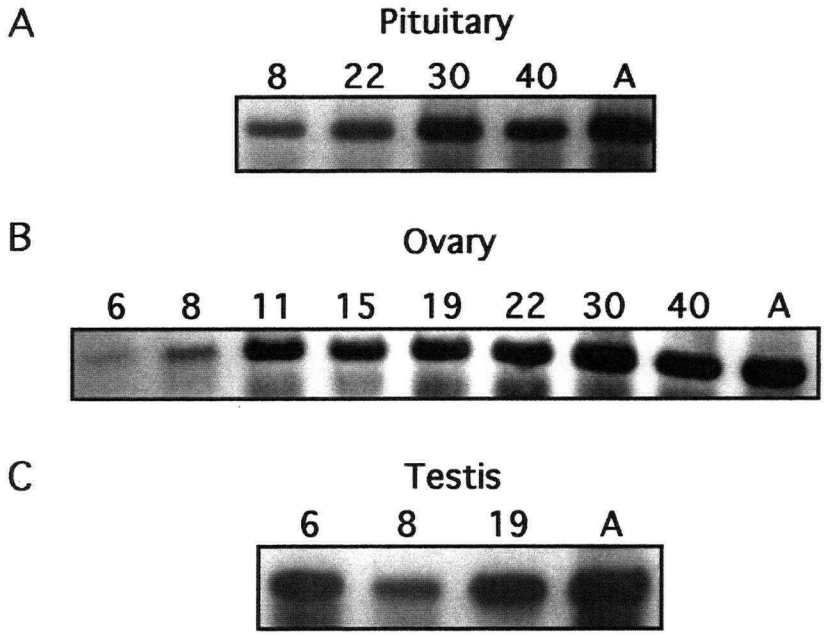


Figure 26. GnRH-receptor ontogeny - Southern blot of pituitary, testis and ovaries. Southern blots of pituitary, ovary and testis samples from rats of different ages (in days; A = adult) showed a progressive increase in GnRH-R expression in the pituitary, ovary and testis.

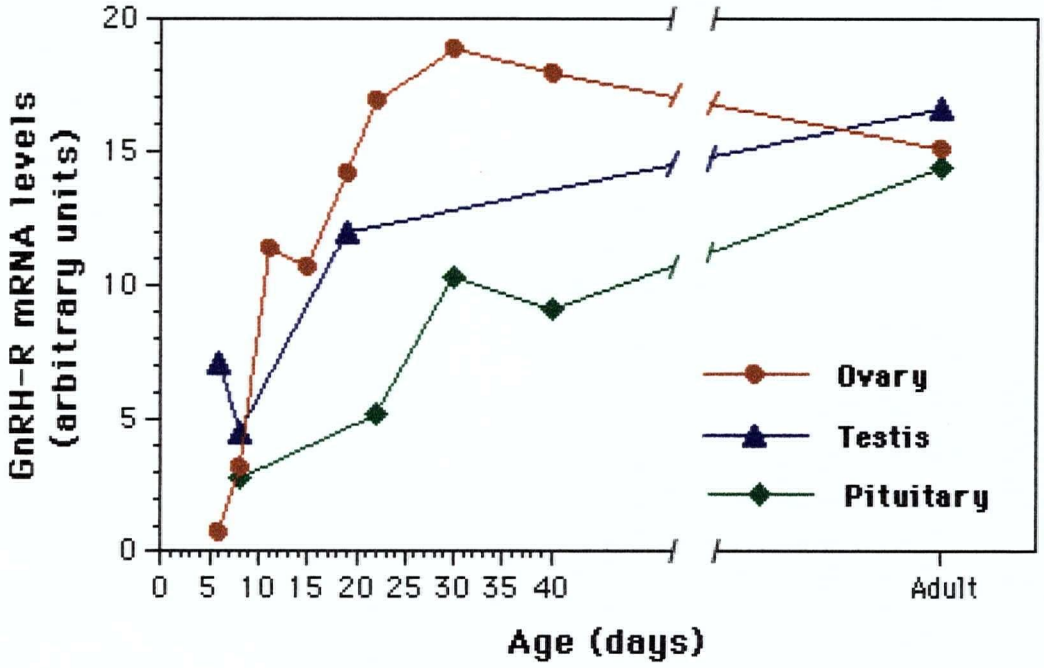


Figure 27. GnRH-receptor ontogeny - Graph of scanned autoradiogram. Graphed scan of the autoradiographed Southern blots of pituitary, ovary and testis samples from rats of different ages (in days) showed a progressive increase in GnRH-R expression in the pituitary, ovary and testis.

RESULTS - CHAPTER III

Quantification of GnRH-receptor mRNA *in vivo*

I. Cycling female Sprague Dawley (SD) rat

A. GnRH-receptor mRNA expression in the ovary and pituitary

The results from 7 different animals per stage (mornings of each day of the cycle) showed that during the estrous cycle, levels of GnRH-R mRNA in the pituitary were found to be at their lowest on the morning of the estrus. Levels rose thereafter, until the morning of the second day of diestrus, up to almost 2 times that of the estrus, and declined again towards the next estrus (Figure 28).

In the ovary, no significant changes in GnRH-R mRNA levels could be detected with the sampling of stages studied (Figure 28).

β-actin mRNA did not display any variations for at any stage in the tissues examined.

B. Serum progesterone and estradiol measurements

Serum P_4 and E_2 levels were determined by radioimmunoassays. Serum P_4 levels were increased by 2.2 to 3 fold between diestrus I and proestrus, and fell down to the level of diestrus 1 by the morning of estrus. Serum E_2 levels were at their highest on the morning of proestrus and dropped by 2 times by the next diestrus period (Figure 29).

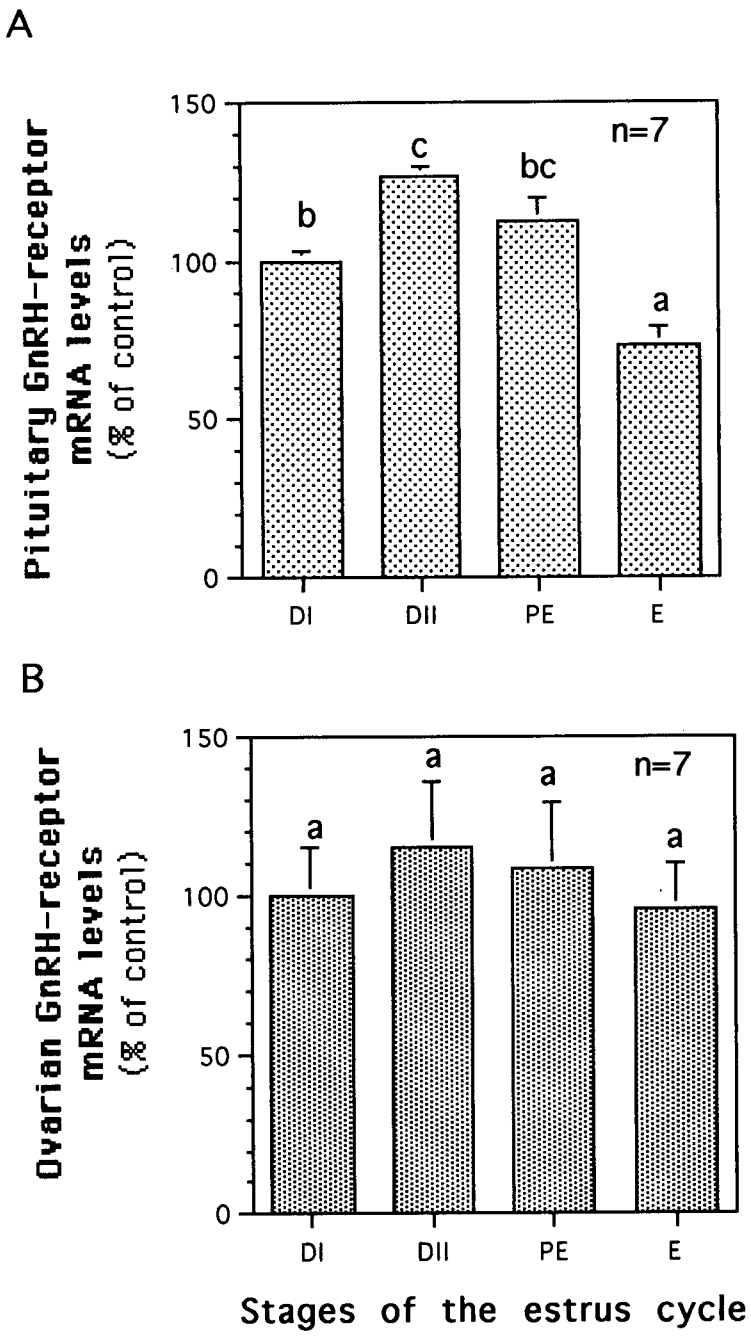


Figure 28. GnRH-receptor mRNA expression in the ovary and the pituitary of the cycling rat. RNA from pituitaries and ovaries was obtained from cycling adult rats at different stages of the estrous cycle, and subjected to RT/PCR and Southern blotting. GnRH-R mRNA levels were quantified from the autoradiograms of the pituitary (**A**) and the ovary (**B**) samples. Diestrus I was arbitrary chosen as the 100% control level. In the pituitary, GnRH-R exhibit lower levels during estrus, but no significant changes were observed in the ovary. ($p<0.05$; $a \neq b \neq c$)

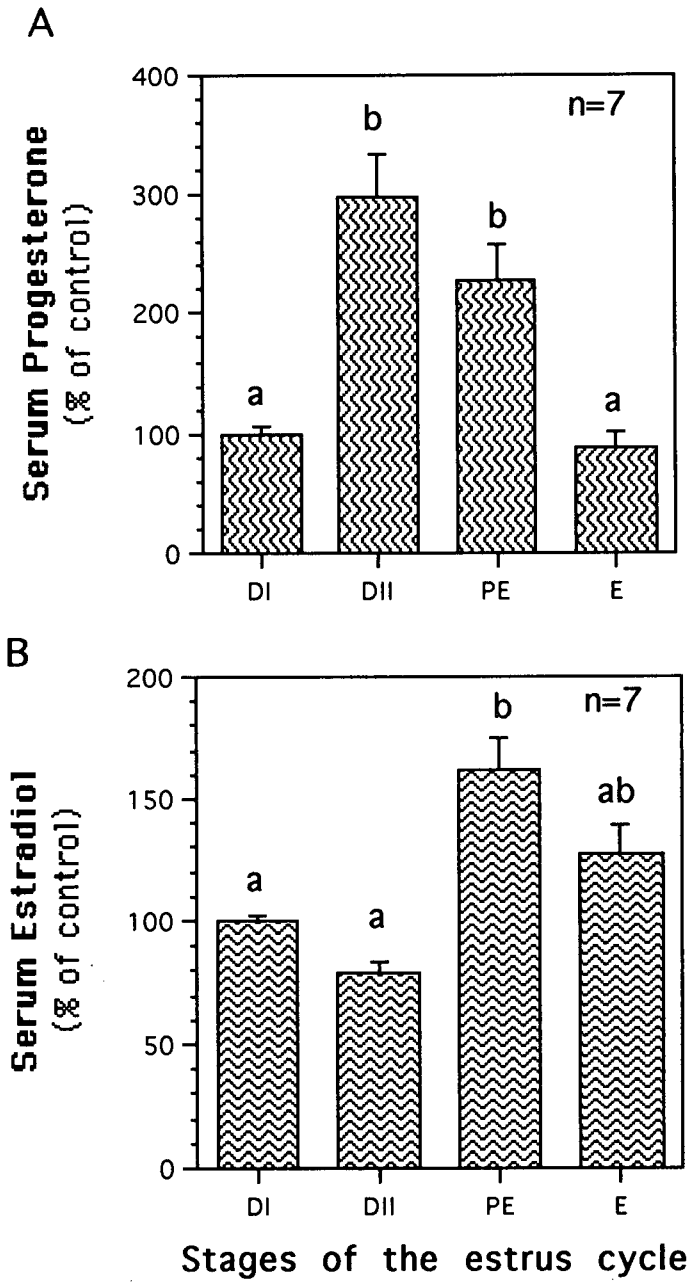


Figure 29. Steroidogenesis in the cycling rat. Normal, cycling adult rats were sacrificed in the morning of each day of the estrous cycle, as determined by vaginal smears. Serum P_4 (**A**) and E_2 (**B**) were measured with a [^{125}I]RIA kit. Diestrus I was arbitrary chosen as the 100% control level. Variations in the serum steroid levels reflect the normal profile of adult cycling rats for the morning of each day, although the P_4 surge was not detected due to the time-points assayed ($p<0.05$; $a \neq b$).

II. *In vivo* studies in the PMSG/hCG synchronized model

A. Ovarian and pituitary GnRH-receptor mRNA levels

In the pre-ovulatory ovary following PMSG induced follicular differentiation, no statistically significant changes in the levels of GnRH-R mRNA levels occurred when compared to saline-treated controls. However, in the ovulatory group (12 hours after hCG administration), a dramatic reduction to levels down to 30 % of those seen in control animals was detected. In the post-ovulatory ovary, GnRH-R mRNA levels rose again to two-fold higher than those seen during ovulation (Figure 30).

In pituitaries of pre-ovulatory animals, levels of GnRH-R mRNA were considerably reduced when compared to controls. hCG treatment did not affect GnRH-R mRNA abundance at 12 hours after hCG injection. However, by 48 hours after hCG injection, the levels returned to those seen in the controls (Figure 30).

B. Serum steroid levels

In order to correlate changes in GnRH-R expression with circulating steroid levels, serum concentrations of P_4 and E_2 were measured (Figure 31). The decrease in pituitary GnRH-R mRNA levels in the pre-ovulatory group occurred despite a significant increase in serum E_2, whereas ovarian GnRH-R expression pattern was found to be inversely related to serum P_4 levels (Figure 31).

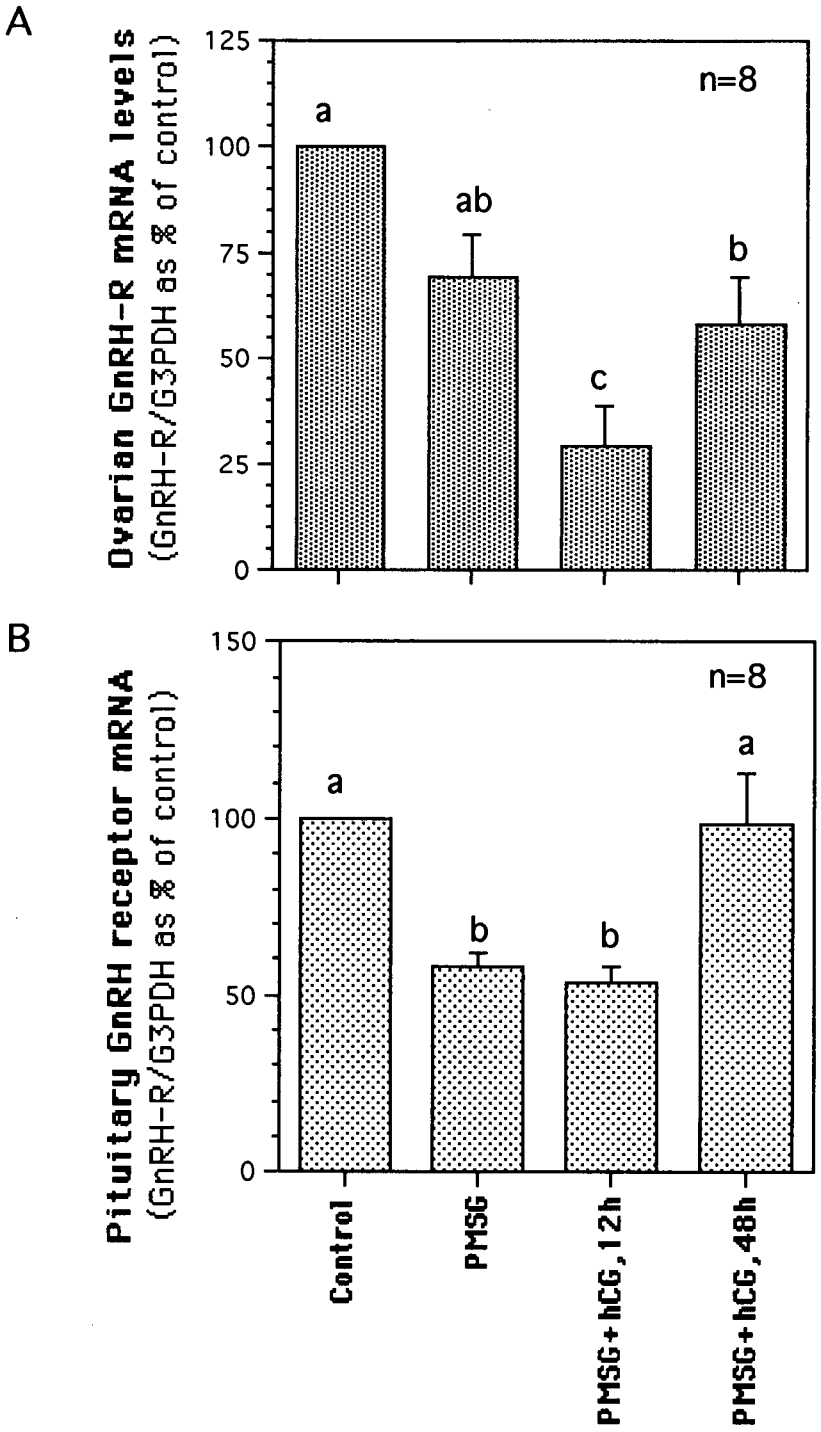


Figure 30. peri-ovulatory expression of GnRH-receptor. Ovarian (**A**) and pituitary (**B**) expression of GnRH-R mRNA during the peri-ovulatory period was studied by RT-PCR/Southern blotting. Groups were obtained as described in materials and methods and summarized in Figure. 13. In the ovary, PMSG/hCG treatment lowered the levels of GnRH-R mRNA down to 30% after 12 hours. After 48 hours following hCG injection, GnRH-R mRNA levels gradually returned to levels closer to the control. In the pituitary, PMSG alone down-regulated GnRH-R mRNA levels to the same degree of PMSG/hCG treatment and GnRH-R returned to control levels by 48 hours after hCG injection. ($p<0.05$; $a \neq b \neq c$)

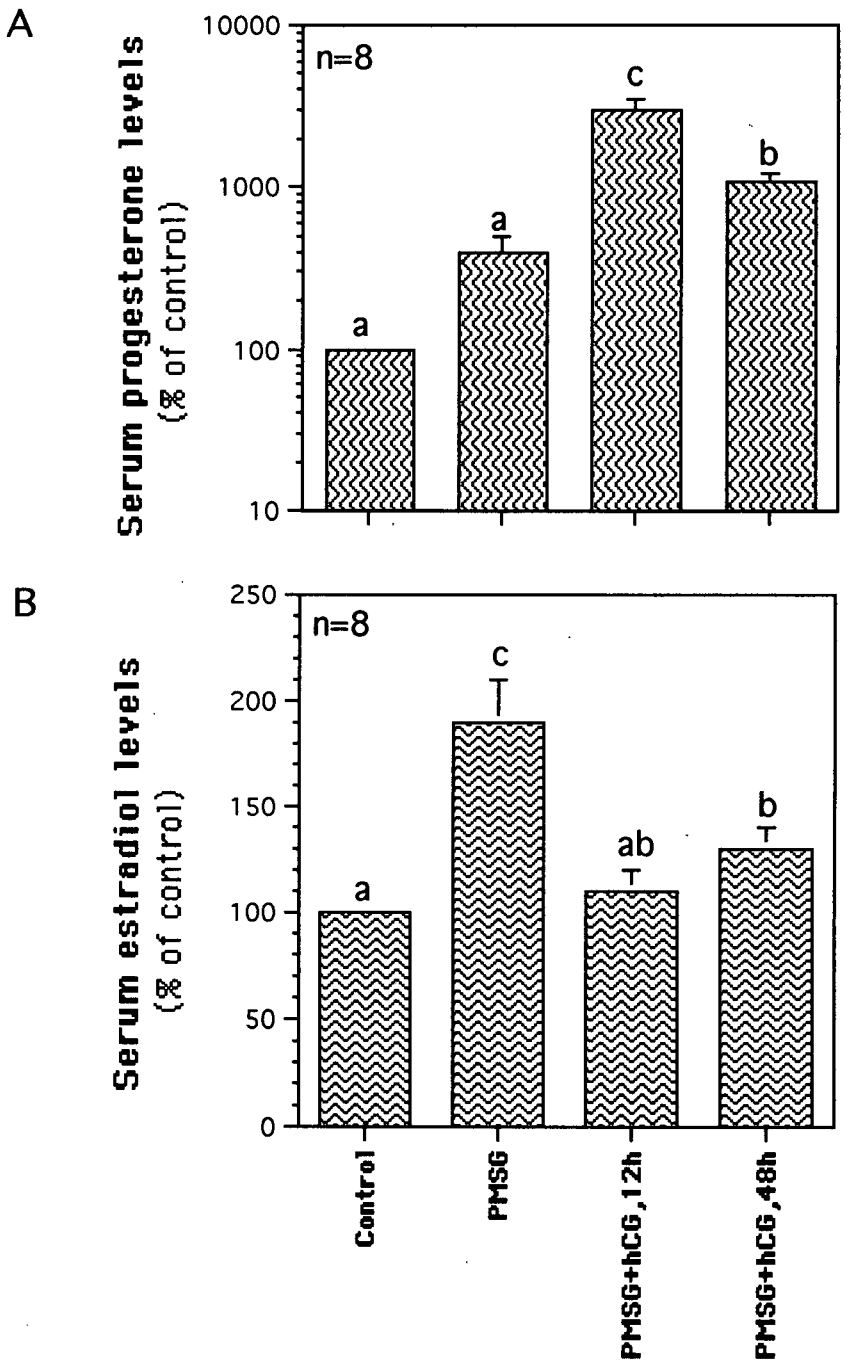


Figure 31. Peri-ovulatory steroid levels. Serum concentrations of P_4 (**A**) and E_2 (**B**) were measured using [^{125}I] RIA kits (Diagnostic Systems Laboratory Inc. Webster, TX). Groups are as in Figure 13. Treatments cause P_4 to rise with a maximum level 12 hours after hCG injection (corresponding to a stimulated ovulation) while E_2 levels peak 48 hours after PMSG injection (corresponding to a proestrus stage). ($p<0.05$; $a \neq b \neq c$)

RESULTS - CHAPTER VI

Quantification of GnRH-receptor mRNA *In vitro*

I. Studies in the granulosa cell model I

A. Effects of LH, FSH and GnRH on progesterone production

To verify that the *in vitro* culture system of rat granulosa cells is sensitive to treatment with FSH (ovine), LH (ovine), and GnRH, cells were isolated from PMSG-primed immature rats and cultured with a low concentration of FSH (50 ng/ml) and androstenedione (10^{-7}M) to stimulate a pre-ovulatory phenotype. Under these conditions, the cell viability routinely assessed by exclusion of trypan blue exceeds 90% in plated and washed cells after a 24 hour pre-incubation period. As expected, these cells responded to treatments with LH and FSH with a 10-fold increase in P_4 output, while GnRH inhibited P_4 production compared to the control. The assumption that GnRH-R is also expressed by those cells is corroborated by the finding that GnRH, when added in conjunction to LH or FSH, completely abolished the response of P_4 synthesis to gonadotrophins. (Figure 32).

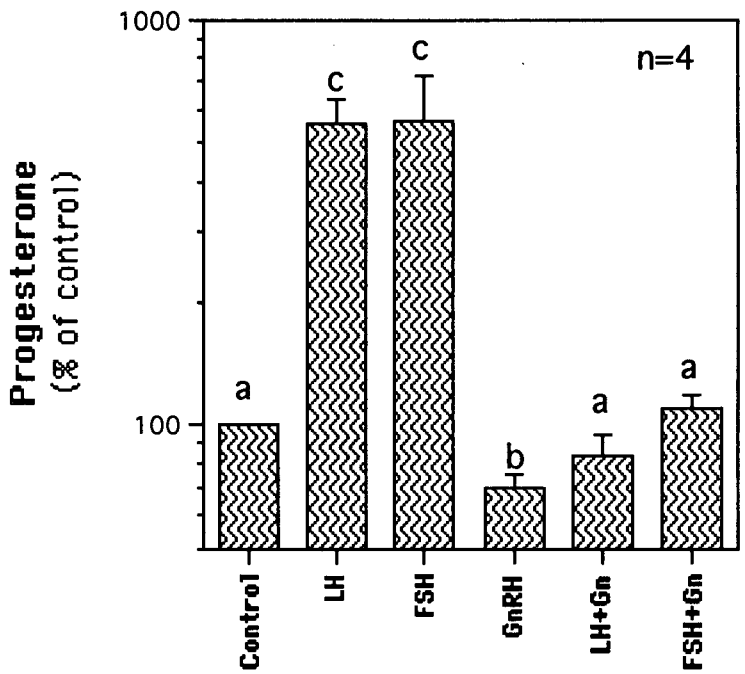


Figure 32. Progesterone output in FSH pre-treated cultured granulosa cells. This graph shows the effects of a 24-hour treatment period with LH, FSH, or GnRH alone or in combination, on P_4 production (measured by [^{3}H] RIA) by FSH-pretreated pre-ovulatory granulosa cells. The gonadotrophins cause a significant increase in P_4 production while GnRH inhibits basal production and gonadotrophin-stimulated production. ($p<0.05$; $a \neq b \neq c$)

B Time- and concentration-dependent effects of LH

To assess the time-course effect of a high concentration (1 μg) of LH, mimicking the situation seen after an endogenous LH peak, total RNA from granulosa cells cultured for periods of 3, 5, 9, 12, 24 and 48 hours were isolated and assayed for abundance of GnRH-R mRNA. The levels of GnRH-R mRNA did not change significantly until 12 hours after stimulation with LH, when they dropped to 25% of the levels seen at 0 hour. The levels of GnRH-R mRNA remained low at 24 hours, and returned to the levels seen at 0 hour by 48 hours after application of the LH treatment (Figure 33).

The decrease in GnRH-R mRNA levels correlated with drastic increases in P_4 output, but preceded the increase in E_2 synthesis occurring at 24 hours (Figure 34). The levels of GnRH-R mRNA were found to decrease with increasing concentrations of LH, reaching their minimum of 40 % compared to the untreated control for a LH concentration of 100 ng/ml (Figure 35). This down-regulation of GnRH-R occurred concomitantly with increases in P_4 and E_2 (Figure 36).

C. Effects of FSH

The possibility of a more general gonadotrophin-induced regulation of GnRH-R mRNA levels was further tested by 24 hour treatments with increasing concentrations of FSH. However, in repeated experiments, no change was seen in GnRH-R transcripts abundance compared to the expression of G3PDH (data not shown), while both P_4 and E_2 responded to FSH treatment with an increase in production, as is it the case with LH treatment (data not shown).

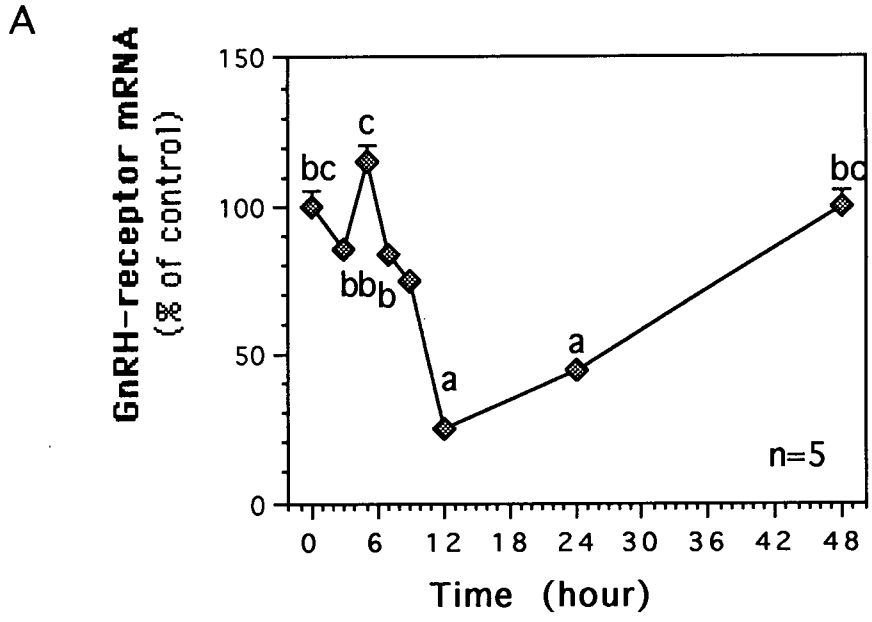


Figure 33. LH time-course effects on GnRH-R mRNA levels. Time dependent effects of LH on GnRH-R mRNA levels, quantified by RT-PCR/Southern blot hybridization. Cells were exposed to LH (1000ng/ml/well) for up to 48 hours. GnRH-R mRNA levels were plotted as a function of time. LH down-regulated the mRNA with a maximum efficacy after 12 hours of treatment. After 48 hours, GnRH-R mRNA returned to control levels. ($p<0.05$; $a \neq b \neq c$)

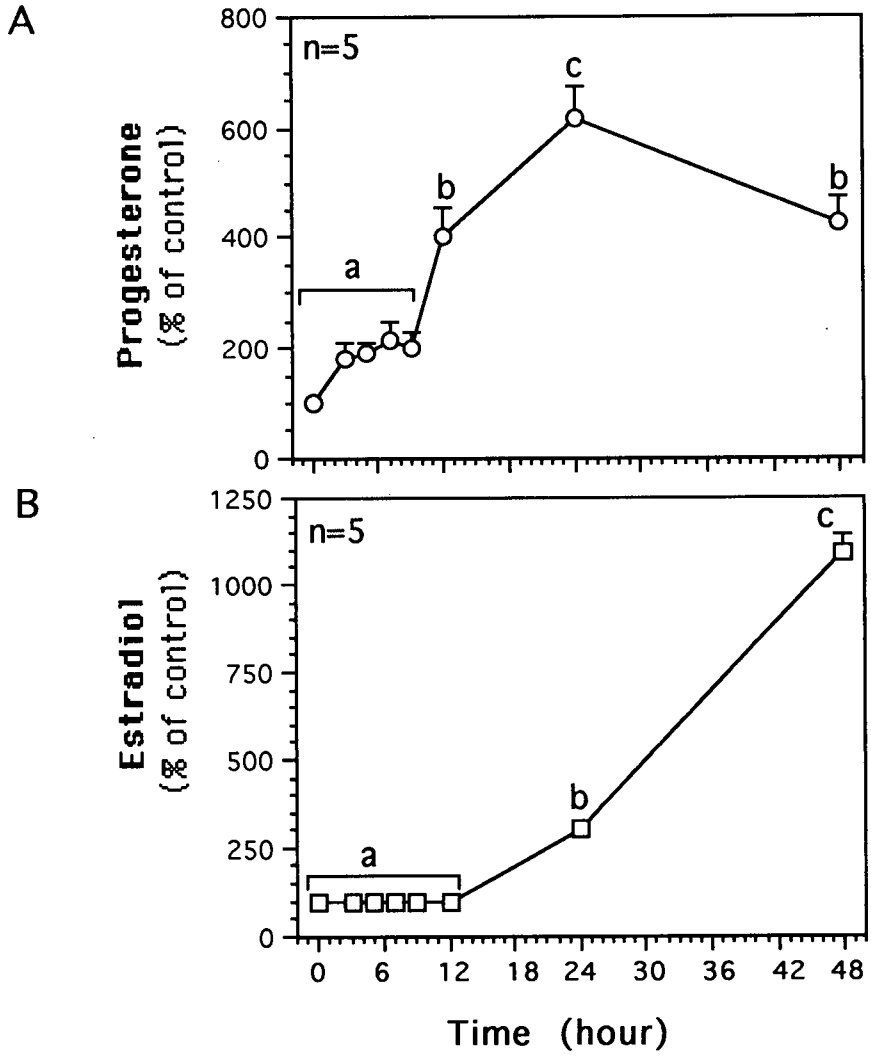
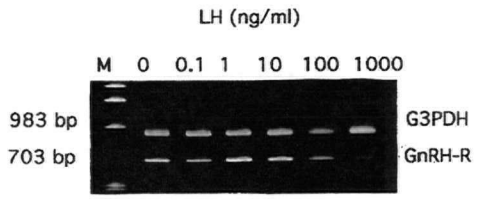


Figure 34. LH time-course effects on steroidogenesis. Time dependent effects of 1000ng of LH on P_4 (**A**) and E_2 (**B**) show a progressive accumulation of both steroids (measured by [^{3}H] RIA). P_4 seems to plateau after 24 hours, and even be partially metabolized or degraded by 48 hours. ($p<0.05$; $a \neq b \neq c$)

A



B

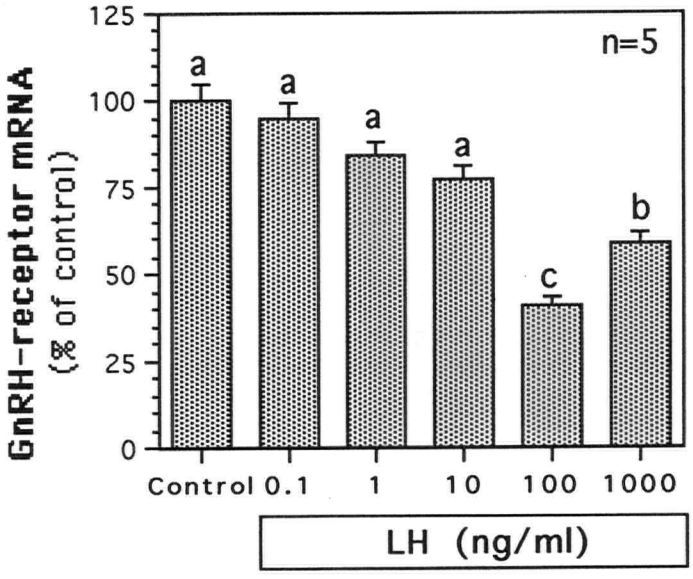


Figure 35. Effects of increasing concentration of LH on GnRH-R mRNA levels. Concentration dependent effects of LH on GnRH-R mRNA level were quantified by RT-PCR/Southern blotting. Cells were exposed to LH (0.1 to 1000ng) for 24 hours. GnRH-R mRNA levels were measured. **A** shows the gel of one representative experiment (GnRH-R and the control gene G3PDH run together on the same gel) and **B** is a graph of the relative quantities of GnRH-R product for the pooled experiments (n=5). Both show a concentration dependent decrease in the levels of GnRH-R. G3PDH does not show any significant variation from one treatment to another. ($p<0.05$; $a \neq b \neq c$)

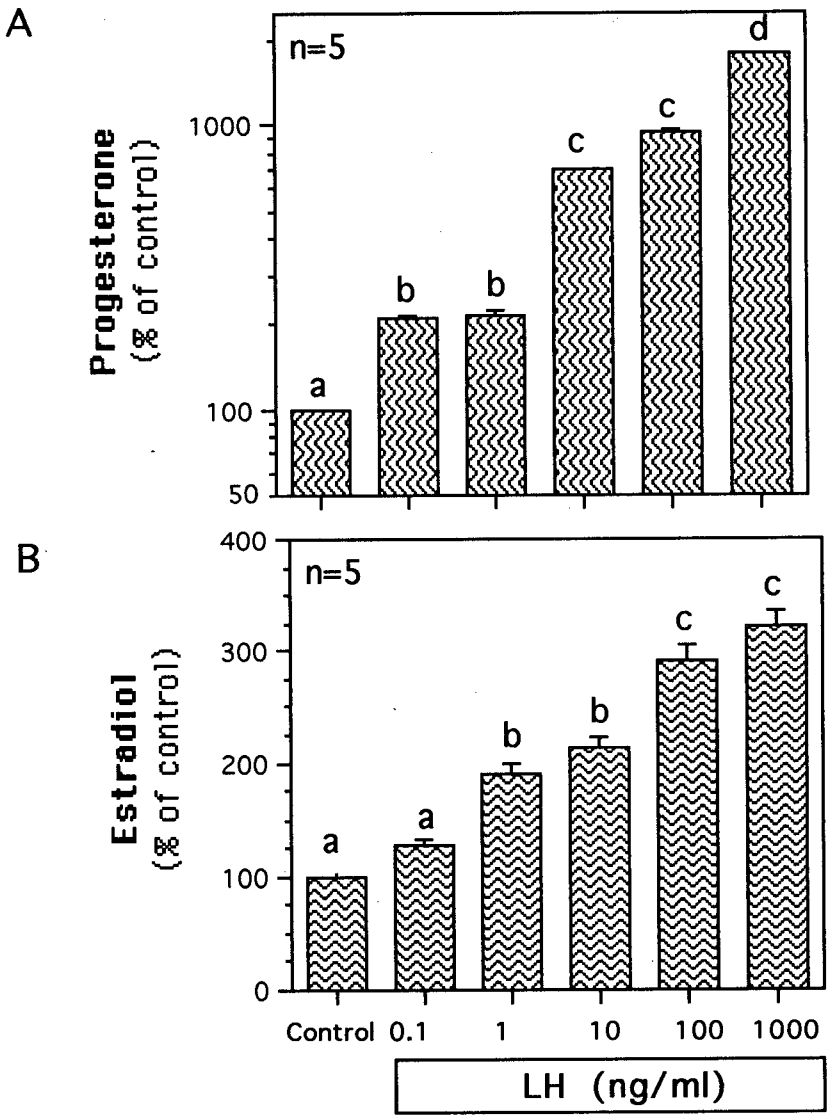


Figure 36. Effects of increasing concentration of LH on steroidogenesis. Cells were exposed to LH (0.1 to 1000ng) for 24 hours and P_4 (**A**) and E_2 (**B**) levels were measured by [^{3}H] RIA. Both P_4 and E_2 exhibited a concentration dependent increase up to 3 times control values for E_2 and 20 times for P4. ($p<0.05$; $a \neq b \neq c \neq d$)

D. Effects of GnRH

GnRH has been postulated to directly influence the synthesis of its own receptor in the pituitary. The possibility of this phenomenon occurring in the ovarian cell was, therefore, also assessed. With increasing concentrations of GnRH, ranging from 10 pM to 10 μM, a concentration dependent up-regulation of GnRH-R gene expression was seen, with a maximal stimulation of 2.4-fold occurring at 1 μM (Figure 37).

GnRH-induced changes in GnRH-R mRNA levels were inversely related to P_4 output for GnRH concentrations up to 10^{-6}M (Figure 38). Estradiol levels were not measured as androstenedione was not provided with the treatments.

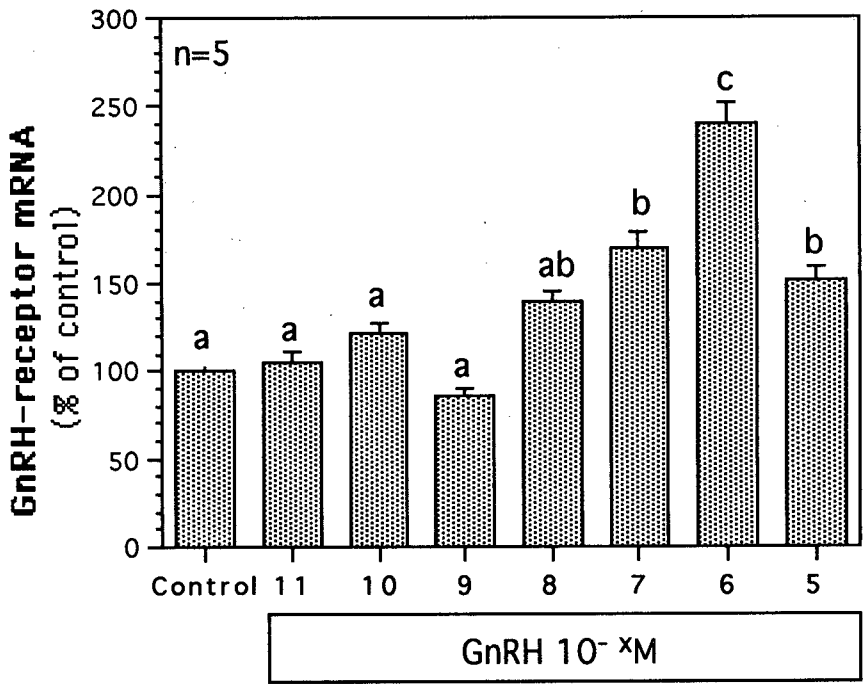


Figure 37. Effects of increasing concentration of GnRH on GnRH-R mRNA levels. GnRH (10^{-11} to 10^{-5}M) effects on GnRH-R mRNA expression were quantified by RT-PCR/Southern blotting after a 24 hour treatment period. GnRH increased the mRNA levels of its own receptor at concentrations from 10^{-7}M. ($p<0.05$; $a \neq b \neq c$)

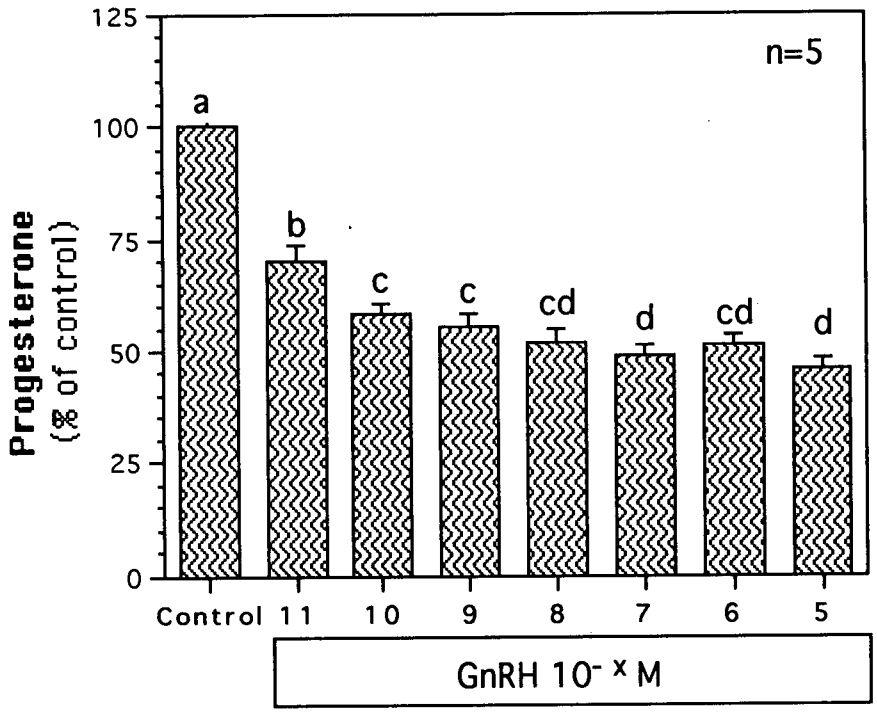


Figure 38. Effects of increasing concentration of GnRH on steroidogenesis. GnRH effects on P_4 production (measured by [^{3}H] RIA) after a 24 hour treatment period are shown. GnRH inhibited basal P_4 production down to 50% of the control. ($p<0.05$; $a \neq b \neq c \neq d$)

II. Studies in the granulosa cell model II

The effects of different hormonal treatments on rat granulosa cells were further investigated in a slightly different cell model. Details of the protocol used for this granulosa cell model are described in the methodology section. Briefly, PMSG primed immature female rats were sacrificed 54 hours after injection (prior to ovulation). Cells were recovered from the ovaries and pre-incubated for 24 hours in the presence of FBS (10%), and antibiotics (Penicillin and streptomycin, 1%) before receiving treatments.

Figure 39 shows the ovaries of control, immature animals compared to the ovaries of a PMSG treated animal.

Figure 40 shows the appearance of granulosa cells (derived from Model II) in culture.

A. Effects of culture time on granulosa cells differentiation

To investigate the effects of time on granulosa cell differentiation, cultures were carried out for 1, 2, 3 or 4 days before receiving treatment for a 24 hour period. The medium was changed daily with DMEM:F12 containing 2% FBS (and antibiotics) for the rest of the pre-culture period. Samples were treated as usual, and RNA extraction was carried out simultaneously. Overall RNA concentrations, as determined by spectrophotometry, were compared between the four days (Figure 41). Over the course of the four days, total RNA levels increased by 33%.

Progesterone output was assayed following a 24 hour treatment period with hCG (1 IU/ml), GnRH (10^{-9} M) or hCG plus GnRH. The cells pre-cultured for one day only responded to all treatments with dramatic increase in P_4 secretion. The cells for which a longer recovery time was allowed did not respond to any of the test treatments (Figure 42). Hence, for further studies, the cells were only allowed a 24 hour recovery period before receiving treatments.

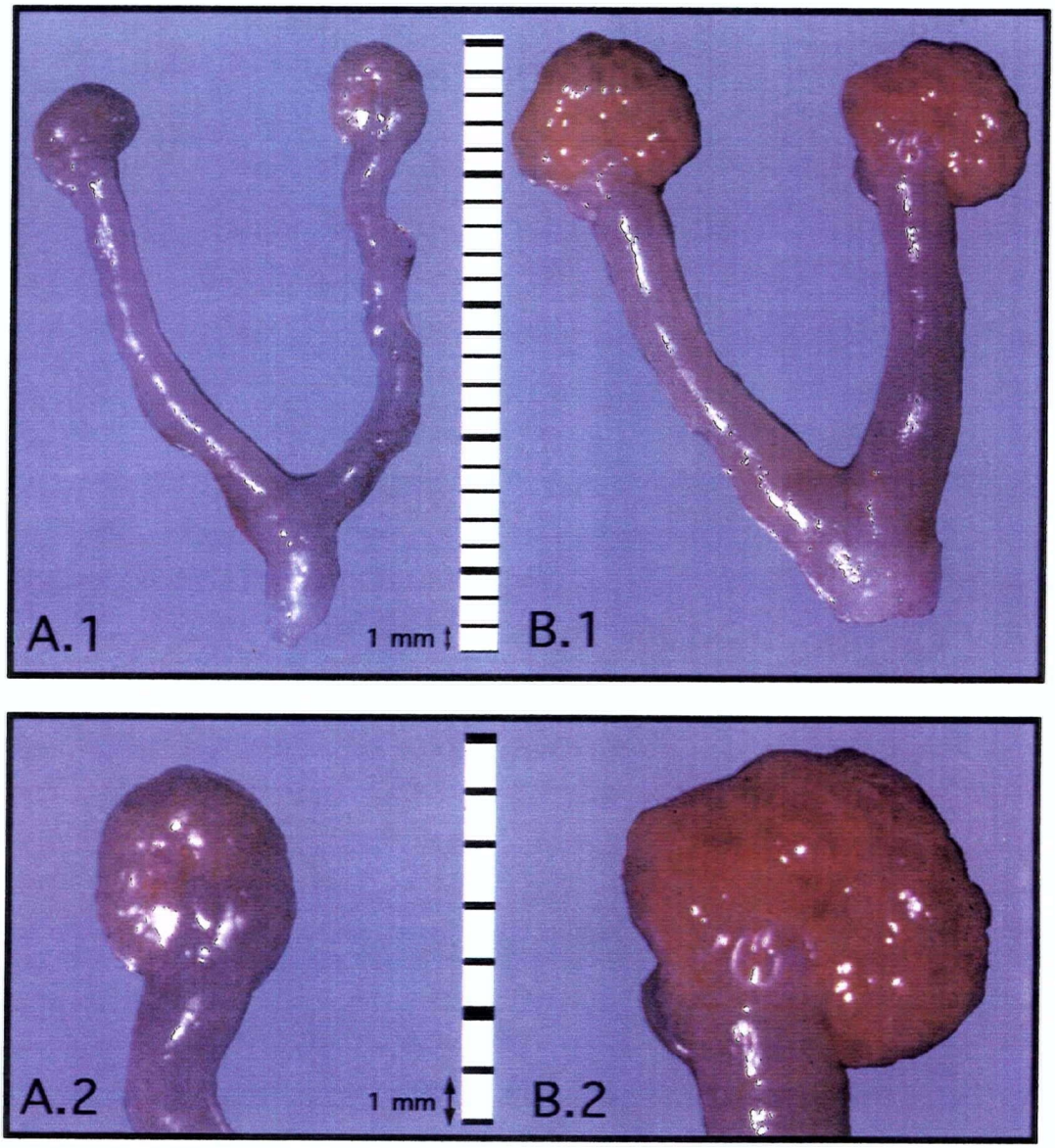


Figure 39. Ovaries of control (immature) and PMSG treated rats. Immature Sprague-Dawley female rats (25 days old) were sacrificed and the genital tract removed for comparison with animals treated with 10 IU PMSG, and sacrificed after 48-54 hours. Pictures show: (**A.1**) the whole genital tract of the control female and (**A.2**) one of her ovaries at higher magnification; (**B.1**) the whole genital tract of the treated female and (**B.2**) one of her ovaries at higher magnification.

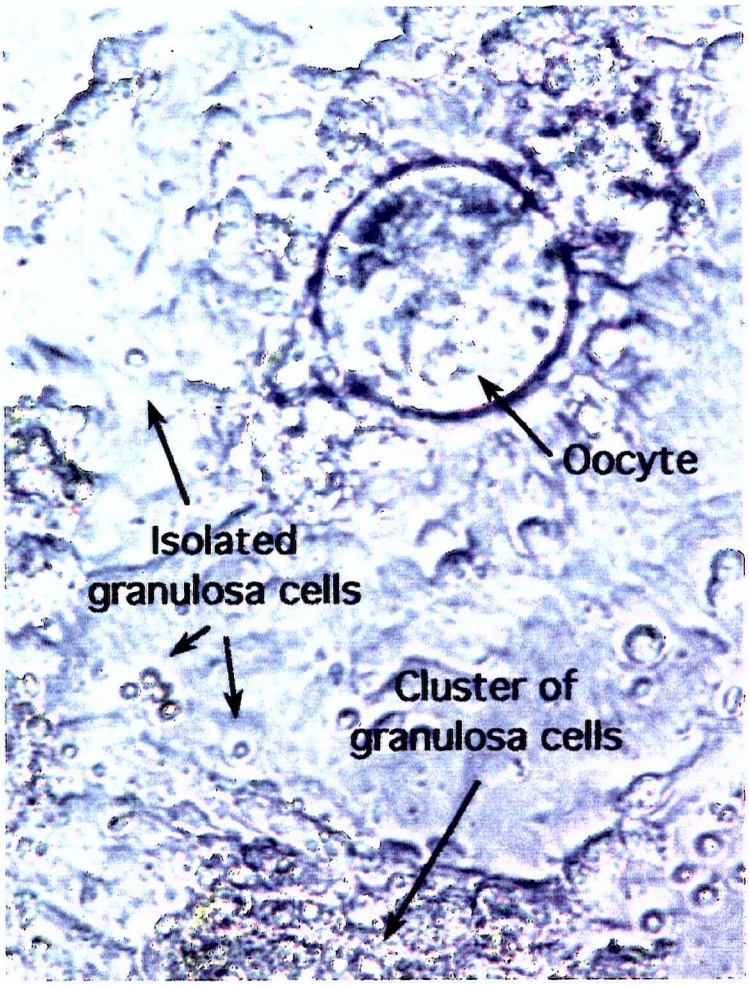


Figure 40. Granulosa cell culture. Granulosa cells were obtained as described in figure 15 (Model II). The appearance of those cells after a 24 hour recovery period is shown here. (Note the presence of an oocyte).

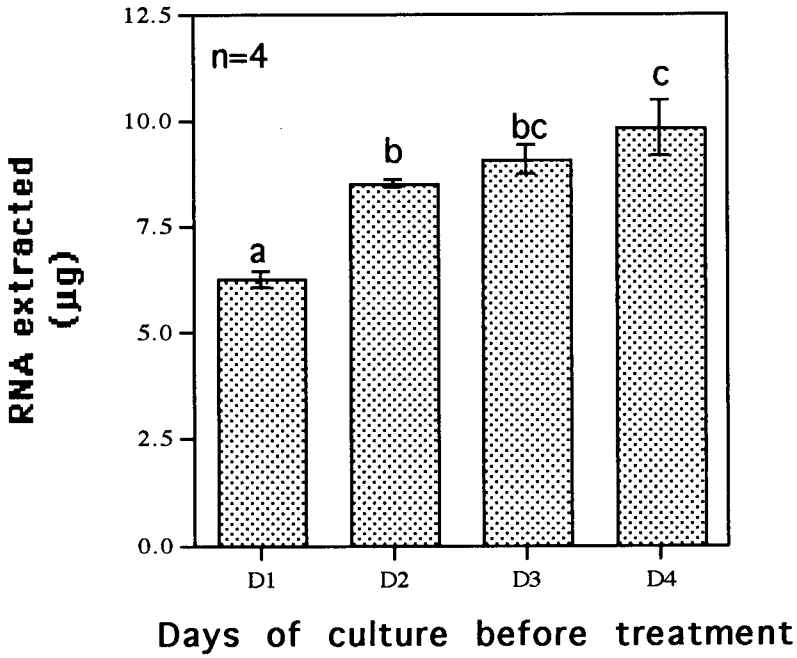


Figure 41. RNA quantification after 1 to 4 days of culture. Granulosa cell cultures were undertaken for different periods of time (1, 2, 3 or 4 days before treatment). Total RNA was extracted and quantified by spectrophotometry. The total amount of RNA extracted increased over the period of culture time for the same cell density initially plated. ($p<0.05$; $a \neq b \neq c$)

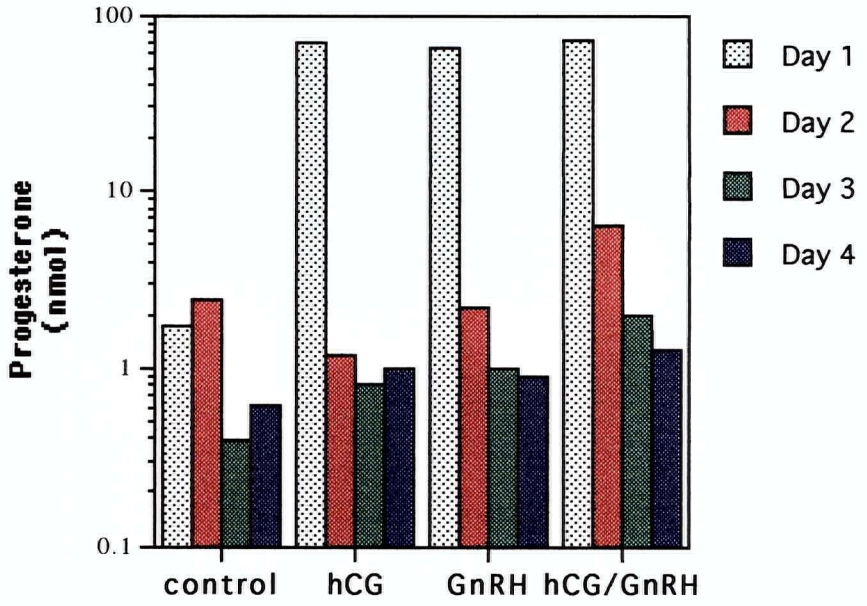


Figure 42. Basal and hCG-stimulated P_4 production after 1 to 4 days of culture. Basal P_4 production, as well as P_4 output from hCG and/or GnRH treated granulosa cells was measured after 1 to 4 days of pre-culture followed by 24 hours of treatment. Progesterone production was stimulated by all treatments only in D1 cultures, while D2 to D4 cultures did not respond to any treatments, and the basal levels of P_4 production even decreased over time.

B. Time-course effects of experimental treatments on mRNA levels

In order to choose the length of treatment that would show when regulation of the mRNA of interest occurs, time-courses of hCG and GnRH treatments were performed. Treatments with hCG were prepared at the concentrations of 0.1, 1 and 10 IU/ml, and GnRH was made up at 10^{-11}, 10^{-9} and 10^{-6} M. Treatments were applied for 3, 6, 18 or 36 hours, after which samples were processed as before.

Figure 43 shows the time-course effects of GnRH on its own mRNA. No effect was seen after 3, or 6 hours, but 18 hours of treatment displayed a strong response in the variation of GnRH mRNA levels, with a maximal increase in expression at 10^{-9} M of GnRH.

A treatment period of 18 hours seemed appropriate for the different mRNA investigated and for the different treatments applied, and was therefore chosen for the rest of the study (other time-course data showed the same tendency).

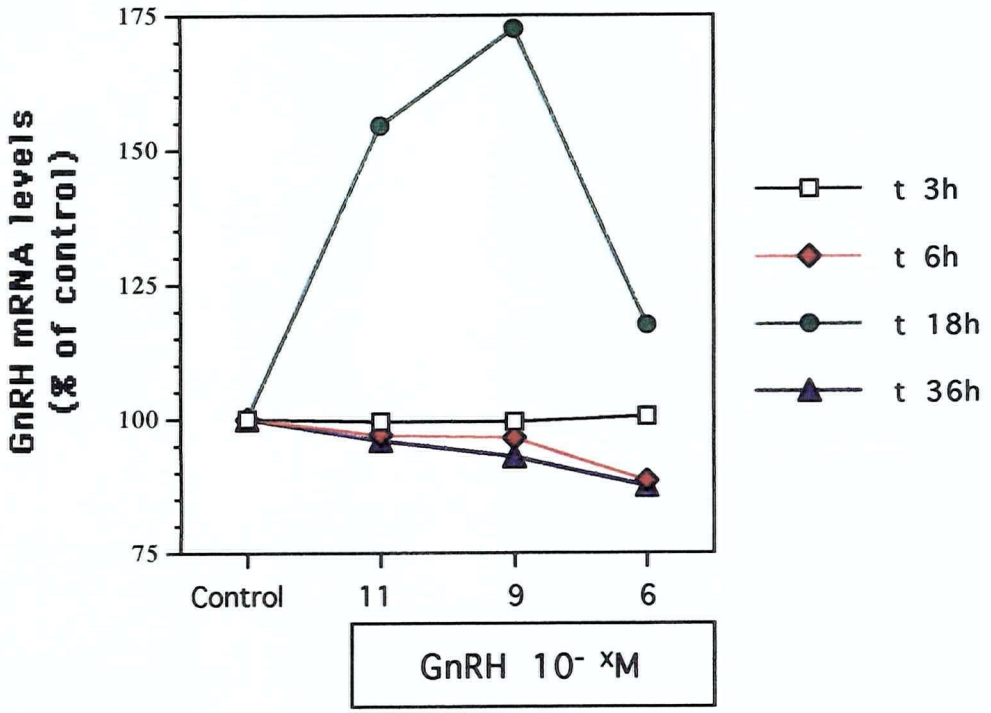


Figure 43. GnRH time-course effects on GnRH mRNA levels. To determine the optimum length of treatment showing the maximum effect on mRNA levels, time-course experiments were undertaken with different concentrations of different treatments over a period of time ranging from 3 to 36 hours. This representative experiment (out of 3) shows that the effects of GnRH on its own mRNA as quantified by RT-PCR are maximum after 18 hours when GnRH mRNA levels increase in a bell curve-like fashion in response to GnRH, with maximum stimulation at 10^{-9}M. After 3 or 6 hours of treatment, no effect could be detected, and by 36 hours of treatment the response had disappeared. For subsequent cultures, 18 hours was chosen as a treatment period.

C. Effects of GnRH, $PGF_{2\alpha}$ and hCG

When cells were exposed to GnRH, $PGF_{2\alpha}$ and hCG, alone or in combination, GnRH-R and $PGF_{2\alpha}$-R mRNA, as well as steroidogenic activity were found to be altered differentially:

Both concentrations of GnRH tested (10^{-9} and 10^{-6} M) increased GnRH-R mRNA levels up to 170% compared to the untreated control. The lower concentration of $PGF_{2\alpha}$ (10^{-9} M), but not the highest (10^{-6} M), inhibited GnRH-R mRNA expression, while the combination of GnRH and $PGF_{2\alpha}$ did not alter control levels. Treatment with hCG (1 IU/ml) greatly increased GnRH-R mRNA levels (by 2.5-fold), but when in combination with GnRH, $PGF_{2\alpha}$ or both, levels returned to those of the control (Figure 44).

The mRNA levels of $PGF_{2\alpha}$-R were unaltered by the lower concentrations of GnRH and $PGF_{2\alpha}$, alone or in combinations. Higher concentrations of GnRH or $PGF_{2\alpha}$, however, increased $PGF_{2\alpha}$-R mRNA levels by 3.2- and 1.6-fold, respectively (Figure 45).

None of the treatments affected β-actin mRNA levels, suggesting that the variations observed for GnRH-R and $PGF_{2\alpha}$-R mRNAs are specific (Figure 46).

Progesterone production was found to be stimulated by GnRH (up to 2.6-fold), but not $PGF_{2\alpha}$. In combination with GnRH, $PGF_{2\alpha}$ did not alter P_4 response to GnRH. hCG, alone or in combination with GnRH and/or $PGF_{2\alpha}$, increased P_4 production (by almost 2.5-fold by itself), although combined treatments were less effective than hCG alone (Figure 47). Estradiol production was unaffected by single treatments of GnRH or $PGF_{2\alpha}$, but was stimulated equally by all combinations of treatments (Figure 48).

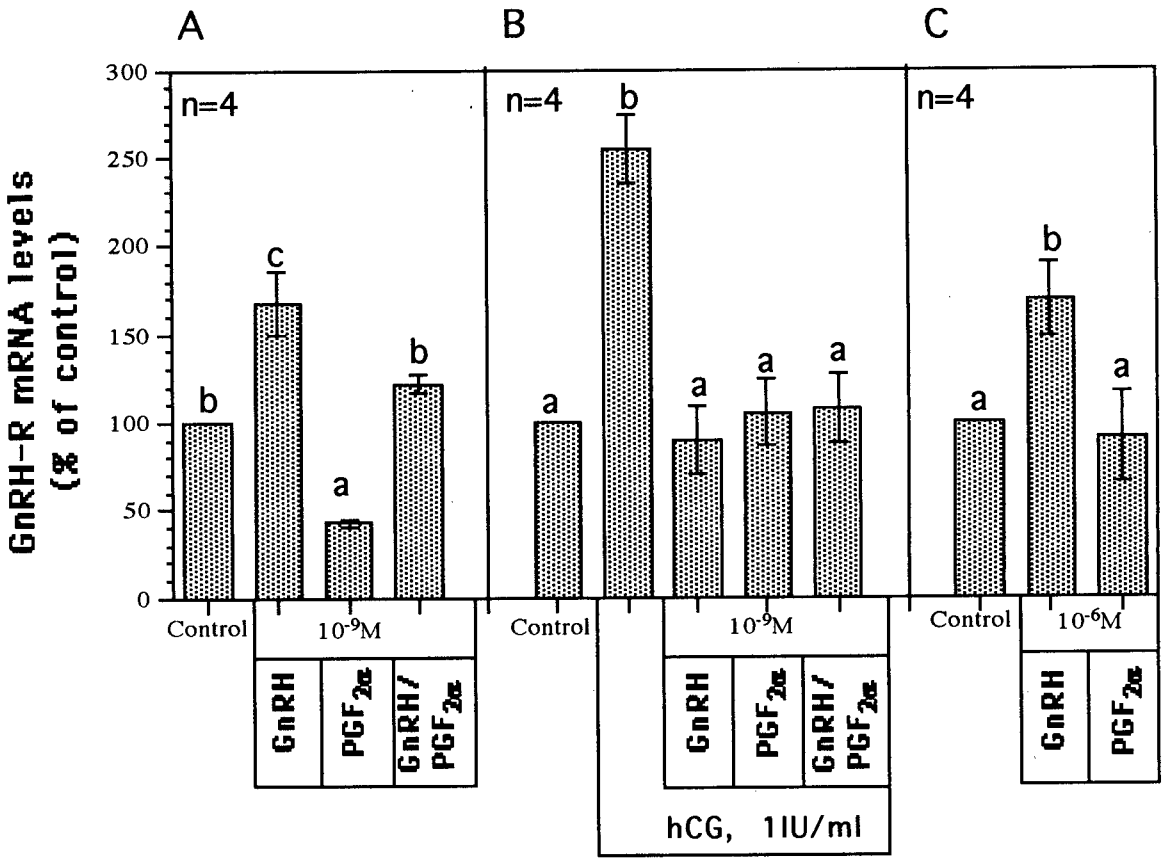


Figure 44. Effects of GnRH, $PGF_{2\alpha}$ and hCG on GnRH-R mRNA levels. Cells were exposed to hCG, GnRH and $PGF_{2\alpha}$, alone or in combination and the effects on GnRH-R mRNA levels were measured by RT/PCR. hCG, GnRH or $PGF_{2\alpha}$ (10^{-9}M) increased the levels of GnRH-R mRNA while a lower concentration of $PGF_{2\alpha}$ had no effect. In combination with hCG, GnRH and/or $PGF_{2\alpha}$ down regulated hCG-stimulated GnRH-R mRNA levels (for each separate graph, $p<0.05$; $a \neq b \neq c$).

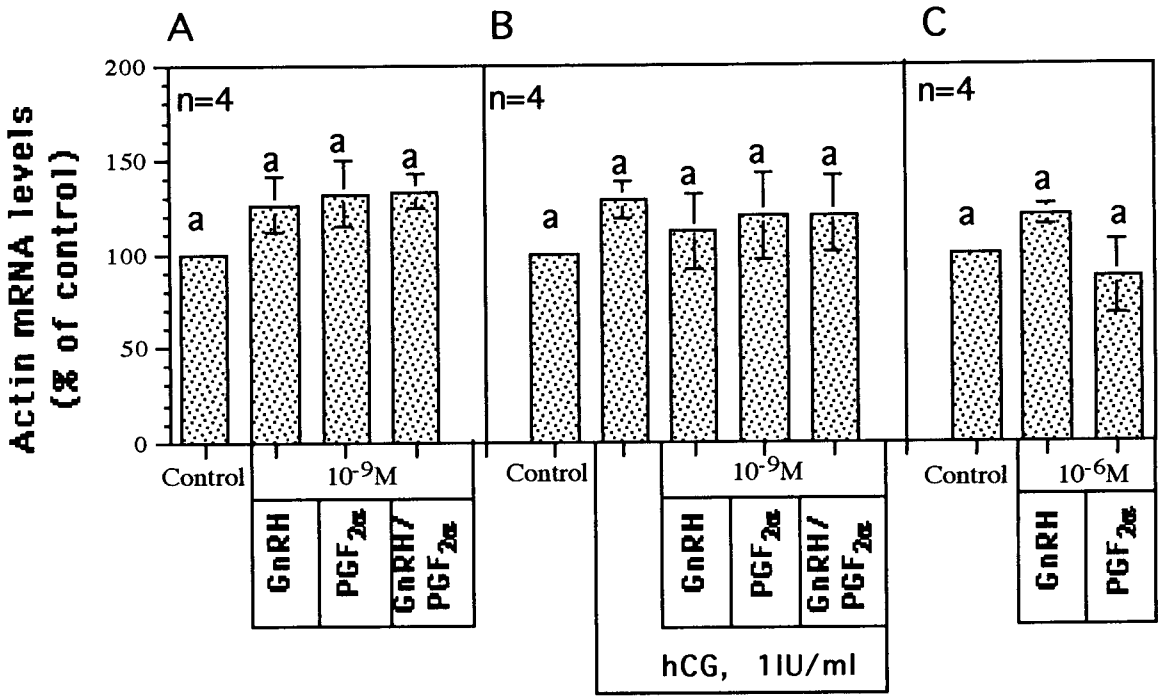


Figure 45. Effects of GnRH, $PGF_{2\alpha}$ and hCG on $PGF_{2\alpha}$-R mRNA levels. Cells were exposed to hCG, GnRH and $PGF_{2\alpha}$, alone or in combination and the effects on $PGF_{2\alpha}$-R mRNA levels were measured by RT/PCR. High concentrations (10^{-6}M) of GnRH or $PGF_{2\alpha}$ increased the mRNA levels of $PGF_{2\alpha}$-R while hCG, alone or in combination with GnRH and/or $PGF_{2\alpha}$ had no effect on mRNA levels. (for each separate graph, $p<0.05$; $a \neq b \neq c$).

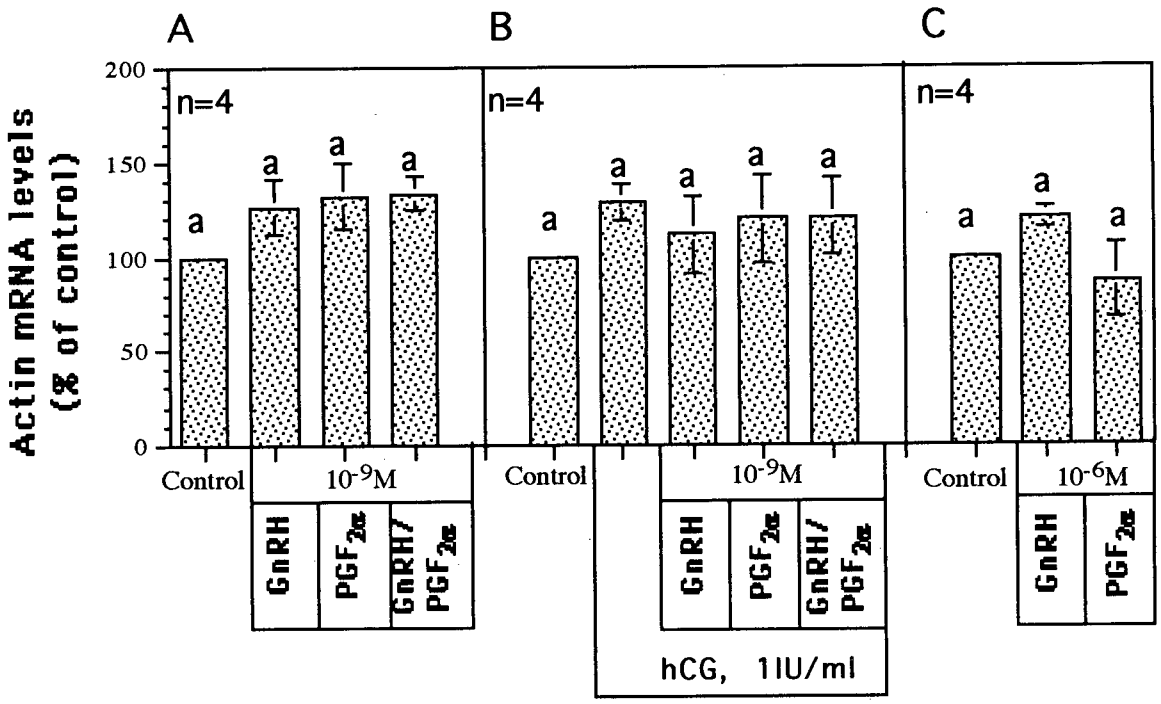


Figure 46. Effects of GnRH, $PGF_{2\alpha}$ and hCG on β-actin mRNA levels. Cells were exposed to hCG, GnRH and $PGF_{2\alpha}$, alone or in combination and the effects on β-actin mRNA levels were measured by RT/PCR. Treatments had no effect on β-actin mRNA levels.

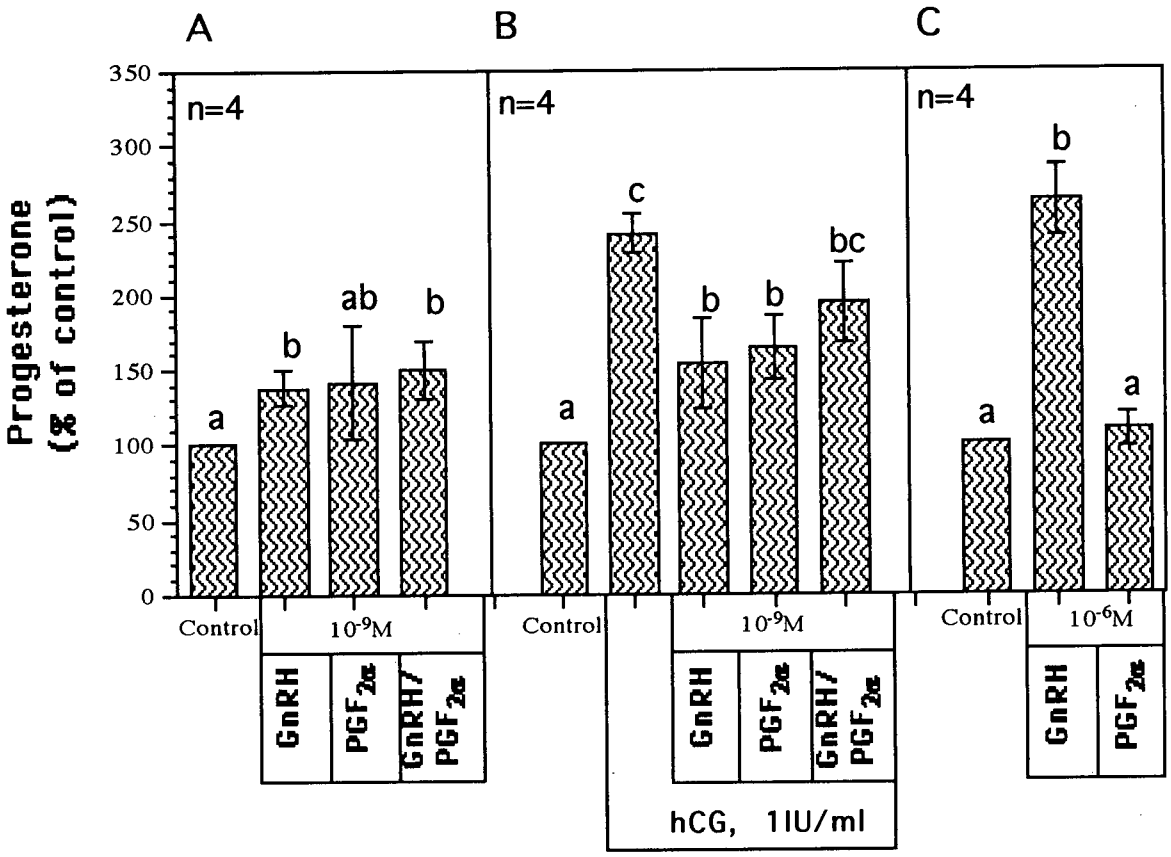


Figure 47. Effects of GnRH, $PGF_{2\alpha}$ and hCG on progesterone production. Cells were treated as in Figure. 44 and the effects on P_4 were measured by RIA. hCG and GnRH, but not $PGF_{2\alpha}$ increased P_4 production while GnRH and/or $PGF_{2\alpha}$ in combination with hCG decreased stimulated P_4 output to levels higher than basal (for each separate figure, $p<0.05$; $a \neq b \neq c$).

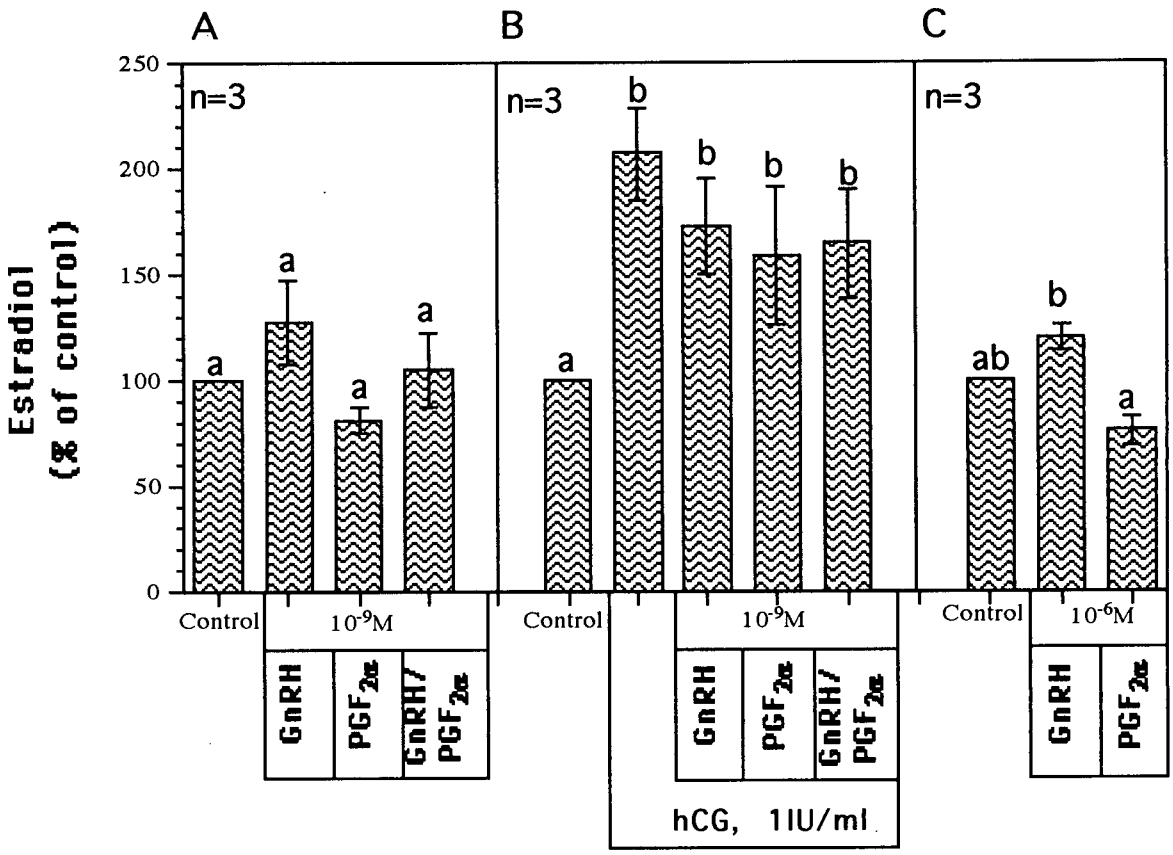


Figure 48. Effects of GnRH, $PGF_{2\alpha}$ and hCG on estradiol production. Cells were treated as in Figure 44 and the effects on E_2 were measured by RIA. Estradiol production was stimulated by hCG, alone or in combination with GnRH and/or $PGF_{2\alpha}$, but GnRH and/or $PGF_{2\alpha}$ had no effect on their own. (for each separate figure, $p<0.05$; $a \neq b$).

D. Effects of hCG

Treatments with hCG increased GnRH-R and $PGF_{2\alpha}$-R mRNA levels, with rises of 3.4 and 2.3 times the control, respectively (Figure 49-50). The levels of β-actin were unaffected by the treatments (Figure 50).

Progesterone and estradiol output rose in response to hCG treatments (Figure 51).

E. Effects of GnRH

When cells were treated with GnRH, stimulation of GnRH-R mRNA was observed. Levels rose up to 160 % in a concentration-related manner (Figure 52). In this set of experiment, the variability of the response to hCG was high, thus not showing the stimulation observed in other experiments because of the large error bar.

Levels of GnRH mRNA were reduced to half the control levels at higher concentrations of GnRH (10^{-6} M), while mid-range concentration (10^{-9} M) slightly simulated them (1.35-fold) (Figure 53).

The mRNA levels of $PGF_{2\alpha}$-R were increased by GnRH treatment up to more than 3-fold at 10^{-6} M (Figure 53). β-actin mRNA levels were unaltered by the treatments (Figure 54).

GnRH was found to stimulate P_4 (Figure 55) up to levels comparable to hCG-stimulated production (close to 3-fold), but not E_2 production (Figure 55).

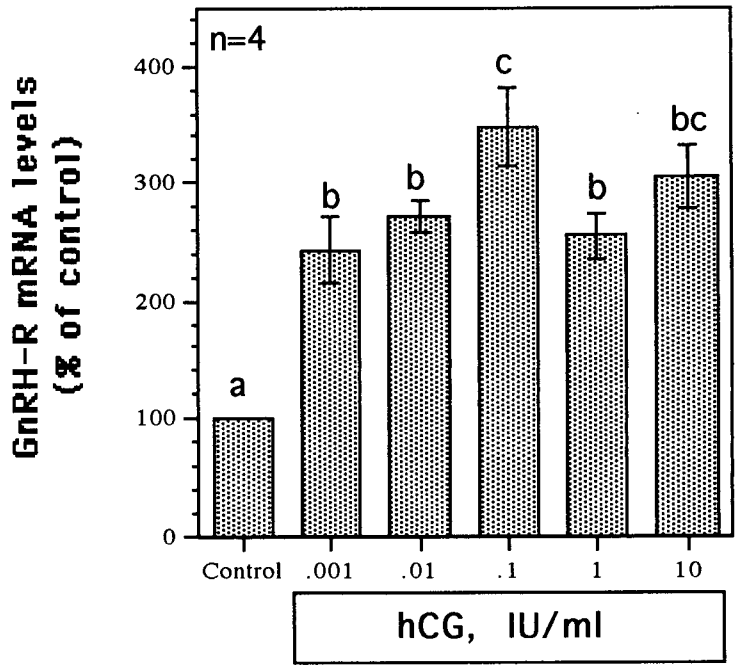


Figure 49 Effects of increasing concentration of hCG on GnRH-R mRNA levels. Cells were treated with increasing concentrations of hCG and effects on GnRH-R mRNA level were quantified by RT-PCR. GnRH-R mRNA levels were increased by increasing concentrations of hCG, with a maximum stimulation at 0.1 IU/ml ($p<0.05$; $a \neq b \neq c$).

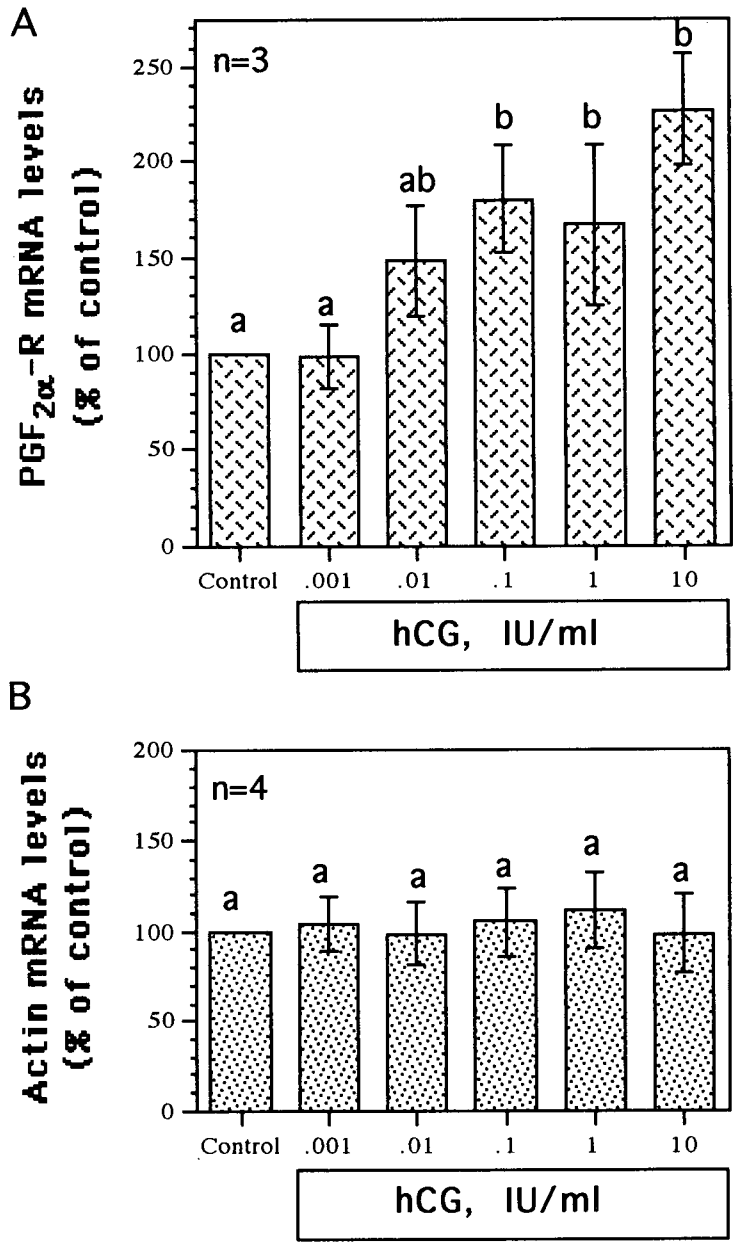


Figure 50. Effects of increasing concentration of hCG on $PGF_{2\alpha}$-R mRNA and β-actin mRNA levels. Cells were treated with increasing concentrations of hCG and effects on $PGF_{2\alpha}$-R (**A**) and β-actin (**B**) mRNA level were quantified by RT-PCR. $PGF_{2\alpha}$-R mRNA levels were increased by increasing concentrations of hCG, while treatments had no effect on β-actin mRNA ($p<0.05$; $a \neq b$).

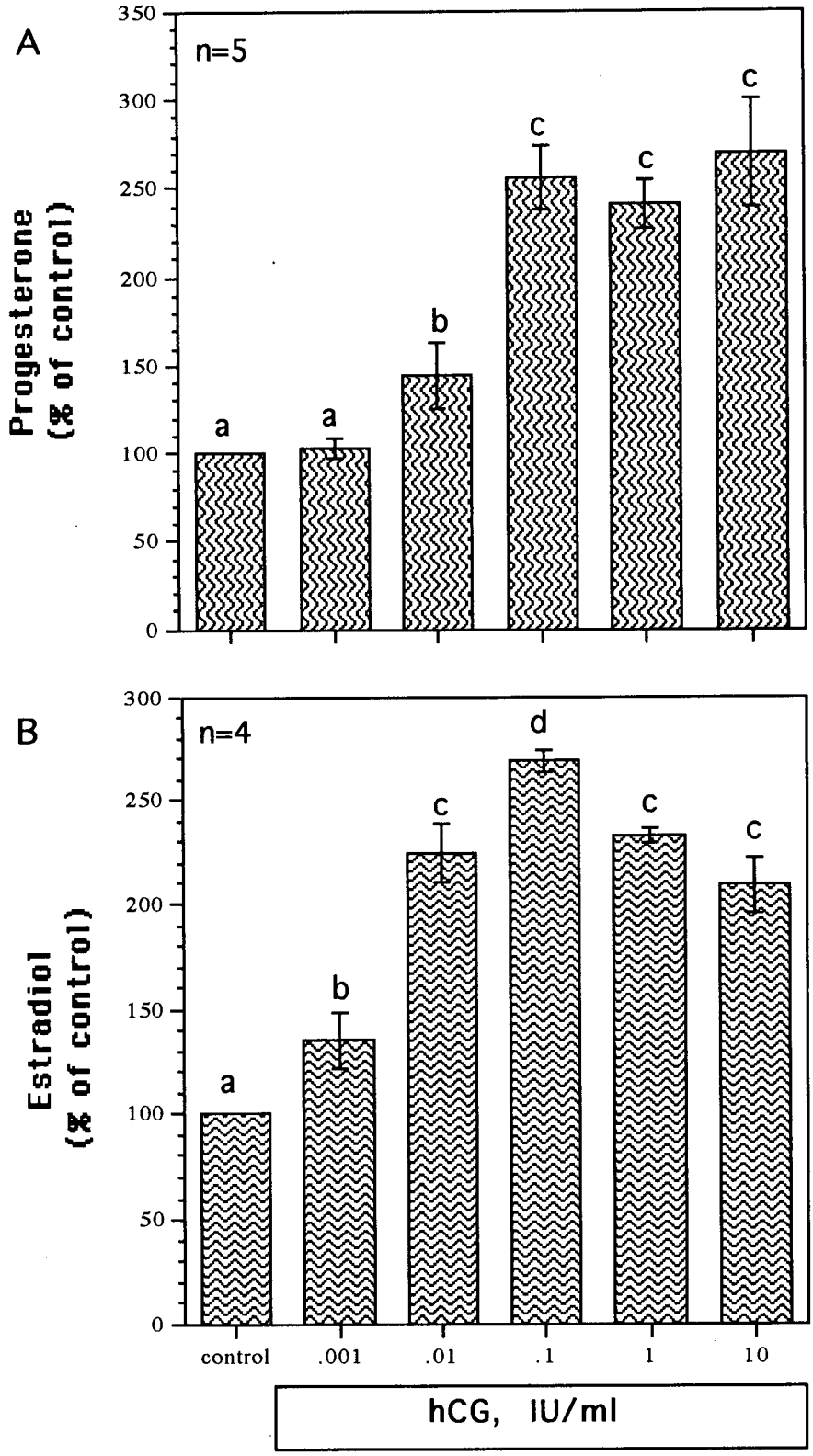


Figure 51. Effects of increasing concentration of hCG on steroidogenesis. Granulosa cells were exposed to hCG (10 μIU/ml to 10 IU/ml) for 18 hours. Concentration dependent effects of hCG on P_4 (**A**), and E_2 (**B**) were quantified by [^{3}H]RIA. hCG increased both P_4 and E_2 in a concentration dependent manner, with stimulations close to 300% for both steroids ($p<0.05$; $a \neq b \neq c \neq d$).

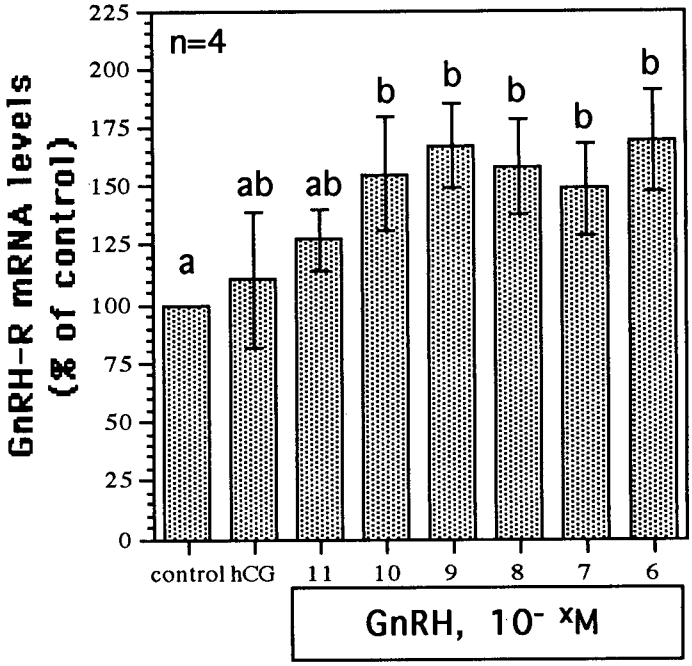


Figure 52. Effects of increasing concentration of GnRH on GnRH-R mRNA levels. Cells were exposed to hCG (1 IU/ml) or GnRH (10^{-11} to 10^{-6}M) and concentration dependent effects of GnRH on GnRH-R mRNA levels were measured by RT/PCR. GnRH increased the expression of its own receptor by more than 50% over the control levels. In this experiment, hCG did not significantly stimulate GnRH-R ($p<0.05$; $a \neq b$).

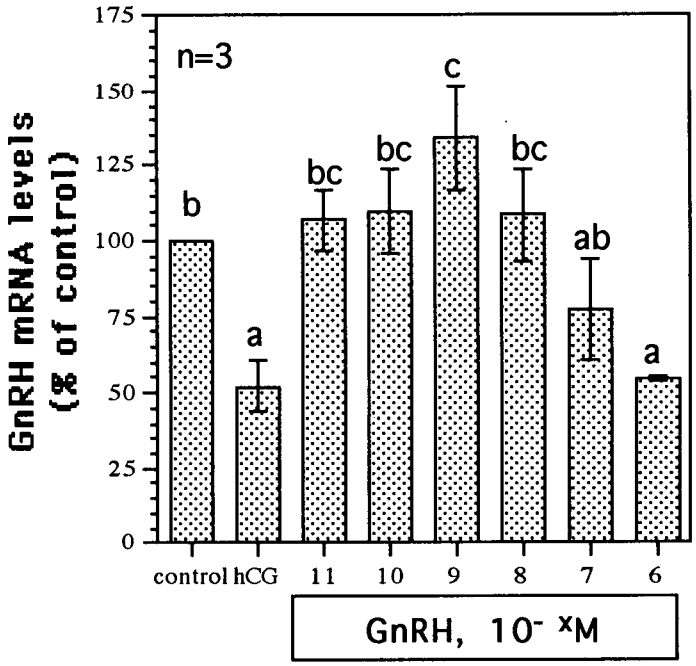


Figure 53. Effects of increasing concentration of GnRH on GnRH mRNA levels. Cells were exposed to hCG (1 IU/ml) or GnRH (10^{-11} to 10^{-6}M) and concentration dependent effects of GnRH on GnRH mRNA levels were measured by RT/PCR. GnRH inhibited its own mRNA. hCG was found to inhibit GnRH mRNA ($p<0.05$; $a \neq b \neq c$).

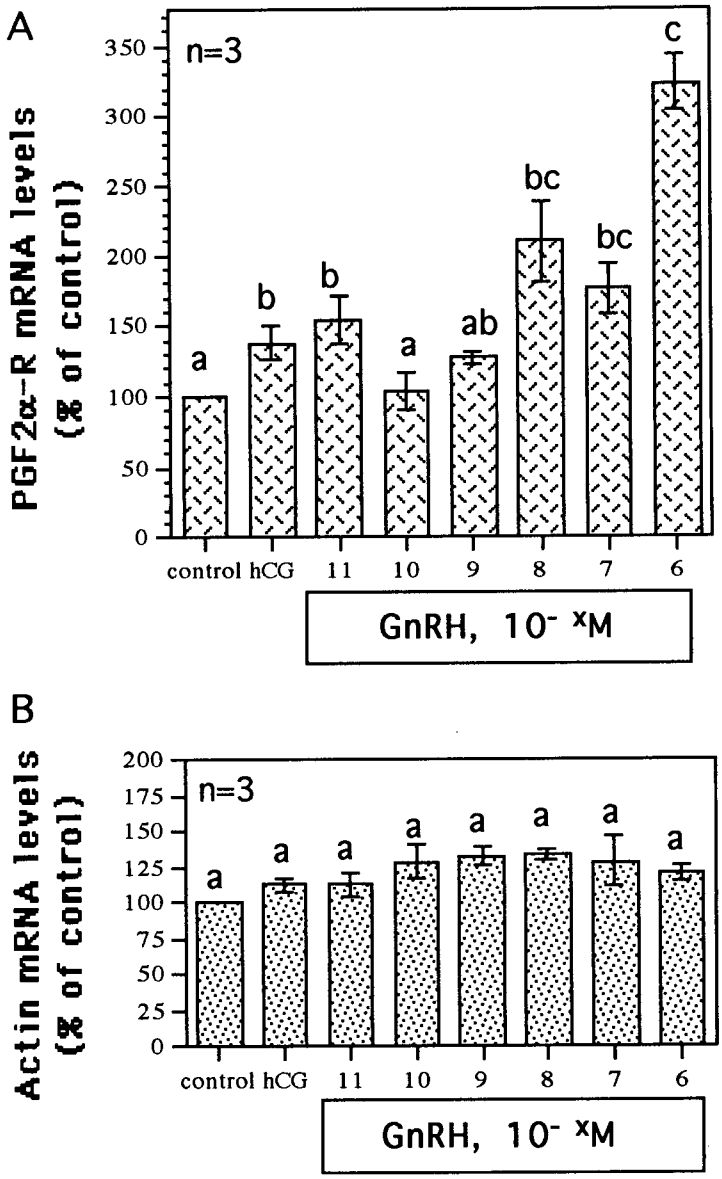


Figure 54. Effects of increasing concentration of GnRH on $PGF_{2\alpha}$-R mRNA and β-actin mRNA levels. Cells were exposed to hCG (1 IU/ml) or GnRH (10^{-11} to 10^{-6}M) and concentration dependent effects of GnRH on $PGF_{2\alpha}$-R mRNA levels (**A**) were measured by RT/PCR. β-actin (**B**) was measured as a control for the different genes quantified. GnRH increased $PGF_{2\alpha}$-R mRNA levels in a concentration dependent fashion while no effect on β-actin mRNA was observed ($p<0.05$; $a \neq b \neq c$).

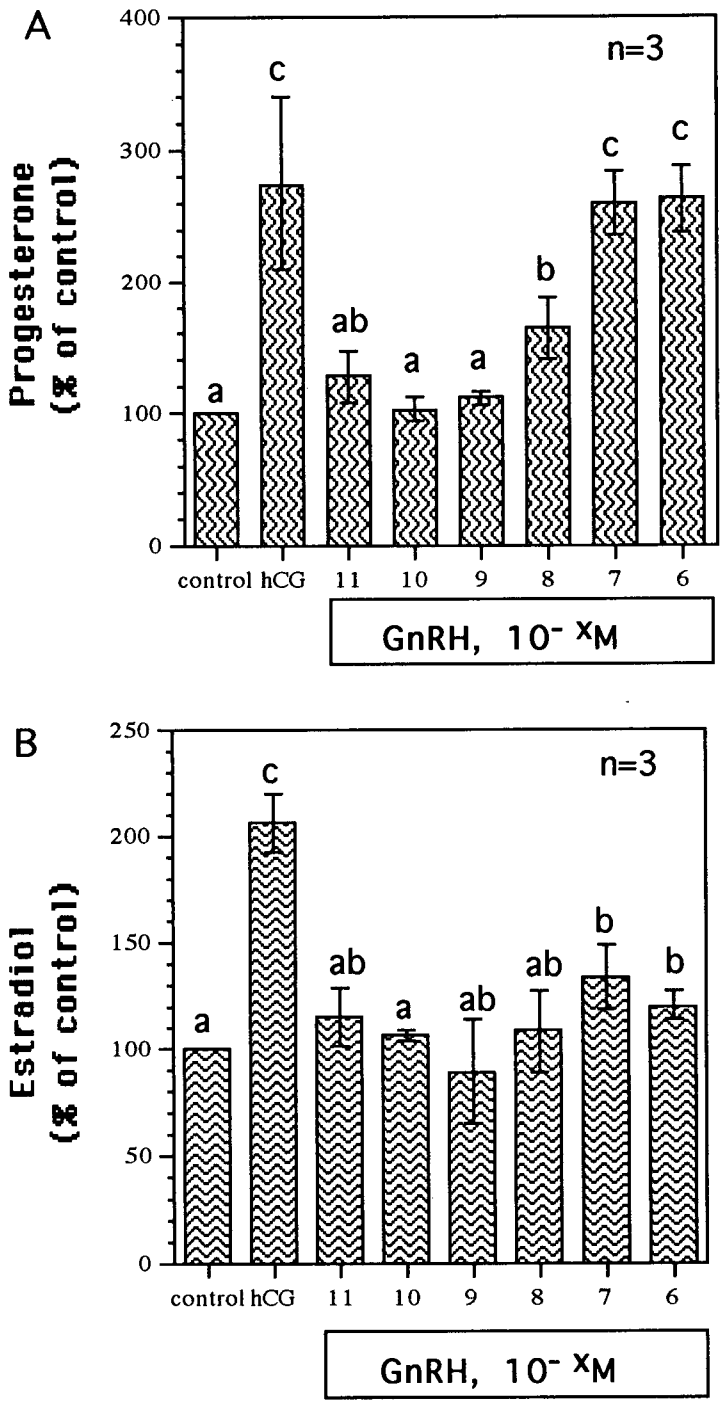


Figure 55. Effects of increasing concentration of GnRH on steroidogenesis. Cells were exposed to hCG (1 IU/ml) or GnRH (10^{-11} to 10^{-6}M) and concentration dependent effects of GnRH on P_4 (**A**) and E_2 (**B**) production were measured by [^{3}H]RIA. GnRH stimulated P4, but not E_2 production ($p<0.05$; $a \neq b \neq c$).

F. Effects of $PGF_{2\alpha}$

Treatments with $PGF_{2\alpha}$ exhibited a concentration-dependent inverse bell curve-like inhibition of GnRH-R mRNA levels, with mid-range concentration of 10^{-9} M inhibiting mRNA levels by more than 2-fold (Figure 56).

An inverse bell curve-like response of $PGF_{2\alpha}$-R mRNA was also observed in response to $PGF_{2\alpha}$, but in this case, 10^{-9} M had no effect while lower and higher concentrations were stimulatory by 2-fold (Figure 57). Once again, β-actin mRNA levels did not show any variations (Figure 57).

Progesterone and estradiol were not affected by $PGF_{2\alpha}$ treatments (Figure 58).

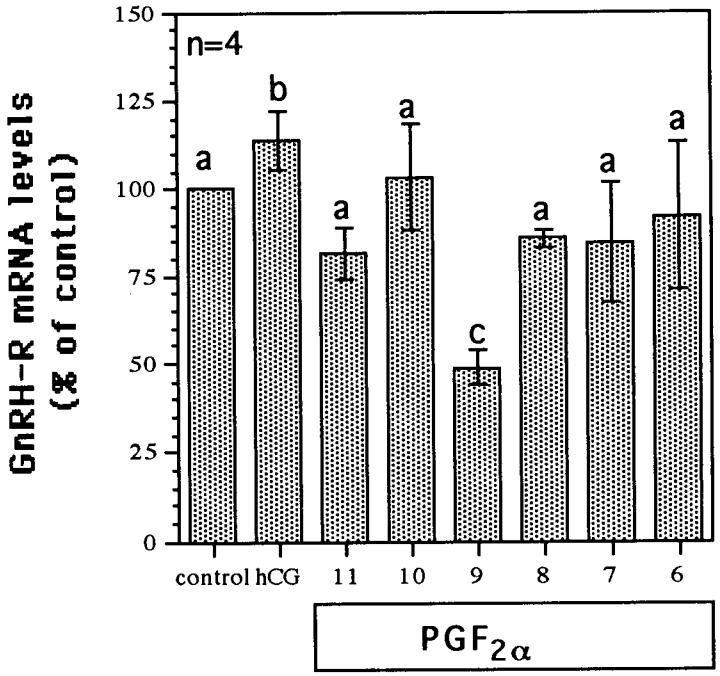


Figure 56. Effects of increasing concentration of $PGF_{2\alpha}$ on GnRH-R mRNA levels. Cells were exposed to $PGF_{2\alpha}$ (10^{-10} to 10^{-6}M) and concentration dependent effects on GnRH-R mRNA levels were measured by RT/PCR. GnRH-R was inhibited at 10^{-9}M of $PGF_{2\alpha}$ reducing the levels of mRNA to half the one of the control ($p<0.05$; $a \neq b \neq c$).

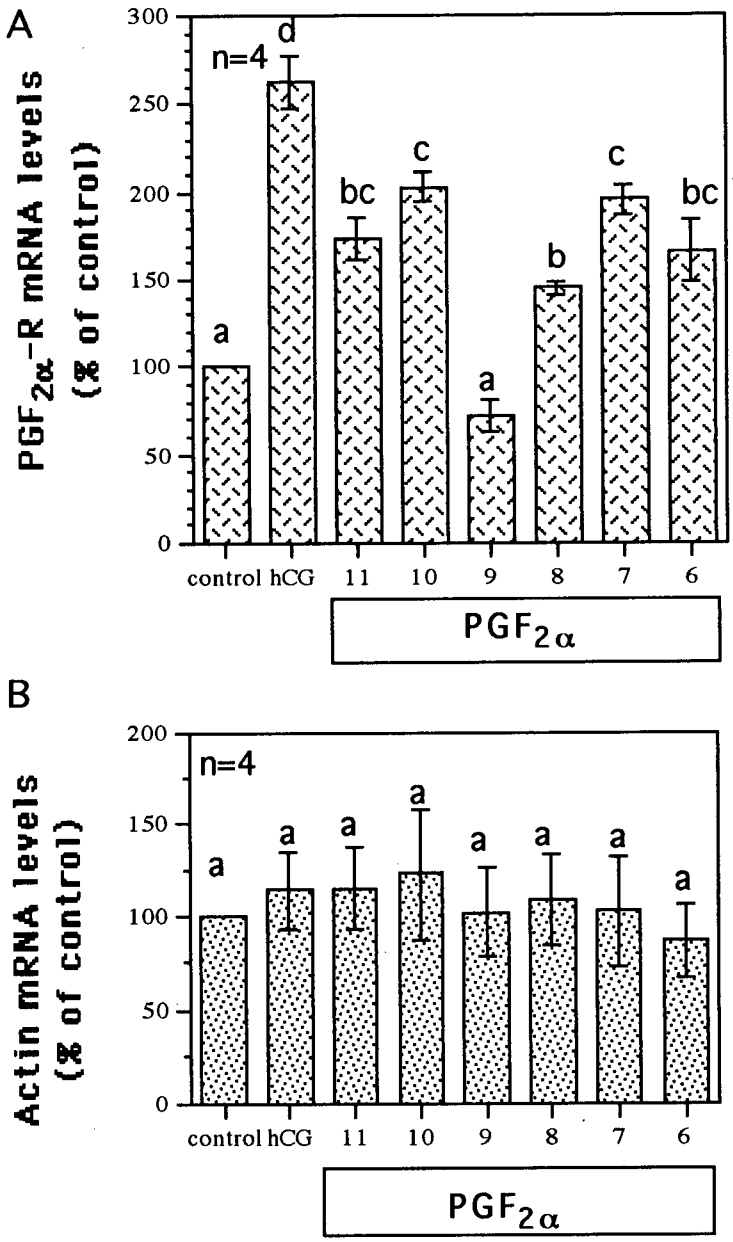


Figure 57. Effects of increasing concentration of $PGF_{2\alpha}$ on $PGF_{2\alpha}$-R mRNA and β-actin mRNA levels. Cells were exposed to $PGF_{2\alpha}$ (10^{-10} to 10^{-6}M) and concentration dependent effects on $PGF_{2\alpha}$-R (**A**) and β-actin (**B**) mRNA levels were measured by RT/PCR. $PGF_{2\alpha}$-R exhibited a bimodal stimulation with 10^{-9}M of $PGF_{2\alpha}$ having no effects, while lower or higher concentrations increased the levels of $PGF_{2\alpha}$-R mRNA by up to two times. Treatments had no effect on β-actin mRNA ($p<0.05$; $a \neq b \neq c \neq d$).

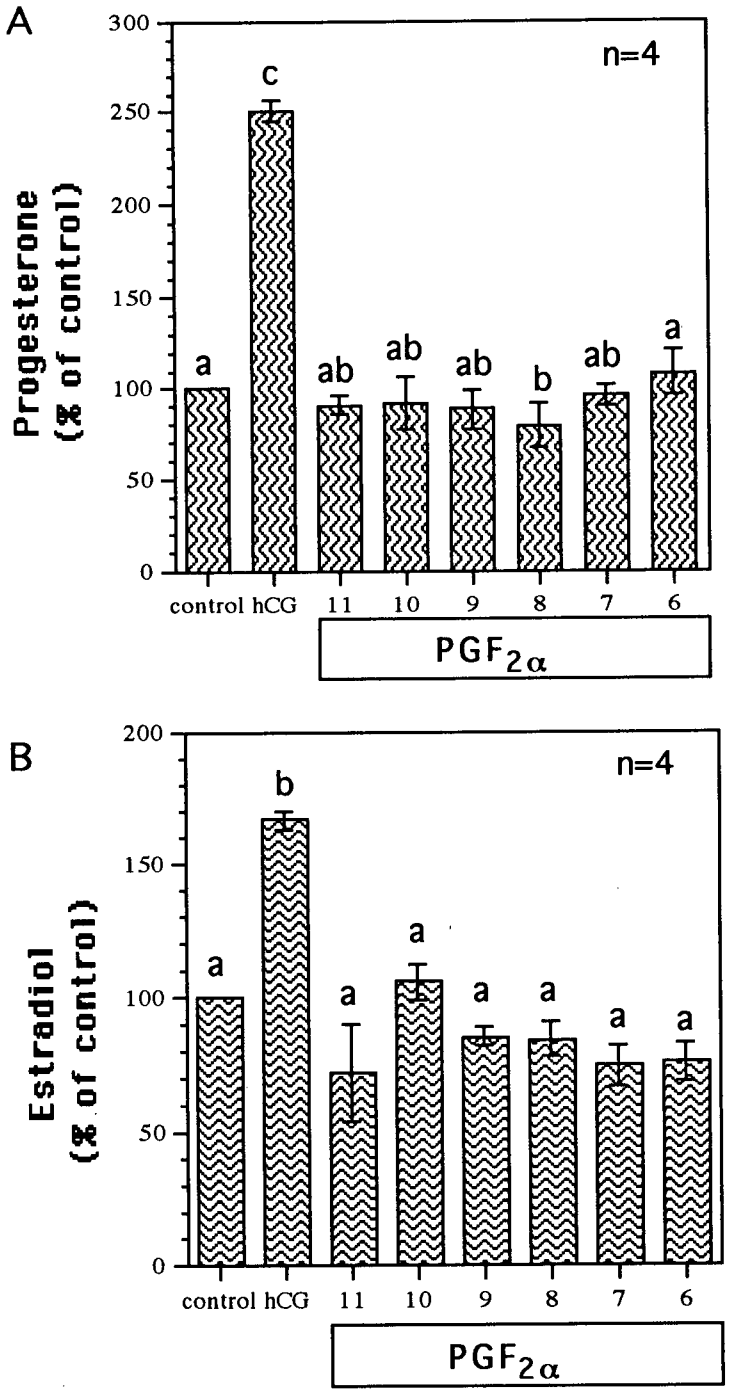


Figure 58. Effects of increasing concentration of $PGF_{2\alpha}$ on steroidogenesis. Cells were exposed to hCG (1 IU/ml) or $PGF_{2\alpha}$ (10^{-11} to 10^{-6}M) and concentration dependent effects of $PGF_{2\alpha}$ on P_4 (**A**), E_2 (**B**) production were measured by [^{3}H]RIA. While the cells were responsive to hCG, $PGF_{2\alpha}$ had no, or little effect on steroidogenesis ($p<0.05$; $a \neq b \neq c$).

DISCUSSION

I. About the studies

A. Project

GnRH is an important regulator of the reproductive function in humans, other mammals, and even lower organisms. Its actions are mediated by a membrane-bound receptor susceptible to regulation as GnRH itself.

In this study, the regulation of GnRH-R mRNA was under investigation. Localization and ontogeny of GnRH-R mRNA were first defined in the rat. The *in vivo* levels of GnRH-R mRNA were then determined in the pituitary and the ovary of the mature cycling female and the PMSG/hCG primed immature rat. Further *in vitro* studies on granulosa cell cultures intended to define the regulators of GnRH-R mRNA expression.

B. Models and techniques

B.1. The animal models

The ideal subject for relating any study to the human would be the human, but *in vitro* studies are limited by the availability of human tissues (for example, human granulosa cells that could be obtained from IVF centres), and invasive *in vivo* studies are practically impossible to conduct. For these reasons, an animal model was chosen for *in vivo*, as well as *in vitro* investigations. The rat presents several advantages that make it a model of choice:

1) *in situ*, *in vivo* and *in vitro* studies can be undertaken, offering a large array of models.

2) The laboratory rat is readily available, easy to breed, rapidly mature, and has a short cycle. Hence, studies can be designed and executed relatively rapidly, without having to wait for seasonal

breeding periods. Moreover, immature rats can be stimulated and synchronized to yield large numbers of animals at the same stage of sexual maturation.

The models designed for the present studies are as follows:

1) Adult male and female rats were used for tissue localization. GnRH-R mRNA detection was important to assess its distribution and localize the potential targets for GnRH. When GnRH analogues are used clinically, they primarily were intended to target the pituitary and alter the reproductive function. GnRH and its analogues are now also known to target the reproductive organs directly, and may be used to achieve this purpose. But the effects of GnRH on the rest of the body are largely overlooked. Thus, determining the potential targets of GnRH, through the presence of GnRH-R, is a starting point for other studies aiming at defining the actions of GnRH on non-reproductive tissues, and the potential secondary effects of a GnRH analogue treatment on the body.

2) Neonatal rats obtained from litters allowed a temporal determination of GnRH-R distribution in the male and female gonads and in the female pituitary. GnRH is known to play a role in the regulation of the reproductive cycle, but what may be its fate in the sexually immature animal ? If the receptor for GnRH is present before puberty, then a role for GnRH can be postulated, and further studies designed to examine this possibility are discussed further.

3) Mature cycling females seemed an obvious choice for detecting potential physiological alterations in GnRH-R mRNA levels during the estrous cycle, in the pituitary and possibly in the ovary. Although variations were not detectable in the ovary, this model was expected to reflect true physiological phenomenon, being that it was not altered by any exogenous treatments.

4) The immature PMSG/hCG primed *in vivo* model was chosen to substitute for the mature cycling female because of the synchronized properties of the follicles. In this model, all the recruited follicles are maturing at the same rate, and during the first cycle induced by PMSG/hCG treatment, all recruited follicles are at the same stage of development. The detection of GnRH-R mRNA

variations in the ovary was made possible in this model, providing answers that the normal cycling rat was masking.

5) *in vitro* granulosa cell models were designed to detect regulations of GnRH-R mRNA by various endogenous or exogenous candidates at the cellular level.

A first model, referred up to now as model I and described in details in the methodology section, received FSH and androstenedione pre-treatment with the belief that this would promote and maintain a pre-ovulatory differentiation state. It is difficult to judge and estimate what state these cells are at, as various further treatments might also differentiate the cells further. In this model, GnRH is displaying anti-steroidogenic effects.

A second model was developed in an attempt to simplify the procedure employed in obtaining these cells, and avoid the use of a pre-treatment that renders interpretation of the results more difficult. Since a pre-ovulatory state was sought, it seemed more logical to let the cells differentiate further *in vivo*, rather than pushing maturation *in vitro*. At the time of collection, it is safe to assume that the cells were indeed in a pre-ovulatory state, since they were collected very shortly before ovulation. At the time of treatment, and after treatment, it is again difficult to pretend that the cells are at a particular state. In this case, the steroid response to GnRH displayed luteotrophic actions on basal P_4 production.

B.2. The techniques

For the purpose of these studies, detection of mRNA species and measurement of steroid levels were required. To measure these different parameters, several cellular and molecular biology tools were used:

1) Radioimmunoassay, or RIA, was the technique of choice for the measurement of steroids, both from serum and culture media.

2) RNA extractions were performed to isolate total RNA that were to be further assayed for the mRNA(s) of interest.

3) These studies required the use of several detection and quantification methods:

-For detection and verification only, Northern or Southern blots hybridization was performed. These techniques are specific, and the product detected is likely to be the one of interest.

-For quantification, RT/PCR followed by Southern blotting, hot PCR, or simply cold PCR followed by counts or densitometry were used. Southern blotting was not necessary when the product looked at was already shown to be correct. Any other technique listed above was expected to be equally satisfactory.

C. Problems and problem solving

During the course of these studies, a few problems were encountered with the models and the techniques, but could often be overcome.

C.1. The animal models

1) The normal cycling female failed to show if there was any variation in the ovary, because of the multi-stage nature of the ovarian follicles. Another *in vivo* model, the immature PMSG/hCG primed rat, was substituted to overcome this problem, and successfully showed regulation of GnRH-R mRNA levels between different stages of follicular development.

2) The first granulosa cell model received a pre-treatment that rendered dating (i.e. determining the state of development) and interpretation of the results more difficult. A second model, with no FSH pre-treatment, was less manipulated and yielded more cells. Although it turned out to be quite different from the first model, it gave rise to more results per animal and was accepted as a different model.

C.2. The techniques

1) Two RIA techniques had to be used to measure P_4 and E_2 from serum, or from medium. A relatively easy and inexpensive RIA using tritium-labeled P_4 and E_2 was employed for assaying steroids in medium. This assay was not effective for serum, most certainly due to the interactions between serum and buffer, affecting the buffer properties, found to be very important for the quality of this assay. Thus, a different RIA, using ^{125}I label, was commercially purchased as a kit, and reserved for serum.

2) For large amounts of tissues, a cesium-chloride / butanol:chloroform extraction method was used. This extraction protocol is rather long, although not very labor intensive as a 12 hour centrifugation step is responsible for most of the time required for the extraction. But when expected RNA yields were low, this technique was not efficient enough and could not be performed. A kit, although more expensive and more labor intensive, even if it was less time consuming, was used for those samples, as it could yield sufficient amounts of total RNA to work with (0.5 to 5 μg per sample).

3) Several detection and quantification methods were used, each of them presenting advantages and drawbacks:

-a. Northern blotting would have been the technique of choice, both for verification of the nature of the mRNA and for quantification, as the number of intermediate steps is limited, thus reducing the chance of experimental error or assay variability. Furthermore, Northern blot provides a direct mean of quantification. But in most cases, the RNA yield was limited, and not sufficient for Northern blots. Moreover, the level of expression of the gene of interest, GnRH-R, was low in the ovarian granulosa cells, and may not always be detectable by Northern blot analysis.

-b. Southern blot hybridization was preferred over Northern to remedy the problem of the low RNA yields. Although more time consuming because of the added RT/PCR step, the amplification process will detect even the weakest signal. However, it has the potential of

introducing intra- and inter-assay variations from one sample to the other, and measurements are only semi-quantitative, due to the non-linear nature of the PCR step.

-c. PCR is a good amplifying tool, but over-amplification will lead to a plateau effect. This is why cycle experiments were performed to determine the optimal cycle number for a given cDNA species, at the quantities examined. Although not always perfectly linear, PCR amplification reflects relative variations with enough accuracy for a restricted range. Measurements of the genes of interest were tested against a housekeeping gene (β-actin or G3PDH) chosen according to the more constant expression for each cDNA source.

-d. Hot PCR is easier and faster than Southern, but is not necessarily as specific. Cold PCR is in all respects as good as hot PCR without the inconvenience of the isotope, and only the potential of not detecting weak products as well as a labeled method. However, as long as the PCR product was shown to be correct, PCR is a reliable method.

As all techniques yield similar results, PCR was utilized, as it avoids unjustified use and exposure to isotopes, and reduced the number of steps (hence, reducing potential experimental errors) for quantification compared to a Southern blot hybridization. Furthermore, PCR still maintained the amplification advantage.

For all of the techniques mentioned above, the limiting parameter is the accuracy of the counts (for the methods using isotopes) or the densitometry readings. These quantifications methods are not linear (see profile of standard curves, on Figure 10 and 22) and would all require the use of standard curves specific to each assay. However, for relatively small variations (up to 2-3 times), direct readings can be considered accurate enough for relative quantification, even though standard curves were not used.

II. Tissue specificity

A. Synopsis

The mRNA for GnRH-R was detected in the brain, pituitary of the cycling female, lung, kidney, adrenals, uterus of the cycling female, ovary, corpus luteum, testis, ventral prostate, epidydimis, peri-ovarian fat, and oviduct (Figure 59).

It was not detected in the pituitary of the pregnant female, uterus of the pregnant or lactating female, cerebellum, hypothalamus, heart, mammary gland, duodenum, ileum, colon, spleen, liver, placenta, pancreas and iliopsoas (muscle).

All samples were positive for β-actin mRNA. This study did not atempt to quantify GnRH-R in the different tissues, since tissues were not all proccessed simultaneously.

B. General implications

The presence of GnRH-R and GnRH among a wide variety of tissues and species suggests that it is ancient and ubiquitous [King et al., 1995].

GnRH is present in multiple isoforms in the vertebrate [White et al., 1994]. Multiple variants of GnRH derived from alternate splicing and/or post-transcriptional modifications are identified, including the two forms encoded by two different genes and found in vertebrates (mammalian GnRH and chicken GnRH II). The large number of isoforms suggests that GnRH is an ancient gene, and that during evolution, the peptide was subjected to structural changes and gene duplication. GnRH was first recruited as a neurotransmitter in the CNS, and with evolution, as a paracrine modulator of gonadal function, and an autocrine regulator in tumor cells. In most species, the ancient chicken GnRH II acts as a neurotransmitter, while the second form which varies across classes is responsible for the physiological regulation of gonadotrophins [King et al., 1995 (review)].

C. GnRH-receptor mRNA in the central nervous system

As expected, the GnRH-R was found in the pituitary of the cycling female rat. The gonadotropes, which represent 5 to 10% of the cell population in the pituitary, are the cell type expressing GnRH-R. Multiple reports exist, demonstrating the presence and regulation of GnRH-R in the mouse and rat pituitary [Naik et al., 1985; Ban et al., 1990; Laws et al., 1990a, 1990b; Eidne et al., 1992; Perrin et al., 1993; Kakar et al., 1992, 1993]. Pituitary GnRH-R levels are under gonadal control, and auto-regulation. GnRH-R mRNA was not detected in the pituitary gland of the pregnant rat , suggesting a down-regulation of the mRNA during this period.

Furthermore, GnRH-R gene expression was also found in the brain outside of the pituitary, confirming earlier reports [Crumeyrolle et al., 1994; Jennes et al., 1994]. Other studies also localized GnRH-R binding sites in the rat dorsal hippocampus [Leblanc et al., 1988; Ban et al., 1990], amygdala, septum and subiculum [Badr et al., 1987].

This study did not detect GnRH-R mRNA in the preoptic area of the hypothalamus, while the same samples were positive for GnRH mRNA. The presence of GnRH-R mRNA in the arcuate nucleus and the ventromedial hypothalamus was however reported by Jennes (1994), and binding studies detected very low amounts of the receptor in the hypothalamus [Badr et al., 1987]. In the brain, GnRH acts as a neurotransmitter and is, possibly, involved in the expression of reproductive behaviors [Jennes, 1994]. The reason for this discrepancy is unclear, but it may be that the tissue isolated as the hypothalamus did not contain the proper nuclei. Furthermore, the number of binding sites are reported to be low [Badr et al., 1987]. Thus it could also be that the levels of mRNA in the samples used for this study were under the detection limit.

GnRH and GnRH-R have been shown to be colocalized in different areas of the brain. The finding of GnRH-R mRNA being expressed in several areas of the brain strongly suggest that GnRH has multiple sites of action and acts as a neurotransmitter/neuromodulator in the central nervous system. As for the pituitary receptors, hippocampus GnRH-R appears to be under sex steroid modulation [Badr et al., 1988]. In the brain, GnRH-R is under more complex regulatory systems involving other neurotransmitters of the POMC, GABA, opioids [Seong et al., 1995].

D. GnRH-receptor mRNA in the reproductive organs

The finding of GnRH-R transcripts in the rat ovary, and testis lend support to previous binding [Pieper et al., 1981], and autoradiographic studies [Latouche et al., 1989, Millar et al., 1982].

D.1. In the female reproductive organs

While these previous autoradiographic studies suggested that GnRH binding sites were on the surface of the ovarian granulosa cell [Latouche et al., 1989], this present study did not attempt, at this point, to isolate and examine this cell type. However, the presence and regulation of GnRH-R mRNA in cultured granulosa cells was also demonstrated in subsequent in vitro (model I) experiments [Olofsson et al, 1995].

This study also detected the presence of GnRH-R mRNA in the oviduct and in the peri-ovarian fat. No previous report of these occurrences were found in the literature, although GnRH has been detected in the porcine oviduct [Li et al., 1993]. The role of GnRH, if it is colocalized with its receptor in these tissues, is not clear. Among the possibilities, GnRH could also affect steroid metabolism in peri-ovarian fat, since it contains all the necessary precursors for steroid production. However, GnRH has not been reported to be present in fat, although it could come from the ovary itself. Another distinct possibility is that the fatty cushioning tissue, certainly acting as a mechanical barrier, could also act as a biochemical barrier, binding any GnRH or GnRH-like material that could escape the ovary in an active form, preventing any effects on other potential targets.

In the reproductive tissues, GnRH-R presence suggest that GnRH (also expressed) exerts local actions that are not restricted to the gonads.

D.2. In the male reproductive organs

The presence of GnRH-R was detected in the testis, but also the ventral prostate and the epididymis of adult male rats. Although there are other reports demonstrating the presence and/or regulation of GnRH-R in the testis [Pieper et al., 1981; Ban et al., 1990], or on the testicular Leydig cell [Millar et al., 1982], there are few reports of GnRH-R on accessory male sex organs.

GnRH-R presence was detected on the prostate by Kakar (1992), but no binding sites on normal prostates were found by other authors [Hierowski et al., 1983; Srkalovic et al., 1990]. However, these authors reported binding sites on prostate tumors. Regulation studies demonstrated effects of GnRH on the epididymis [Hatier et al., 1994] and the prostate [van Minnen, 1988], but there are no other report of GnRH-R presence (or absence) in the epididymis.

As it is the case in the ovary, GnRH affects steroidogenesis in the testis. GnRH is produced by Sertoli cells and spermatogenic cells, and acts in a paracrine fashion on the neighboring GnRH-R located on the interstitial and Leydig cells [Bahk et al., 1995]. Testicular GnRH regulates its own binding sites, a sub-population of FSH-induced prolactin receptors, and testosterone production. GnRH also indirectly regulates LH and FSH serum levels [Clayton et al., 1986]. In the testis, GnRH production is also regulated by testicular opioids, synthesized by the Leydig cell, and acting on the Sertoli cells which carry receptors for them [Saint et al., 1988].

D.3. Reproductive related organs where GnRH-receptor mRNA was not detected

It is interesting to notice that GnRH-R expression may be altered in tissues when a tumor develops (e.g. mammary gland or prostate carcinomas). These tumors are often found to be steroid sensitive, and GnRH analogues are often considered as a treatment of choice, because it controls the steroid levels, hence controlling the development of such tumors [Auclair et al., 1981; Corbin, 1982; Sandow, 1983]

Although this study did not demonstrate the presence of GnRH-R mRNA in the mammary gland of the cycling rat, other studies reported that GnRH-R mRNA was present in the mammary gland of virgin, pregnant and lactating animals [Palmon et al., 1994; Levi et al., 1996]. However, Levi reported that GnRH mRNA was not expressed in the mammary gland of virgin animals, while it was expressed in that of pregnant and lactating ones. GnRH-R was also reported in the breast of the human [Kakar et al., 1994]. The absence of GnRH-R mRNA where binding may have been reported may be due to down-regulation of mRNA expression.

E. GnRH-receptor mRNA in the non-reproductive organs

The finding of GnRH-R mRNA expression in tissues other than the central nervous system and the reproductive tissues is not too surprising, if those tissues are categorized by their function. Lung, kidney and adrenals are all steroid sensitive and/or steroid producing organs, just as the pituitary and the gonads. GnRH binding sites have been reported in liver, spleen, renal cortex, lung and cardiac muscle [Heber et al., 1978], and also adrenals [Pieper et al., 1981]. These findings differ from the present molecular study in that GnRH-R mRNA was not detected in the heart, spleen and liver, while other Northern blot and RT/PCR studies did not detect GnRH-R mRNA in liver and spleen [Kakar et al., 1992]. In another binding study on mice, liver, kidneys, heart, lungs, spleen, gastrointestinal tract, adrenal glands, thymus, thyroid gland, muscle, and adipose were found to be unreactive [Murdoch, 1995]. Thus, there appear to be a number of contradictions that could be attributed to the techniques used to detect GnRH-R, the species studied, the stage of the organ looked at, or maybe the GnRH-R isoform that might be different in different tissues.

It is interesting to note that, all tissues where GnRH-R was found were either steroidogenic or steroid dependent. Thus, GnRH appears to be important not only in the regulation of steroid secretion, but also at the site of steroid action. Therefore, it can be postulated that in these organs, GnRH may act as a local regulator of steroidogenesis, and/or steroids can regulate the expression of GnRH-R, as is the case in the pituitary [Gregg et al., 1989]. A role of the adrenal in the regulation of gonadal steroid regulation has been advanced [Kalra et al., 1977; Jayatilak et al., 1980]. Anti-steroidal effects of GnRH were reported in the kidney [Prasad et al., 1985]. Other possibilities explaining the presence of GnRH-R in these tissues could be that they are involved in the metabolic clearance and/or the degradation of GnRH. Lung, liver and kidney are indeed known to be active sites of catabolism. This hypothesis is supported by several reports investigating this possibility [Heber et al., 1978; Carone et al., 1987; Berger et al., 1993].

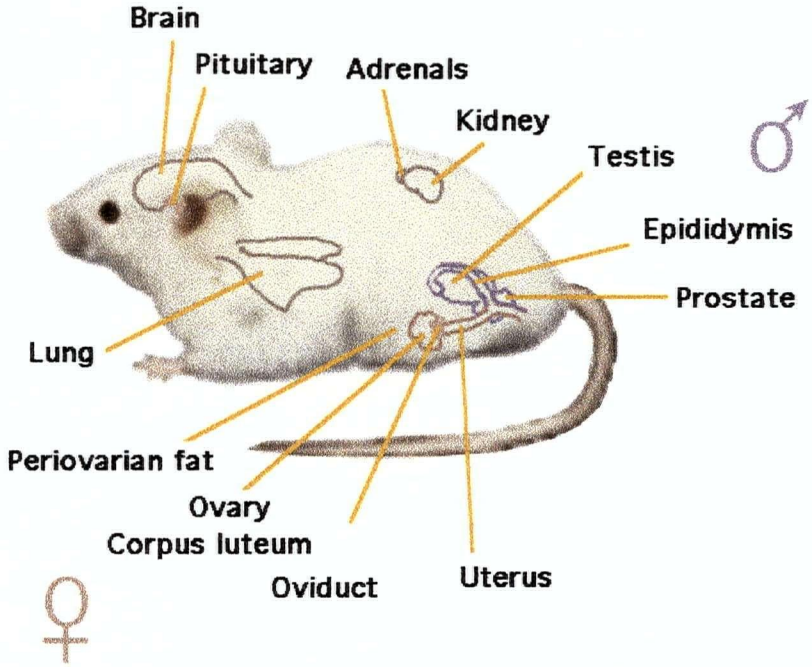


Figure 59. Tissue localization of GnRH-receptor mRNA in the rat. GnRH-R mRNA was detected by RT/PCR in the brain, pituitary of the cycling female, lung, kidney, adrenals, uterus of cycling female, ovary, corpus luteum, testis, ventral prostate, epidydimis, peri-ovarian fat, and oviduct. It was not detected in cerebellum, hypothalamus, heart, mammary gland, duodenum, ileum, colon, spleen, liver, placenta, pancreas and muscle.

III. Ontogeny

A. Synopsis

In the pituitary and the gonads, the expression of GnRH-R mRNA was found to vary with age. Hardly detectable in the ovary before the sixth day of life, GnRH-R transcripts increased until puberty, and then, drop slightly in adulthood. The expression of the GnRH-R transcript in the testis appears to follow a similar pattern to that of the ovary, increasing with age. The pituitary GnRH-R mRNA was also detectable at an early age, and progressively increased with age.

B. General implications

The role of GnRH and its receptor is more or less established in the adult, but the period of sexual maturation before and at puberty is critical for the establishment of a normal reproductive function.

GnRH has been found to modulate PRL and GH release *in vitro* in neonatal pituitaries [Andries et al., 1995], and stimulate lactotrophe differentiation in explanted fetal pituitaries [Begeot et al., 1983]. In neonatal rats, GnRH is also suspected to play a role in the differentiation of the immune functions [Morale et al., 1991]. The differentiation of GnRH neurons, earlier in fetal development, is under the influence of such factors as bFGF, TGF [Voigt et al., 1996], and the proopiomelanocortin (POMC) may be responsible for the pulsatility of GnRH secretion [Wiemann et al., 1989].

C. GnRH-receptor mRNA in the ovary

In the ovary, GnRH-R mRNA levels were found to rise with age, and drop slightly at the onset of puberty, when a reduction in GnRH binding sites has been reported to occur [Smith-White et al., 1981].

In the ovary, LH receptors are induced by GnRH-stimulated LH production, whereas FSH receptor appearance is not dependent of GnRH-stimulated FSH [Sokka et al., 1990]. Although ovarian GnRH biosynthesis does not begin before 2 weeks of postnatal life [Lamprecht et al., 1976], GnRH-R is already present, and GnRH can be provided to developing pups through the mother's milk [Smith, 1984, 1986]. Later during development, the drop in GnRH-R during the first proestrus following puberty has been correlated to a reduction in GnRH inhibitory function, thus enhancing ovarian steroid secretion [Smith et al., 1980; White et al., 1981]. GnRH itself has been reported to drop on the morning of the first estrus [Ojeda et al., 1976]. Thus, the pattern of GnRH-R expression detected during ontogeny in the ovary supports the postulated roles of GnRH in the establishment of a functional reproductive system in the rat.

D. GnRH-receptor mRNA in the testis

The presence of GnRH-R mRNA was demonstrated here in the testis of immature rats. Previous binding studies also found GnRH-R in the testis of newborn and immature rats [Huhtaniemi et al., 1985]. Testicular GnRH-R mRNA increased with age, a trend which also parallels previously reported binding studies [Dalkin et al., 1981].

GnRH binding sites were not detectable prenatally in the testis [Nemeskeri et al., 1986], but appeared in culture where they were responsive to GnRH analogues. GnRH-R presence in cultured fetal and neonatal Leydig cells support the role of GnRH or GnRH-related peptides in the modulation of gonadotrophin actions in these cells [Dufau et al., 1985]. Other indirect evidence suggest a role of GnRH in testicular growth and Sertoli cell maturation [Vogel et al., 1983].

E. GnRH-receptor mRNA in the pituitary

In the pituitary, the age groups studied revealed an increase in the expression of GnRH-R mRNA, finding supporting by earlier GnRH binding studies reporting an increase in binding sites, after birth [Dalkin et al., 1981; Aubert et al., 1985]. Binding studies previously reported that the

number of pituitary GnRH-R increased during sexual maturation. In the female, this number rises to a plateau between 15 and 30 days of age, and increase further to roughly double by 50 days. In males, the number rises gradually until 35 days (reaching levels comparable to those seen at day 50 in the female) and remains constant until 60 days [Duncan et al., 1983]. The rise observed in the present study did not reveal a plateau between day 15 and 30, but it is a strong possibility that GnRH-R mRNA and GnRH-R protein do not follow exactly identical patterns, as it has been demonstrated in the case of the male hypothalamic GnRH mRNA, GnRH precursor and GnRH. During fetal development in the male rat, GnRH mRNA increased and remained elevated, while GnRH continued to increase and GnRH precursor decreased after an initial increase [Dutlow et al., 1992].

GnRH binding sites have been reported in the fetal pituitary as early as day 12, although high affinity sites do not appear before day 17 of gestation, coinciding with the start of LH release [Aubert et al., 1985]. At this time, it is certainly GnRH of amniotic origin, capable of reaching the fetal pituitary, that is responsible for the first release of LH [Jennes, 1990].

GnRH takes part in the differentiation and maturation of the pituitary. Its presence, and that of its receptor, can be expected during ontogeny. During fetal development, GnRH has been reported to induce pituitary differentiation of LH and TSH cells [Heritier et al., 1994], and induce LH production and release after 17 days [Aubert et al., 1985]. GnRH was first detected in rat brain at day 15 of fetal life, and by day 19, GnRH neuronal connections are established with most of the target areas in the brain [Jennes, 1989].

IV. Cycling rat

A. Synopsis

GnRH-R mRNA levels were found to be regulated in the pituitary of the cycling female rat, but no detectable changes were observed at the level of the whole ovary on the different days of the estrous cycle.

B. GnRH-receptor mRNA in the pituitary

The maximum levels found in the pituitary were observed on the morning of diestrus II. However, previous studies reported maximum binding sites during the proestrus period, prior to the ovulatory surge. This is a time point that was not assayed in the study undertaken here, and the apparent temporal shift in these results may be due to the fact that the present study missed this particular point in time. Another possibility is that the expression of the protein receptor is delayed compared to the expression of the mRNA, since protein and mRNA patterns can be quite different [Dutlow et al., 1992].

Pituitary GnRH-R is under the direct control of endogenous GnRH [Naik et al., 1985]. There also appears to be a close correlation between E_2 and GnRH-R mRNA, rising and declining simultaneously (Figure 60). The initial slow rise in E_2 could increase the release of hypothalamic GnRH, in turn increasing the expression of its own receptor. This mechanism is supported by previous binding studies [Clayton et al., 1980, Lloyd et al., 1988].

C. GnRH-receptor mRNA in the ovary

The fact that the ovarian stages did not display any variation in the levels of GnRH-R mRNA is not surprising, since the number of agonist sites as determined from radioreceptor assays do not change during the estrous cycle when related to whole ovaries [Pieper et al., 1981; Marchetti et al., 1988], or exhibit only a slight increase on proestrus when expressed as binding

sites per ovary [Koves et al., 1989]. In the ovary of the adult cycling female, follicles at all different phases of development are present at any stage of the estrous cycle. These include immature follicles of all sizes (i.e. advancement in maturation), and also luteolytic corpora lutea from a previous cycle. Since the presence of GnRH binding sites has been shown to be differentially localized in the whole ovary [Latouche et al., 1989], an assay at the level of the whole ovary is likely to represent only average levels of the GnRH-R mRNA. Thus, visualizing GnRH-R mRNA variations between different stages of the estrous cycle would be possible using *in situ* hybridization.

D. Steroid levels during the estrous cycle

As for the steroid levels measured for this study, the variations in E_2 levels are in accordance with the expected fluctuations, with levels rising during the proestrus period, until they reach a threshold responsible for initiating the ovulatory peak of LH, at which point they decline again. The frequency of sampling was not appropriate to detect the peak of P4, which is supposed to happen at the precise time of ovulation, shortly after midnight on the day of estrus. The levels observed before estrus were on the rise (on diestrus II and proestrus), but they already fell by the morning of estrus.

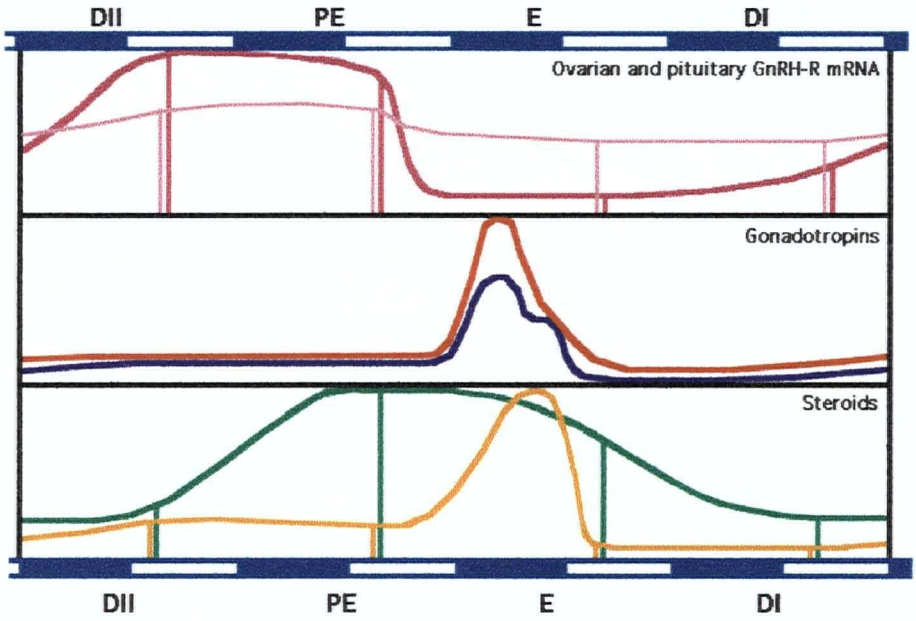


Figure 60. Extrapolated GnRH-receptor, gonadotrophins and steroid levels in the cycling rat. Levels of the different hormones and receptors were extrapolated from the present studies and/or the known hormonal patterns. The horizontal axis correspond to the days (═) and nights (■) of the estrous cycle. The vertical lines or the curves represent levels and fluctuations of corresponding hormones or receptors:

— ovarian GnRH-R mRNA; — pituitary GnRH-R mRNA; | levels of pituitary GnRH-R mRNA in the cycling rat at a particular point in time; — predicted LH; — predicted FSH; — P_4 levels in the cycling rat; — E_2 levels in the cycling rat.

V. PMSG/hCG synchronized model

A. Synopsis

In view of seemingly unchanged expression of the GnRH-R gene during the estrous cycle in whole ovaries, where follicles at various stages of maturity and corpora lutea from previous ovulations are present, the immature rat model was chosen to more precisely examine the period prior to, during and following the first ovulation. Pituitary and ovarian GnRH-R mRNA were found to be transiently down-regulated by PMSG/hCG treatment.

B. GnRH-receptor mRNA in the pituitary

The finding of a significant decrease in pituitary GnRH-R mRNA expression prior to hCG injection, or the endogenous LH surge, which occurs at 56-60 hours after PMSG injection in this model [Hillensjö et al., 1974], can be compared to the decreased GnRH-R mRNA levels seen prior to the onset of LH surge in the early morning of proestrus [Bauer-Dantoin et al., 1993]. hCG injection does not affect GnRH-R mRNA levels further after 12 hours. The rebound in GnRH-R mRNA observed after 48 hours is more likely due to a return to control levels rather than a direct effect of hCG treatment.

C. GnRH-receptor mRNA in the ovary

A striking feature in the present finding of ovarian peri-ovulatory GnRH-R mRNA expression pattern is the close correlation to the reported decline in numbers of GnRH ligand-binding sites 3 days after PMSG injection and also hCG treatment in gonadotrophin-primed immature rats [Harwood et al., 1980 a,b]. In keeping with the known down-regulation of both LH and FSH receptor mRNAs following an ovulatory dose of hCG [LaPolt et al., 1990, 1992], findings herein provide preliminary evidence for yet another member of the G-coupled receptor family which is down-regulated at both the mRNA and protein level by LH/hCG [Piquette et al.,

1991]. Interestingly, this down-regulation is transient, since increased mRNA levels of GnRH-R were detected in the early luteal phase of immature rats (Figure 61).

D. Steroid levels

The P_4 levels were found to be higher in the PMSG/hCG group, corresponding to the time of ovulation. This increase matches perfectly the situation seen in cycling animals, displaying a P_4 peak that coincides with ovulation. Estradiol levels were maximum in the PMSG group, correlating to the high levels observed during proestrus.

E. General implications

GnRH-R mRNA levels were found to be highly regulated in the PMSG/hCG synchronized immature rat, underlying the importance of gonadotrophins in the modulation of GnRH/GnRH-R function in the ovary. The levels of GnRH-R mRNA observed in the pituitary of the PMSG/hCG model seemed to correlate well with those observed in the cycling animal at comparable points in time. Thus, a general pattern for pituitary GnRH-R throughout the estrous cycle could be extrapolated from the data generated by those two series of experiments. This pattern is shown in Figure 62. The increase in pituitary GnRH-R parallels the increase in E_2, suggesting that E_2 might be indirectly responsible for this rise. The biphasic effect of E_2 suggested by Naik (1985) can also be observed here with a decline of pituitary GnRH-R mRNA at the time when E_2 reaches a plateau. This point in time, before the night of estrus, also correspond to the initiation of the LH and FSH surge, and the rise in serum P4.

However, the normal cycling rat and the PMSG/hCG model differ greatly in one respect: The E_2 levels in the PMSG/hCG synchronized model drop earlier than in the normal rat, but to a lesser extent. After ovulation, the steroid levels in the primed immature rat remain elevated, while those of the normal rat decrease to basal levels. The elevated steroid levels of the PMSG/hCG model suggest that luteinization follows ovulation, which is certainely induced by the prolonged effects of hCG.

The pattern of ovarian GnRH-R mRNA could not be detected in the ovary of the normal cycling rat, for reasons already described, but a profile could be established in the PMSG/hCG model, whose follicles are all at the same stage of development. As mentioned above, the decrease in GnRH-R coincides with the hCG treatment, leading to the suggestion that ovarian GnRH-R may be down-regulated by LH/hCG. In the normal cycling rat, if such a drop occurs in the recruited follicles ready for ovulation, it may be attributed to the rising levels of LH and/or FSH. From *in vitro* studies presented below, it is likely that LH is responsible for the down-regulation of GnRH-R mRNA in granulosa/luteal cells, but FSH has no effect on GnRH-R mRNA levels [Olofsson et al., 1995]. Another feature of GnRH-R profiles is the apparent parallel, although shifted, patterns between the pituitary and the ovarian mRNA levels. The time lapse between the decline of those to mRNA populations roughly equals the time laps between the E_2 plateau (responsible for the pituitary GnRH-R decline) and the onset of the LH surge (responsible for the ovarian GnRH-R decline). In summary, there are two GnRH/GnRH-R systems modulating the reproductive function. The hypothalamo-pituitary system controls gonadotrophins and is controlled by steroids (E_2), while the gonadal system controls steroid production and is under gonadotrophin control (LH).

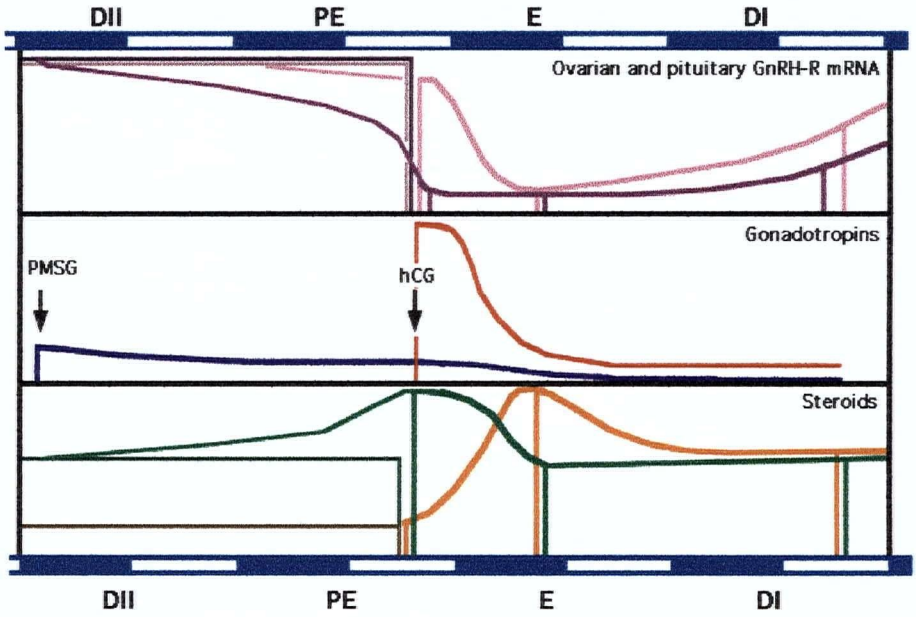


Figure 61. Extrapolated GnRH-receptor, gonadotrophins and steroid levels in the PMSG/hCG primed rat. Levels of the different hormones and receptors were extrapolated from the present studies and/or the known hormonal patterns. The horizontal axis correspond to the days (▬) and nights (▬) of the estrous cycle. The vertical lines or the curves represent levels and fluctuations of corresponding hormones or receptors:
— ovarian GnRH-R mRNA; — pituitary GnRH-R mRNA; — progestrone levels in the PMSG/hCG rat; — E_2 levels in the PMSG/hCG rat.

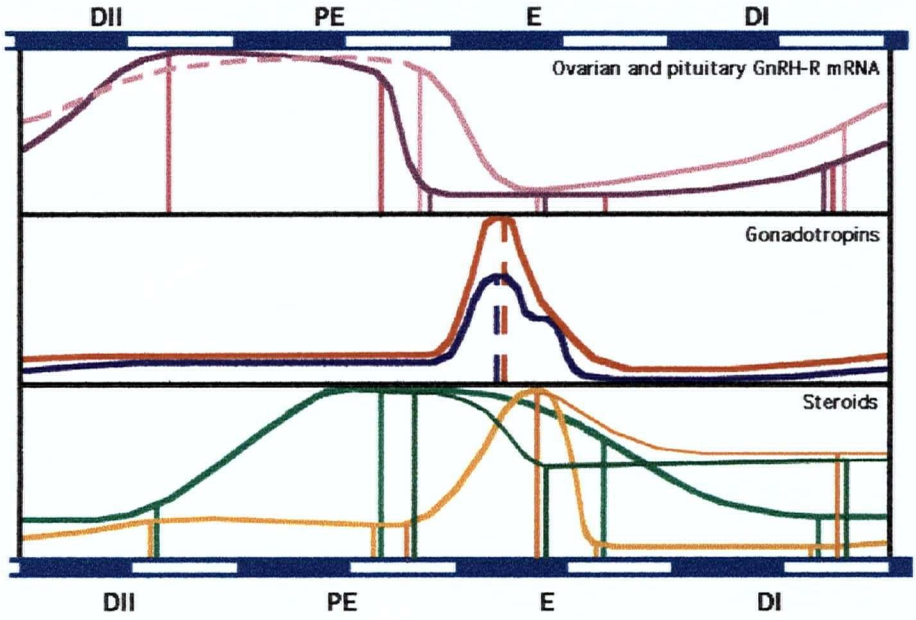


Figure 62. Extrapolated GnRH-receptor, gonadotrophins and steroid levels in the cycling or PMSG/hCG primed rat. Levels of the different hormones and receptors were extrapolated from the present studies and/or the known hormonal patterns. The horizontal axis correspond to the days (▭) and nights (■) of the estrous cycle. The vertical lines or the curves represent levels and fluctuations of corresponding hormones or receptors:

— ovarian GnRH-R mRNA; — pituitary GnRH-R mRNA; | levels of pituitary GnRH-R mRNA in the cycling rat at a particular point in time; — predicted LH; — predicted FSH; — P_4 levels in the cycling rat; — progestrone levels in the PMSG/hCG rat; — E_2 levels in the cycling rat; — E_2 levels in the PMSG/hCG rat.

VI. Granulosa cell - Model I

In vitro studies were conducted in two different granulosa/luteal cell models. Pre-ovulatory granulosa cells were obtained from PMSG primed immature rats, and pre-cultured for 24 hours before receiving treatments. In the first model, granulosa cells received FSH/androstenedione during the pre-culture period, while the second model did not.

A. Synopsis

The studies undertaken in this model demonstrated that GnRH-R mRNA is detectable in pre-ovulatory rat granulosa cells and that the levels of transcripts are hormonally regulated by LH and GnRH. These findings add further support to the hypothesis of a potential role for GnRH as a local factor involved in the regulation of ovarian function.

B. LH effects

Treatment with exogenous LH, mimicking the endogenous LH surge, induced a down-regulation of GnRH-R transcripts which was time and concentration dependent. Not surprisingly, the down-regulation of GnRH-R transcripts in the *in vitro* system is similar both in magnitude and temporal occurrence to an *in vivo* ovulatory dose of hCG which decreases ovarian GnRH-R mRNA levels, 12 hours after hCG injection [Olofsson et al., 1994]. The decrease in GnRH-R transcripts is transient, as transcripts return to pre-treated levels after another 36 hours, corresponding roughly to the time required for luteinization. This *in vitro* experiment correlates well with the *in vivo* experiment described previously. In the cultured pre-ovulatory cells, LH displays identical effects to those exerted by hCG on the *in vivo* ovary, with a down-regulation of GnRH-R mRNA, also reported in binding studies [Harwood et al., 1980]. As could be expected, levels of P_4 and E_2 rose in response to LH treatments.

On the other hand, FSH did not elicit any response in GnRH-R mRNA levels in those cells, although there is a report of FSH altering the distribution of GnRH-R mRNA in hypophysectomized rats *in vivo* [Whitelaw et al., 1995]. FSH was biologically active, since P_4 production increased following FSH treatment. It is possible that the FSH pre-treatment applied to the granulosa cell culture somehow played a role in 'desensitizing' GnRH-R mRNA response, although levels could still be altered in a negative fashion by LH. Since the receptors for FSH were not down-regulated by FSH, and were still present and functional, as shown by the steroid response, it is more likely that the state of differentiation, at which these granulosa cells culture are at, is not permissive for responding to FSH.

C. GnRH effects

It is well documented that the number of GnRH-R is both up- and down-regulated by GnRH in the pituitary of many mammalian species [Conn et al., 1986]. The situation in the gonads, however, is still largely unclear. In the present study, a 24 hour treatment with GnRH, concentration-dependently increased GnRH-R mRNA levels. This is in accordance with the report that the GnRH-R binding capacity increased in the ovary after stimulation by GnRH [Pieper et al., 1981]. This up-regulation mechanism may serve to amplify and accelerate the effects of GnRH; for example, inhibition of basal P_4 production in response to increasing GnRH concentrations was observed, and supports the anti-steroidogenic function of GnRH previously reported [Massicotte et al., 1984]. In this system, GnRH also inhibited gonadotrophin-stimulated P_4 production, exhibiting anti-steroidogenic effects on both basal and stimulated P_4 production.

D. General implications

The *in vitro* FSH-pretreated granulosa cell model closely resemble the *in vivo* PMSG/hCG model described above. In this model, GnRH-R mRNA was found to be transiently down-regulated by LH and up-regulated by GnRH. These findings were similar to the reported regulation in human granulosa-luteal cells by these hormones [Peng et al., 1994].

In this study, GnRH appeared to be primarily anti-gonadotrophic, inhibiting both basal and gonadotrophin stimulated P_4 production.

VII. Granulosa cell - Model II

A. Synopsis

These studies demonstrate the ability of hCG, GnRH and $PGF_{2\alpha}$, alone or in combination, to regulate GnRH-R mRNA transcripts in the rat granulosa/luteal cell. These results support the concept of GnRH as a local regulator in the ovary.

In this granulosa/luteal cell model, GnRH-R mRNA levels were found to be up-regulated by hCG and GnRH, and down-regulated by mid-range doses of $PGF_{2\alpha}$ (10^{-9}M). In contrast, GnRH mRNA levels were down-regulated by GnRH. More like GnRH-R mRNA, $PGF_{2\alpha}$-R mRNA levels were up-regulated by hCG, GnRH and $PGF_{2\alpha}$.

B. Preliminary results

B.1. Culture time

It was observed that total RNA increased by close to 30% over a period of four days of culture. On the other hand, progesterone profiles were not affected by hormonal treatment after day 1 of culture. Thus, it appears that the increase in RNA combined with the loss of responsiveness over time may reflect dedifferentiation of the cells, under these culture conditions.

B.2. Time-course

The greater variations in mRNA levels were seen after 18 hours of treatment. Shorter and longer treatment periods resulted in lesser or no effect on mRNA levels. The time dependent response to treatments reflect transient effects, with a maximal regulation around 18 hours and a return to basal levels by 36 hours.

C. hCG effects

Although previous studies showed a down-regulation of GnRH-R mRNA levels in response to hCG *in vivo*, and LH *in vitro* [Harwood et al., 1980], interestingly, up-regulation in response to hCG was demonstrated in this system. This discrepancy is likely attributable to the state of differentiation of these cells. Unlike the model I cells, these cultures have not received FSH pre-treatment, but instead, have been carried to a further state of differentiation *in vivo*. Being collected shortly before ovulation, these cells have probably been subjected to the endogenous surge of LH preceding ovulation [Hillensjö et al., 1974].

Human CG was also found to increase $PGF_{2\alpha}$-R mRNA levels. *In vivo* reports demonstrated an increase in $PGF_{2\alpha}$ binding for 7 days after hCG administration in PMSG/hCG primed rats, and a decrease until day 21 [Brambaifa et al., 1984]. These results correlate with the present finding. However in luteal cells of the pregnant rat, it was reported that treatment with hCG did not produce changes in receptor concentration [Bussmann et al., 1989]. Thus, it could be postulated that the cultured cells of this model do not correspond to a differentiated state equivalent to a corpus luteum of pregnancy. Another possibility could be that during pregnancy, $PGF_{2\alpha}$-R are somehow maintained at high levels and cannot be further stimulated by hCG. In that case, the up-regulation observed presently could bring the cultured cell to this state, and the cells could be induced to differentiate into luteal cells of pregnancy, after hCG treatment.

D. GnRH effects

GnRH had a stimulatory effect on its own receptor mRNA levels, an effect comparable to the up-regulation that can be observed in the pituitary [Conn et al., 1994], and also observed in the previous model studied [Olofsson et al., 1995]. On the other hand, GnRH negatively auto-regulates GnRH transcripts levels, possibly as part of a negative feedback system.

Elevation of $PGF_{2\alpha}$-R mRNA in response to GnRH treatment was also seen. To the best of the author's knowledge, there have been no previous reports of $PGF_{2\alpha}$-R mRNA regulation by GnRH.

GnRH also increased P_4 production on its own, while only slightly inhibiting hCG-stimulated P_4 production. These findings support the idea that GnRH can be steroidogenic [Massicotte et al., 1984, Srivastava et al., 1994], as well as anti-steroidogenic [Massicotte et al., 1984], as shown in the previous study ('model I').

E. $PGF_{2\alpha}$ effects

The effects of $PGF_{2\alpha}$ on GnRH-R mRNA abundance were inhibitory at 10^{-9}M, although other concentrations had no effect.

Mid-range concentration (10^{-9}M) was without effect on $PGF_{2\alpha}$-R mRNA levels, but lower or higher concentrations stimulated $PGF_{2\alpha}$-R transcripts levels. The regulation of $PGF_{2\alpha}$-R transcripts levels is very comparable to the regulation of GnRH-R. Both GnRH- and $PGF_{2\alpha}$-R mRNA levels were stimulated by hCG and their respective ligand, while co-treatment with any combination of hCG with GnRH and/or $PGF_{2\alpha}$ reduced hCG-stimulated mRNA down to untreated control levels.

In the present study, $PGF_{2\alpha}$ did not display any steroidogenic [Suginami et al., 1976; Khan et al., 1989; Webley et al., 1989; Michael et al., 1993] nor significant anti-steroidogenic actions that it is known to have [Korda et al., 1975; Grinwich et al., 1976; Richardson et al., 1980; Hanzen et al., 1984; Moon et al., 1986; Jalkanen et al., 1987; Michael et al., 1991], exept for a

slight inhibition at 10^{-8}M. In this model, this lack of response occurred although the cells were shown to be responsive to hCG, as both P_4 and E_2 production were stimulated.

F. General implications

If the GnRH-R mRNA levels reflect the binding capacity for GnRH, gonadotrophin seems to be conducive to stronger effects for GnRH, since GnRH-R should be more abundant in their presence. Thus, the presence of GnRH seems to lead to an amplification of its effects through stimulation of its own receptor. In this case, both gonadotrophin and GnRH up-regulate GnRH-R mRNA. With the steroidogenic effects of GnRH, if GnRH and its receptor are present at this stage, they are likely promoting further maturation, rather than preventing it. A negative feedback system in this model is the inhibitory action of GnRH on its own synthesis, for concentrations greater than 10^{-9}M. Hence, dual regulation of GnRH and GnRH-R mRNA constitute a balanced system that may allow a fine-tuned regulation that maintains homeostatic GnRH effects.

Likewise, $PGF_{2\alpha}$-R mRNA varies in the same manner as GnRH-R mRNA, in response to the same stimuli. The presence of GnRH or $PGF_{2\alpha}$ is thus conducive for a potential amplification of $PGF_{2\alpha}$ effects though increased receptor availability. However, in the combined presence of hCG, GnRH and $PGF_{2\alpha}$ (in any possible combination), $PGF_{2\alpha}$-R mRNA levels were unstimulated as opposed to any of these treatments alone. This absence of effect could represent a normal physiological state as GnRH and $PGF_{2\alpha}$ are more likely to be both present than alone. However, if one of these hormones happened to be lacking, the other one could compensate by increasing its own receptor mRNA, thus possibly increasing its effects.

VIII. Comparison between Model I and Model II

A. Implications for differential findings

GnRH-R transcripts were found to be differentially regulated in the rat granulosa/luteal cells, depending on the model examined. The granulosa cell models studied here are likely

representative of different levels of differentiation, since different steroidogenic and molecular responses were observed in the two models, and different yields were obtained.

As a reminder, results of the two sets of in vitro studies are summarized in Table X and Figure 63. Although the cells obtained by the two protocoles appeared morphologicaly similar, the yields obtained from the second model were in average higher. The ovaries, after treatment, appeared larger, and carried more follicles, also bigger than in model I. The extra time allowed for follicular maturation in model II is likely to be responsible for this difference.

B. Gonadotrophin effects

The effects of hCG and LH on GnRH-R mRNA receptor were found to be different in the two granulosa/luteal cell models, with down-regulation by LH in the first model while hCG, sharing the LH receptor, consistently up-regulated GnRH-R mRNA levels in the second. These findings represent the most striking difference between these two models.

To attempt to explain this difference, it should be considered that the hormonal environment seen by those two populations of cells before treatment was quite different: The granulosa cells of the first model (I) did not see the elevated E2 levels preceding the ovulatory signal, but instead, were under continuous gonadotrophin environment. *In vivo* PMSG treatment (with FSH activity) was directly followed by *in vitro* FSH pre-treatment. Thus, treatment with a high dose of LH is comparable to the endogenous LH surge that the cells would see *in vivo*. The GnRH-R mRNA response is in accordance with this hypothesis, since the levels dropped transiently in response to LH, as they did *in vivo* in response to hCG in the PMSG/hCG primed model described earlier.

The cells obtained from the second protocol (model II) were also exposed to PMSG *in vivo*, but since the cells were collected only shortly before ovulation, they certainly have been exposed to higher and more sustained levels of E_2, due to the FSH activity of the PMSG environment. Furthermore, after collection, the cells were not exposed to any gonadotrophin environment for the 24 hours of the recovery period.

C. GnRH effects

The effects of GnRH on its receptor mRNA were found to be identical in the two models, with a positive auto-regulation by GnRH. Thus, unlike what was observed (and described earlier) in the pituitary [Conn et al., 1994], auto-regulation of GnRH-R by GnRH did not seem to depend on the stage of the cycle, at least not for the two present stages.

However, the two models differed again by their steroidogenic response to GnRH. In the first model, GnRH was found to have anti-steroidogenic properties on both basal and stimulated P_4 production, while in the second model, GnRH demonstrated steroidogenic effects on basal P_4 production. This again reflects the different levels of cell differentiation, and the precarious balance between stimulatory or inhibitory effects.

Table X. Recapitulative table of the studies on model I and model II.

	Effects on:				
	Progesterone	**Estradiol**	**GnRH-R**	**GnRH**	**$PGF_{2\alpha}$-R**
Treatment	**MI / MII**	**MI / MII**	**MI / MII**	**MII**	**MII**
LH	++ / NA	+ / NA	- / NA		
FSH	++ / NA	+ / NA	none / NA		
hCG	NA / ++	NA / +	NA / ++	-	+
GnRH	- / +	NA / (+)	+ / +	+ -	+
$PGF_{2\alpha}$	NA / (-)	NA / none	NA / -		+ none +
GnRH+$PGF_{2\alpha}$	NA / +	NA / none	NA / none		none
LH/FSH+GnRH	none / NA				
hCG + GnRH	NA / +	NA / +	NA / none		none
hCG+$PGF_{2\alpha}$	NA / +	NA / +	NA / none		none
hCG+Gn+PG	NA / +	NA / +	NA / none		none

Shaded areas or 'NA': Non Applicable; '+' : stimulation over the untreated control levels; '-' : inhibition over the untreated control levels; 'none' : no effect compared to the basal levels.

D. Difference in hormonal environment:

In the PMSG primed immature rat, PMSG is responsible for inducing follicular maturation, as would be FSH during a normal cycle. Concomitant with follicular development, estradiol levels rise (as observed in the *in vivo* PMSG/hCG model (Figure 61). In the immature rat, however, E_2 does not get a chance to down-regulate FSH, since stimulation is achieved by PMSG.

The second granulosa cell model was matured in vivo for a longer period, giving E_2 the opportunity to raise further, and even reach the plateau phase before declining by the time of autopsy. E_2 is known for its positive, as well as its negative feedback effect on the pituitary. It could be that E_2 has a similar dual function on GnRH/GnRH-R in the ovary, which could lead to a difference in the number of basal GnRH-R at the time of treatment of the cultured granulosa cells. The basal levels of GnRH-R could be conducive to a difference in response to GnRH and how the cells respond to gonadotrophin in regard to GnRH-R mRNA variations.

In conclusion, the differential effects observed in these two models illustrate the importance of maintaining awareness of the ability of subtle differences in models to result in profound changes in culture differentiation and cellular responses. Thus, the multitude of different models presented in the literature constitutes a barrier for the interpretation of the results, but may also provide a valuable pool of unexploited information.

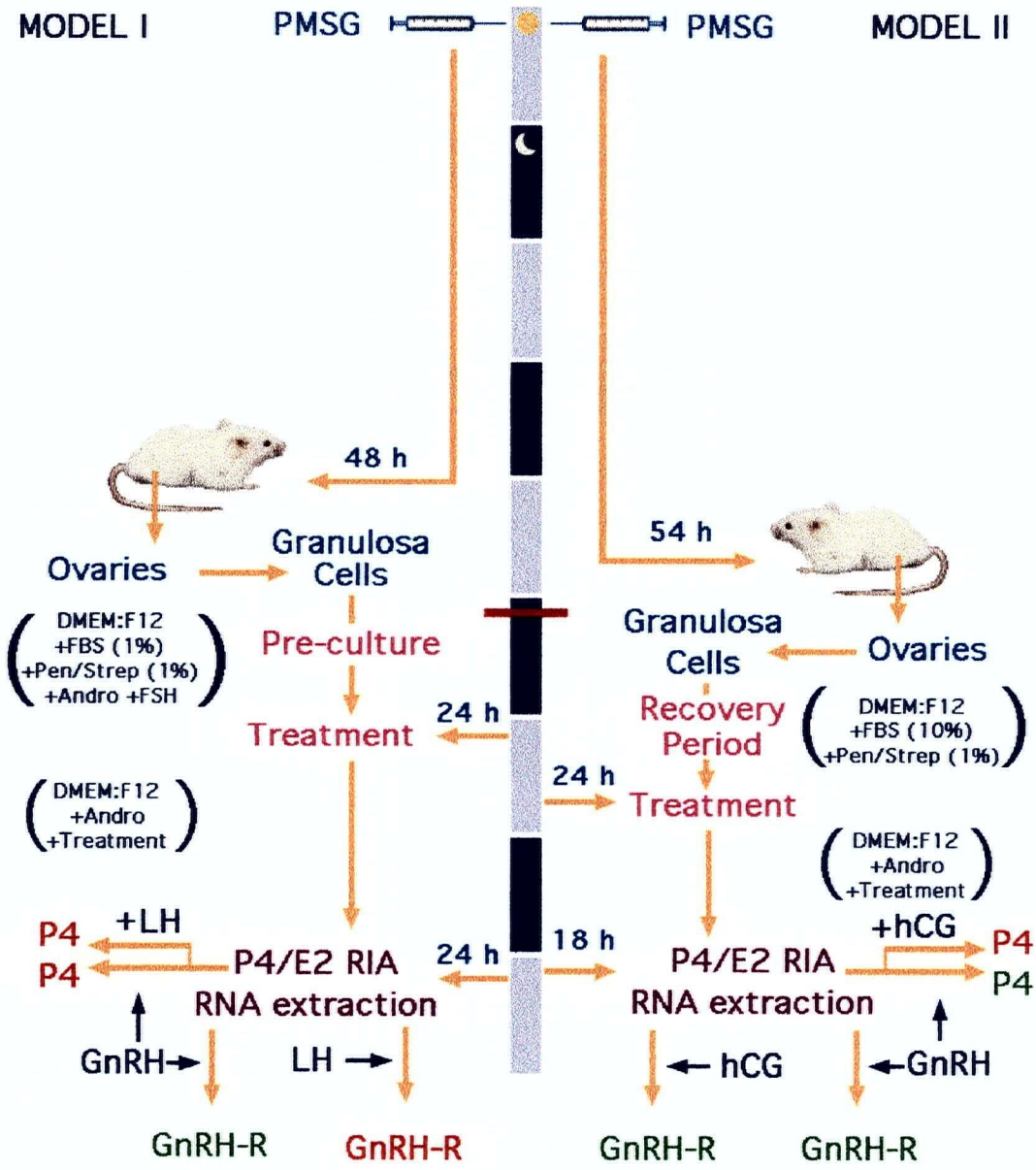


Figure 63. Comparison between Model I and Model II, and their outcome. (Red is for inhibition; green is for stimulation; — indicates the approximate time of ovulation). Subtle differences in the protocol used to obtain granulosa cells from PMSG primed immature rats resulted in significant differences in the outcome of the treatments. The major differences were seen in the effects of GnRH on steroidogenesis and the GnRH-R mRNA response to gonadotrophins.

E. Suggested studies to clarify the issue of dual responses

The present study revealed a number of differential responses to some treatments depending on the initial conditions. These dual responses were discussed earlier.

To better understand the number of dual effects observed during the course of these studies, deeper regulation studies at the level of the gene would be necessary. A system involving regulatory elements in the regulatory region of the GnRH-R gene would certainly be able to explain the complexity of the patterns observed. Regulatory elements that could be expected to be found include different hormone response elements (HRE) such as estrogen response element (ERE) and cAMP response element (CRE).

Transfection of the promoter region of the GnRH-R gene in a CAT (Chloramphenicol Acetyl Transferase) system, and directed deletions and mutations would certainly yield some answers in the regulation of the GnRH-R gene expression. In short, in a CAT assay, a putative regulatory sequence is cloned into a plasmid containing CAT. Different regulatory elements can be tested, and the presence of a response element is revealed and measured by expression of CAT. Directed deletion suppresses the response.

Regulatory regions can be complex, and the level of complexity could explain the multiple outcomes in cellular responses.

SUMMARY

The effects of luteinizing hormone (LH), follicle stimulating hormone (FSH), human chorionic gonadotrophin (hCG), gonadotrophin-releasing hormone (GnRH) and prostaglandin F_2 alpha ($PGF_{2\alpha}$) on the regulation of GnRH-R mRNA levels were investigated *in vivo* and *in vitro*. Progesterone (P_4) and estradiol (E_2) production were measured routinely. The rat models used in these studies included mature cycling female Sprague Dawley rats, as well as immature rats primed with pregnant mare serum gonadotrophin (PMSG) alone, or PMSG followed by hCG. Animals primed with PMSG/hCG were used as an *in vivo* model for the peri-ovulatory period. Animals primed with PMSG alone were used to obtain granulosa cells. Cells were cultured *in vitro*, subjected to experimental treatments and assayed for the mRNA of interest and steroids.

GnRH-R mRNA expression was localized by Southern blotting to the reproductive tissues of both male and female Sprague Dawley rat, as well as in steroidogenic and steroid sensitive tissues. GnRH-R mRNA could be detected in the gonads of both male and female shortly after birth. The intensity of the signal detected by Southern blotting increased progressively towards adult life. In the pituitary, the signal was already present in young animals (D8), and increased with increasing maturity.

In the cycling adult female, pituitary GnRH-R mRNA levels were found to be at their highest levels on the morning of the second day of diestrus. Levels declined towards estrus, reaching lows up to 2-fold below the diestrus II levels by the morning of the estrus, and rising again to 75% of the diestrus II levels by the morning of diestrus I. In the ovary, no apparent changes could be detected between the mornings of each day of the cycle. Serum P_4 and E_2 levels followed previously reported profiles, thus validating the model.

Since GnRH-R mRNA variations were not detectable in the ovary of the cycling adult, a second *in vivo* model was chosen to represent the peri-ovulatory period. Briefly, 28-day old female Sprague Dawley rats were injected subcutaneously (s.c.) with saline (group 1) or 10 IU

PMSG (groups 2 to 4). After 48 hours, groups 3 and 4 received 10 IU hCG s.c. Group 1 (control) and two (pre-ovulatory) were sacrificed 48 hours after the first injection. Group 3 (ovulatory) was sacrificed 12 hours after the second injection, and group 4 (post-ovulatory) was sacrificed 48 hours after the second injection. Ovarian and pituitary GnRH-R mRNA levels were measured by Northern blots and/or RT/PCR followed by Southern blots. In the pituitary, GnRH-R mRNA levels were reduced by 2-fold in the pre-ovulatory and the ovulatory group compared to control and the post-ovulatory group. In the ovary, GnRH-R mRNA levels decreased by 75% in the ovulatory group compared to the control, and were 50% of the control in the post-ovulatory group. Serum P_4 levels increased over the course of the treatment, to reach a high 30 times the control levels at ovulation, followed by a relatively slight decrease to 10 times the control levels for the post-ovulatory group. Estradiol levels were found to be raised only in the pre-ovulatory group, where they were twice that of the control.

Another series of experiments examined the effects of experimental treatments of cultured granulosa cells on GnRH-R mRNA expression. Cells were obtained from the ovaries of PMSG primed immature rats, and plated in the presence of androstenedione and FSH. Following the 24 hours pre-culture period, cells were treated with various concentrations of LH, FSH and GnRH, alone or in combination, in time courses or 24h concentration responses experiments. Progesterone profiles, measured by RIA showed a steroidogenic effect of high concentrations (1000 ng) of LH and FSH, with 5- to 6-fold stimulation over basal P_4 production. GnRH (10^{-6} M) inhibited basal P_4 production by 30%, and inhibited gonadotrophin-stimulated P_4 output down to basal levels. Time course effects of LH (1000 ng), as revealed by RT/PCR followed by Southern blotting, demonstrated a strong reduction of GnRH-R mRNA levels 12 hours after application of LH, with levels returning to control by 48 hours. LH concentration response exhibited the same down-regulation of GnRH-R mRNA levels by more than two times. GnRH was shown to stimulate its own receptor mRNA for concentrations ranging from 10^{-7} to 10^{-6}M. FSH had no effect on GnRH-R mRNA levels.

In a last series of experiments, the effects of hCG, GnRH and $PGF_{2\alpha}$ on GnRH-R mRNA levels were investigated. GnRH and $PGF_{2\alpha}$-R mRNA levels were also measured. Granulosa cells obtained as previously described, were plated in plain medium for 24 hours, and exposed to various combinations of treatments for 18 hours. In this model, hCG was found to elevate GnRH-R and $PGF_{2\alpha}$-R mRNA levels in a concentration response manner, as demonstrated by RT/PCR. Progesterone and estradiol, measured by RIA, also increased (up to 3 times) in response to hCG treatment. A concentration related increase of GnRH-R and $PGF_{2\alpha}$-R mRNA levels could be observed in response to GnRH treatment, while GnRH mRNA was inhibited by higher concentrations of GnRH (10^{-6} M), and stimulated in the mid-range concentrations (10^{-9} M). Progesterone production, but not estradiol, was stimulated by GnRH at the higher concentrations (10^{-8} to 10^{-6} M). Finally, $PGF_{2\alpha}$ was found to inhibit GnRH-R mRNA levels in a bimodal fashion, with 10^{-9} M lowering the mRNA levels by half, while lower and higher concentrations were without effect. The response of $PGF_{2\alpha}$-R mRNA to $PGF_{2\alpha}$ treatment was also bimodal, with an increase in mRNA levels at the low and high concentrations, while the mid-range concentration (10^{-9} M) had no effect on mRNA levels.

The present study demonstrated that:

1. GnRH-R mRNA expression was localized to steroidogenic or steroid-dependent tissues, supporting a general role of GnRH as a modulator of steroidogenesis.

2. GnRH-R mRNA expression was found to increase in the ovary, the testis and the pituitary during ontogeny of the neonatal to pre-pubertal rat, suggesting that GnRH is implied in the sexual maturation, as well as the adult reproductive functions of the rat

3. The ovary of the cycling adult female did not display any variation in GnRH-R mRNA levels, possibly because of the multi-stage nature of the follicles.

4. A. Ovarian and pituitary GnRH-R mRNA variations were detected in response to PMSG/hCG treatment of immature rats.

B. Together with the pituitary pattern observed in the adult female, these results outline the dual role of GnRH as a hypothalamic regulator of gonadotrophin secretion, controlled by steroids, and a gonadal regulator of steroidogenesis, controlled by gonadotrophins.

5. LH/hCG and GnRH were found to have dual effects on GnRH-R and steroidogenesis, depending on the in vitro granulosa/luteal cell studied. Subtle differences in models result in profound changes in cellular differentiation and response to stimuli. Multitude of different models presented in the literature constitutes a barrier for the interpretation of the results, but may also provide a valuable pool of unexploited information.

In conclusion, GnRH-R mRNA levels were found to be altered by a number of experimental treatments, suggesting that the control of GnRH actions in the ovary can also be exerted through control of its receptor mRNA levels.

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