Luteinizing Hormone-Regulated Genes and the Corticotropin Releasing Hormone/Urocortin-Receptor-Binding Protein System in the Primate Corpus Luteum during the Menstrual Cycle

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This book is dedicated to my parents, husband, and daughter.
Their love, understanding, faith, and support encourage me forever.

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ABBREVIATIONS

3 beta-hydroxysteroid dehydrogenase	HSD3B
Adenosine triphosphate	ATP
Adenylate cyclase	AC
Adrenocorticotropic hormone	ACTH
Albumin from bovine serum	BSA
Aminolevulinate, delta-, synthase 1	ALAS1
Androgen	A
Angiopoietin	ANGPT
Antide	ANT
Antral follicle	F
Aromatase	CYP19
Atretic follicle	AF
Base pair	bp
Body weight	BW
Calcium	Ca^{2+}
Central nervous system	CNS
Cholesterol	C
Cholesterol side chain cleavage cytochrome P450	CYP11A
Chorionic gonadotropin	CG
Complement component 4A	C4A
Control	CTRL
Corpus luteum	CL
Corticotropin releasing hormone	CRH
Corticotropin releasing hormone binding protein	CRHBP
Corticotropin releasing hormone receptor 1	CRHR1

Corticotropin releasing hormone receptor 2	CRHR2
Corticotropin-releasing factor	CRF
Cyclic adenosine monophosphate	cAMP
Cyclophilin A	CYCA
Cysteine dioxygenase, type I	CDO1
Cytochrome P450 17α-hydroxylase/17,20-lyase	CYP17
Diacylglycerol	DAG
Digoxygenin	DIG
DL-Dithiothreitol	DTT
DNA base pair markers	M
Early luteal phase	ECL
Estradiol	E
Estrogen receptor	ER
Fas Ligand	FasL
Follicle stimulating hormone	FSH
G protein q	Gq
G protein s	Gs
Glutathione S-transferase	GST
Gonadotropin releasing hormone	GnRH
Granulosa cell	GC
Hematoxylin-eosin	H & E
Hypothalamo-pituitary-adrenal	HPA
Immunohistochemistry	IHC
In situ hybridization	ISH
Inositol triphosphate	IP3
Integrated Molecular Analysis of Genomes and their Expression	IMAGE
Interstitial tissue	IT
Late luteal phase	LCL
Lectin, galactoside-binding, soluble, 7 (galectin 7)	LGALS7
LIM domain only 7	LMO7

Luteal phase	LP
Lutectomy	LX
Luteinizing hormone	LH
Luteinizing hormone receptor	LHR
Matrix metalloproteinases	MMPs
Metallothionein 1	MT1
Mid luteal phase	MCL
Midlate luteal phase	MLCL
No Change	NC
Not applicable	N/A
Open reading frame 1	ORF1
Oregon National Primate Research Center	ONPRC
Ovarian surface epithelium	OSE
Phosphate buffered saline	PBS
Phosphatidic acid selective phospholipase A1	PASPA1
Phosphatidylinositol bisphosphaste	PIP2
Phosphoinositide-3-kinase, regulatory subunit, polypeptide 1	PIK3R1
Phospholipase C	PLC
Polymerase chain reaction	PCR
Polyposis locus protein 1-like 1	DP1L1
Preantral follicle	PAF
Preovulatory follicle	POF
Progesterone	P
Prostaglandin	PG
Prostaglandin dehydrogenase	PGDH
Prostaglandin E2	PGE2
Prostaglandin F2α	PGF2α
Protein kinase A	PKA
Protein kinase C	PKC
Reverse transcription	RT

Sodium Chloride-Sodium Citrate buffer	SSC
Sodium dodecyl sulfate	SDS
Sorbitol dehydrogenase	SORD
Standard error of the mean	SEM
Steroidogenic acute regulatory protein	StAR
Sterol-C4-methyl oxidase-like	SC4MOL
Streptavidin-horseradish peroxidase	HRP
Terminal deoxynucleotidyl transferase nick end labeling	TUNEL
Testes	T
Theca cell	TC
Threshold level	C_T
Transmembrane domain	TMD
Trizma hydrochloride	Tris-HCl
Tumor suppressor deleted in oral cancer-related 1	DOC-1R
Urocortin	UCN
Urocortin 2	UCN2
Urocortin 3	UCN3
Vascular endothelial growth factor	VEGF
Very late luteal phase	VLCL

ABSTRACT

Luteinizing Hormone-Regulated Genes and the Corticotropin Releasing Hormone/Urocortin-Receptor-Binding Protein System in the Primate Corpus Luteum during the Menstrual Cycle

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Luteinizing hormone (LH) is essential for the development/function of the primate corpus luteum (CL) during the menstrual cycle. However, the cellular/molecular processes whereby LH controls luteal structure/function are poorly understood. Therefore, studies were initiated to investigate systematically the genes that are regulated by LH in the monkey CL during the menstrual cycle. Rhesus monkeys (*Macaca mulatta*) were either untreated or received the gonadotropin releasing hormone antagonist to inhibit pituitary LH secretion. RNA from the CL was analyzed using a human gene spotted microarray. A total of 206 cDNAs exhibited a ≥2-fold change after treatment. Sixty-four of them were up-regulated and 142 were down-regulated. Selected cDNAs were validated via semi-quantitative RT-PCR. Twenty-two of 25 cDNAs exhibiting a ≥6-fold change were validated as differentially expressed in the treatment group. Eleven of 25 changed correspondingly in CL collected in the late-to-very late luteal phase of the natural menstrual cycle, which is consistent with the concept that the proteins encoded by up-regulated genes following LH withdrawal may serve as initiators or mediators of luteal

regression. Genes down-regulated following the loss of LH support may promote luteal activities.

Microarray analysis provided unexpected evidence that the level of mRNA specifying CRH-binding protein (CRHBP) increased significantly in the primate CL following LH withdrawal. Therefore, further experiments were designed to determine if other components of the CRH/urocortin (UCN)-receptor (R)-BP system are expressed in the macaque CL and regulated by LH. Components were quantitated for mRNA by real-time PCR and for protein by western blotting. The mRNA and protein was localized by in situ hybridization and immunohistochemistry. All genes encoding the CRH/UCN-R-BP components, except *UCN3*, were expressed in the monkey CL. Suppressing LH secretion reduced ($P < 0.05$) *UCN2* and increased ($P < 0.05$) *CRHBP* mRNA levels. The mRNA and protein levels of *UCN*s, *CRHR1*, and *CRHR2* were maximal ($P < 0.05$) in CL at early-to-mid luteal phase, whereas *CRHBP* peaked ($P < 0.05$) in the late luteal phase. The mRNAs for *CRHR*s and *CRHBP*, but not the ligands, were detected in the granulosa and theca cells of the preovulatory follicle. However, intense staining for the ligands *CRH*, *UCN*, and *UCN2*, as well as for *CRHR*s and *CRHBP*, was evident in the CL, notably in the steroidogenic luteal cells. Ligand and receptor proteins were localized to the granulosa-lutein cells of the CL, whereas CRHBP was limited to the theca and theca-lutein cells of the preovulatory follicle and CL.

The role of the CRH/UCN-R-BP system was investigated by injecting a CRHR antagonist directly into the preovulatory follicle of monkeys. CRHR antagonist treatment had no effect on ovulation. However, estradiol and progesterone levels were significantly less ($P < 0.05$) in the treatment group during the following luteal phase, compared to controls. Histologic evaluation provided preliminary evidence that CRHR antagonist treatment also suppressed luteal structure, as judged by a luteal cavity and TUNEL-labeled cells.

Thus, a local CRH/UCN-R-BP system exists in a cell-specific manner in the primate CL that is dynamically expressed and LH-regulated during the luteal phase of the menstrual cycle. Initial evidence is consistent with the concept that ligand-receptor activation during the early-to-mid luteal phase promotes luteal development and/or structure/function, whereas its loss in the late luteal phase contributes to luteal regression.

CHAPTER 1
INTRODUCTION

The reproductive life of mammalian species, including primates, is characterized by an ovarian cycle. Old World monkeys (*e.g.*, rhesus monkeys or macaques), apes, and women have an ovarian or menstrual cycle that portrays the events culminating in the release of one oocyte at midcycle and the dynamic pattern of sex steroid hormone synthesis and secretion that is essential for preparation of the reproduction tract for fertilization and initiation of intrauterine pregnancy. During the follicular phase, the first half of the cycle, one small antral follicle is selected for further growth and maturation, thereby secreting the steroid hormone, estradiol. The midcycle estradiol peak elicits a surge of luteinizing hormone (LH) and follicle stimulating hormone (FSH) secreted from the pituitary gland, which causes ovulation to occur and expulsion of the oocyte. The LH surge also stimulates the remaining cells in the follicle wall to differentiate into the luteal cells of the corpus luteum (CL); the CL enlarges due to cell hypertrophy as well as vascularization. As CL size and vascularity reach maximum, the developed CL functions and produces significant amount of steroid hormone, progesterone, as stimulated by the "tonic" release of pituitary LH. A functional CL is necessary for the uterine endometrium to differentiate to a point at which an embryo can implant and initiate a successful pregnancy. If pregnancy does not occur, the CL will regress and the progesterone level declines. Without progesterone support the endometrium also regresses and menses starts. The second half of the cycle, from the LH surge to menses, is termed the luteal phase.

It is well established that the proper development and maintenance of a functional CL in many primate species during the menstrual cycle requires the actions of gonadotropin hormone, LH. The inhibition of LH synthesis or action results in reduced progesterone production and premature luteal regression as evidenced by menstruation (Asch *et al.*, 1981; Ellinwood *et al.*, 1984; Groff *et al.*, 1984; Hutchison *et al.*, 1984;

Collins *et al.*, 1986). Previous studies also demonstrated that ablation of endogenous LH production via the administration of a gonadotropin releasing hormone (GnRH) antagonist, Antide, results in a reduction in the number of large luteal cells and a loss in CL function in rhesus monkeys (Duffy *et al.*, 1999). GnRH is secreted by the hypothalamus and acts on the pituitary gland to stimulate the production and secretion of LH. From such studies, it is clear that gonadotropin, especially LH, is an essential "luteotropic" hormone for the proper structure and function of the primate CL during the menstrual cycle. It is well established that LH has acute actions on steroidogenesis mediated primarily through the second messenger cAMP-protein kinase A pathway (Bogovich *et al.*, 1986; Richards *et al.*, 1986). But there is little information on "luteotropic" actions of LH that control luteal structure and function during CL development and regression. Previous investigators have considered individual genes in the CL that are involved in steroidogenesis or tissue remodeling and are LH regulated, like estrogen receptor (ERβ) (Duffy *et al.*, 2000) and members in matrix metalloproteinase family (MMPs) (Chaffin and Stouffer, 1999; Young *et al.*, 2002). However, very little information exists regarding the complement of genes in the primate CL that are under the regulatory control of gonadotropins. Therefore, studies were initiated to systematically identify LH-regulated genes in the primate CL and to test the hypothesis that the expression of genes that is regulated acutely by LH changes during luteolysis.

The study of LH-regulated genes in the monkey CL unexpectedly revealed that mRNA levels of corticotropin releasing hormone binding protein (*CRHBP*) increased significantly following withdrawal of LH support (Xu *et al.*, 2005b). This protein binds corticortropin releasing hormone (CRH) and the CRH-like peptide, urocortin (UCN), and regulates the amount of ligand available to interact with its receptors and stimulate specific signal transduction pathways in target tissues. The CRH/UCN-receptor-binding protein system (CRH/UCN-R-BP) was originally discovered in the hypothalamus-pituitary-adrenal axis (Vale *et al.*, 1981) and later was found in variety of peripheral tissues, including reproductive system, such as the placenta (Shibasaki *et al.*, 1982). The CRH/UCN-R-BP system plays a major role in coordinating the behavioral, endocrine,

cardiovascular, autonomic and immune mechanisms that allow mammals to adapt under both basal and stressful conditions. The ligands include CRH, UCN, UCN2 (stresscopin-related peptide), and UCN3 (stresscopin). Their actions are mediated through activation of two types of G-protein-coupled receptors, CRH receptor1 and 2 (CRHR1 and 2), encoded by separate genes. Also, each receptor exists as a family of related proteins produced from multiple alternatively spliced forms of mRNA (Dautzenberg *et al.*, 2002). Evidence suggests that ligands, such as CRH or UCN, bind to CRHR1 in the pituitary gland and stimulate adrenocorticotropic hormone (ACTH) secretion during a "fight-or flight" response. In contrast, members of the urocortin family bind to CRHR2 in other tissues and mediate the stress-coping responses occurring during the recovery phase of stress without evoking ACTH release. Whether the stress-coping ligands, UCN2 and 3, bind to CRHBP is unknown. With the discovery of *CRHBP* expression in macaque luteal tissue, studies were initiated to test the hypothesis that a local CRH/UCN-R-BP system exists in the primate CL, and if so, to consider its function during the luteal lifespan in the menstrual cycle and if it is regulated by LH.

CHAPTER 2
REVIEW OF THE LITERATURE

2.1 The Primate Ovary and Menstrual Cycle

2.1.1 Anatomy of the Primate Ovary

The primate ovary is a paired amygdaloid shape intra-abdominal organ whose primary roles are: (1) to release an egg that is fully competent for fertilization and embryonic development during each menstrual cycle, and (2) to produce steroid hormones that will prepare the accessory reproductive organs for intrauterine pregnancy and the birth of a healthy offspring.

The mature primate ovary (Fig. 2.1) consists of an outer zone, the ovarian cortex, and an inner zone, the medulla. The cortex, covered by a specialized mesothelium called the ovarian surface epithelium, contains an outer strip of connective tissue called the tunica albuginea. The resting (nongrowing) follicles are distributed in a relatively avascular layer in the ovarian cortex beneath the connective tissue. Growing follicles and the corpus luteum (CL) are found in the cortical medullary border, which is richly vascularized. Follicles are surrounded by a complex and dynamic milieu of ovarian stroma cells and branches of the vasculature. The medulla contains a variably dense connective tissue that enmeshes stroma cells and autonomic nerves, as well as blood vessels and lymphatics which grow to support developing follicle and CL (Gougeon, 2004).

During embryonic development in primates, the oocytes become surrounded by a layer of epithelial pregranulosa cells to form primordial follicles. Most of these follicles remain in a resting stage until they enter the growth phase and/or degenerate by a process called atresia. When the resting follicles enter the growth phase, they enlarge by proliferation of granulosa cells (GC) and expansion of the oocyte. The first stage of

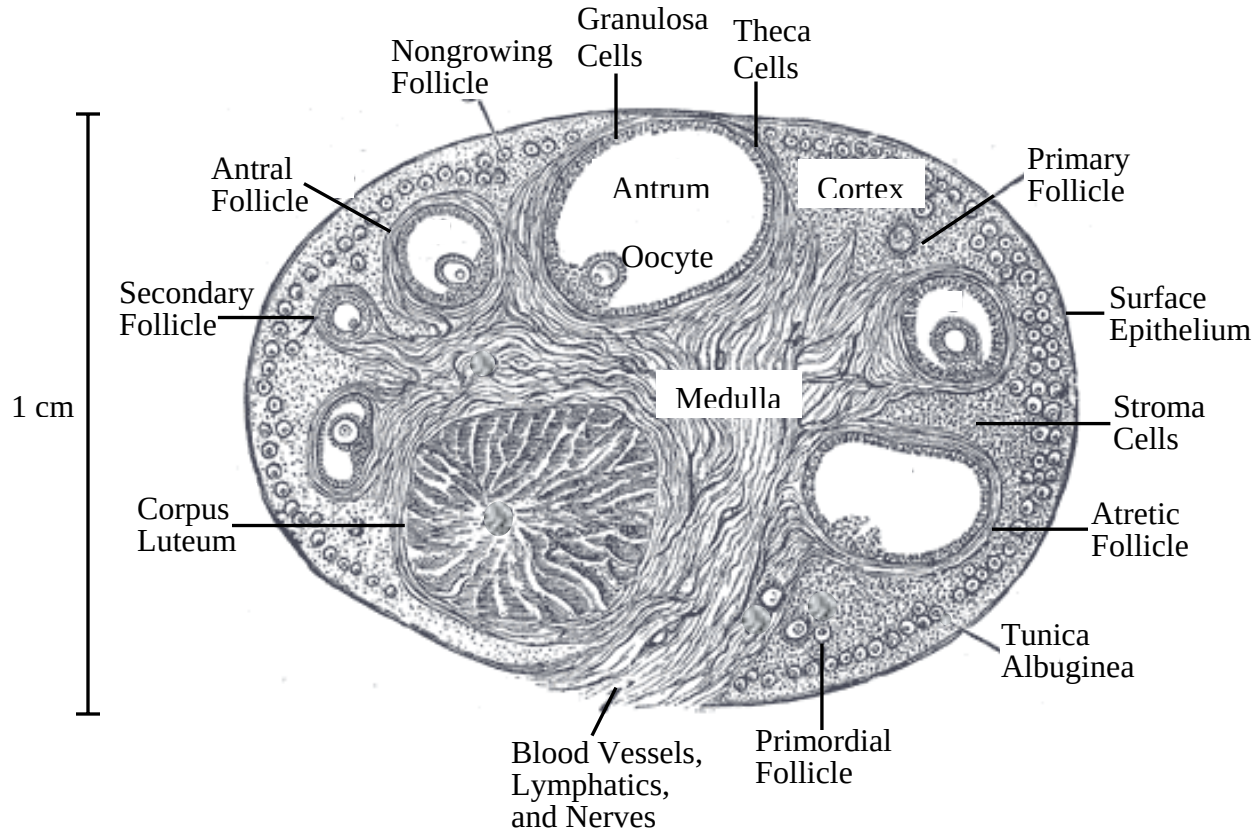

Fig. 2.1 Anatomy of the primate ovary (adapted from Gray, 1918).

follicular growth in primates is the primary follicle in which the granulosa layer is enveloped by a basal lamina that separates the surrounding stromal/thecal elements from GC and excludes capillary, blood cells, and nerves from the granulosa layer. Progressively, follicles become secondary follicles with two or more complete layers of GC surrounding the oocyte. At this stage of development, some stroma cells near the basal lamina become aligned parallel to each other and constitute the theca layer. As the follicle enlarges, the theca layer differentiates into two parts. The outer part, theca externa, consists of cells that are not different from the cells of the undifferentiated theca. In the inner part, theca interna, some fibroblast-like precursor cells assume the appearance of typical steroid-secreting cells. When small fluid-filled cavities in the granulosa layer aggregate to form a single cavity or antrum, the follicle becomes an antral follicle (Gougeon, 2004) (Fig. 2.1).

2.1.2 Menstrual Cycle in Primates

Old World monkeys (*e.g.*, rhesus monkeys or macaques), apes, and women have a menstrual cycle. It is a dynamic process that involves sex steroid hormone synthesis/ secretion and a regular change in anatomy over an approximate monthly time period. In the primate ovary, follicles form during fetal life (Baker, 1963). These primordial follicles constitute the “resting” stock from which growing follicles arise (Gougeon and Chainy, 1987; Koering, 1983). Although some of these follicles start to grow almost immediately, most of them remain in a resting stage until they either degenerate or some signal(s) activate(s) them to enter the growth phase. With increasing age, the population of resting follicles progressively decreases until menopause when few follicles remain. Follicle growth through the preantral stage to the antral stage requires months. In primates, early antral follicles are present in ovaries throughout the follicular and luteal phase and even before the onset of puberty. But after puberty, when menstrual cyclicity is initiated, one antral follicle is selected in each cycle to mature and complete follicular development with the appropriate hormonal support.

The menstrual cycle is usually divided into two parts (Fig. 2.2). In the follicular phase, the first half of the cycle, only one small antral follicle is selected to continue its

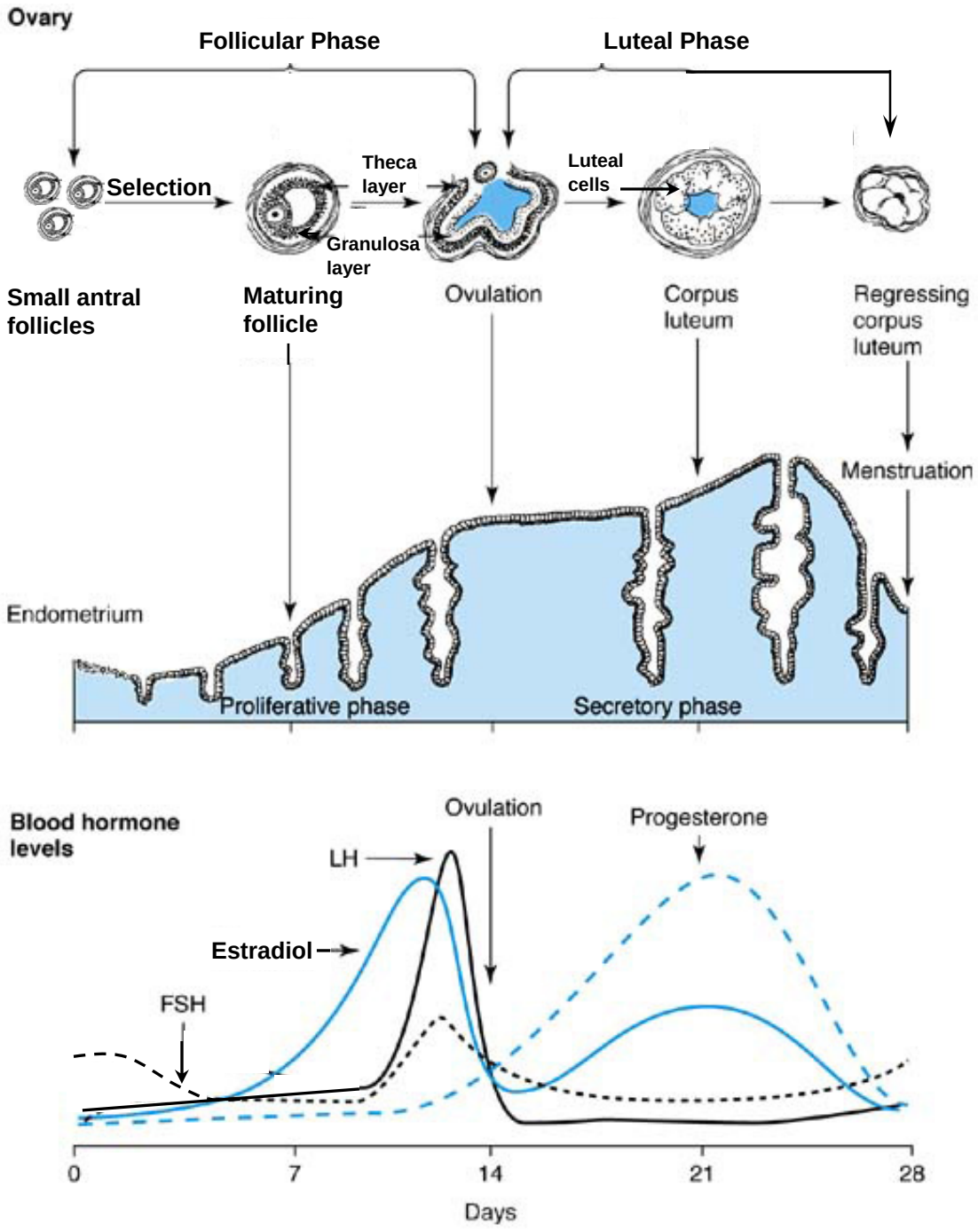

Fig. 2.2 Schematic of events during the menstrual cycle in primates (adapted from Chandrasoma and Taylor, 1997)

growth and to mature into the “dominant follicle”, thereby secreting the steroid hormone, estradiol. Following menses, estradiol promotes the repair and regrowth of the endometrium in the uterus. This regrowing tissue is identified as the proliferative endometrium. The midcycle estradiol peak also elicits a surge of luteinizing hormone (LH) and follicle stimulating hormone (FSH) secreted from the pituitary gland. In the average cycle, this LH and FSH surge starts around cycle day 12 and may last 48 hours. The second half of the cycle, which begins with the LH surge and ends with menses, is termed the luteal phase. The release of LH and FSH matures the oocyte (*i.e.*, reinitiates meiosis) and disrupts the apical wall of the follicle in the ovary, which leads to ovulation and expulsion of the mature ovum. During and after ovulation, the remaining cells comprising the wall of the ovulatory follicle differentiate under the support of the pituitary LH to form the luteal cells of the CL, a solid endocrine gland in the adult ovary (Rothchild *et al.*, 1981; Niswender *et al.*, 1994). Further stimulated by the “tonic” release of LH, luteal formation proceeds in earnest after follicle rupture and continues for a species-specific number of days until the fully-formed CL is present.

As CL size and vascularity reach maximum, the CL functions to synthesize and secrete the steroid hormone, progesterone, for approximately two weeks. Progesterone plays a vital role in the reproductive tract by converting the proliferative endometrium into a secretory lining receptive for implantation of the early embryo and to support a maternal environment that sustains intrauterine pregnancy. In the absence of a pregnancy, the CL will regress and the progesterone level declines. Without progesterone support the endometrium also regresses and leads to menstruation. The first day of low (<100 pg/ml) serum estradiol following the midcycle estradiol peak typically corresponds with the day after the LH surge, and is therefore termed day 1 of the luteal phase (Duffy *et al.*, 1999). There are five luteal phase stages during the natural menstrual cycle, early (day 3-5 post LH surge), mid (day 6-8), midlate (day 10-12), late (day 14-16), and very late (day 18-19; menses) luteal phase. These intervals correspond to the developing, developed functional, on the verge of regressing, regressing and regressed CL, respectively (Young *et al.*, 2002).

Other mammals also have ovarian cycles that are significantly different from that of

primates. Some carnivore species, e.g. dogs, cats, and skunks (Eckstein and Zucherman, 1956; Asdell, 1964), have estrous cycles that occur only at long intervals, such as every 6 to 12 months. A fully functional CL develops after ovulation and its functional life span, from less than 2 weeks to as long as 6 months, does not differ remarkably regardless of whether the CL originates during a fertile or nonfertile cycle (Rothchild, 1981). In rodents, such as the laboratory rat or mouse (Hilliard, 1973; Freeman, 2006), the estrous cycle lasts 4 to 5 days and lacks a true luteal phase. After ovulation, the ruptured follicle takes on some of the characteristics of a CL, but the luteal gland does not fully develop and is considered nonfunctional. A functional CL only develops during pseudopregnancy or pregnancy (Hilliard, 1973). Many of the ungulates, including the domesticated farm animals, also have a CL that forms after ovulation and functions until day 17 to 21 postestrus. If pregnancy does not occur, the CL undergoes luteolysis (Hansel *et al.*, 1973). However, there are differences between primates and ungulates in the activities and processes controlling the length of the ovarian cycle and the luteal phase, as well as in the maternal-ovarian recognition of pregnancy (Fortune, 1994; Knickerbocker *et al.*, 1988). Since macaques have a menstrual cycle comparable to that in women, they are a valuable primate model for basic and applied research relevant to women's health and contraception.

2.1.3 Development of the Primate Corpus Luteum

Luteal formation, also called luteinization, is a remarkable event involving cell proliferation, cell differentiation, and tissue remodeling that is unparalleled in other tissues of the adult mammal (Murphy *et al.*, 2004). It comprises two major processes: (1) the termination of proliferative activity, plus rapid cell hypertrophy and differentiation of the GC of the follicle into luteal cells of the CL with progesterone synthetic capacity; and (2) the rapid growth of blood vessels through angiogenesis/vascularization and, in some species, the lymphatics through lymphangiogenesis (Ichikawa *et al.*, 1987) to replace the previously avascular granulosa layer of the follicle with the extensive vascularized luteal tissue of the CL (Stouffer, 2003).

A common event during collapse of the follicle antrum at ovulation is the infolding

of the follicle wall with breakdown of the basement membrane separating the granulosa and theca layers of the follicle. This is followed by invasion of microvascular cells into the avascular granulosa layer, as well as other cells associated with the vasculature. The follicular infolding and vascular invasion also transport theca cells (TC) toward the center of the follicle cavity. TC may degenerate or be absorbed into the surrounding stroma, become incorporated into the luteal parenchyma as theca-lutein cells, or remain as paraluteal cells clustered along the periphery or along infoldings of the luteal gland. The fate of TC may depend on their differentiated state or proximity to luteinizing GC at ovulation. In addition to the dramatic increase in vascularity, the large increase in mass of the CL is due to hypertrophy of the luteinizing GC (Enders *et al.*, 1973). The GC with a high nuclear-to-cytoplasmic ratio is converted into granulosa-lutein cells with a low nuclear-to-cytoplasmic ratio at a volume increase of up to 10-fold (Smith *et al.*, 1994). In some primate species, such as Old World macaques and humans, cell segregation is evident in the fully developed CL. Theca-derived paraluteal cells located along the periphery and in invaginations of primate luteal tissue are morphologically distinct from granulosa-derived luteal cells in the central parenchyma throughout their life span (Corner *et al.*, 1956). Immunocytochemical evidence suggests that (1) the paraluteal and luteal cells are of theca and granulosa origin in primates, respectively, and (2) the primate CL retains some compartmental analogy to the follicle that facilitates provision of substrate (androgen) from paraluteal cells to luteal cells for continued estrogen production (Sanders *et al.*, 1996; Sasano *et al.*, 1989).

One of the hallmarks of luteinization is the switch from the principal steroid product of the maturing follicle, estradiol, to that of the developing and mature CL, progesterone. This is both a qualitative and quantitative change because the primate CL produces up to 100-fold greater amounts of steroid than the follicle. Thus, circulating levels of estradiol in the presence of the preovulatory follicle reach the picomolar range, whereas circulating progesterone levels produced by the CL can reach the nanomolar range. Studies in several species indicate that the number and size of luteal cells in the subpopulations change during the lifespan of the CL (Stouffer and Brannian, 1993). In general, the numbers and sizes of steroidogenic luteal cells increase as the CL develops during the

early-to-mid luteal phase in primates. It is likely that increases in luteal cell number/size as well as vascular development influence CL activity after ovulation since these events parallel the rise in circulating progesterone levels. Moreover, removal of GC from preovulatory follicles to reduce the source of luteal cells markedly decreased luteal mass and serum progesterone levels, but not luteal lifespan, in primates (Marut *et al.*, 1983). These studies also established the critical importance of granulosa-derived luteal cells as the predominant source of progesterone in the CL during the ovarian cycle.

Although the primary hormone produced by the CL is the steroid progesterone, it is clear that luteal cells synthesize and secrete many peptides and proteins that can serve in a species-dependent manner as local paracrine/autocrine or endocrine factors. Granulosa-lutein cells in primates are considered to be the primary producer of such peptide hormones. For example, although inhibin and activin were initially characterized as modulators of FSH synthesis and secretion by anterior pituitary gonadotropes, they are now considered to be "multicrine" hormones that are expressed and function in the ovary (Findlay *et al.*, 2001). Inhibin and activin (the dimer of inhibin beta subunits) act as paracrine and autocrine modulators of follicle growth and maturation (Woodruff et al., 1990) and also regulate steroidogenesis in the ovary (Sawetawan *et al.*, 1996). Relaxin is synthesized primarily by CL during pregnancy (Stoelk *et al.*, 1991) and acts on myometrium to maintain uterine quiesence in early pregnancy (Szlachter *et al.*, 1980).

A functional CL is necessary for the uterine endometrium to differentiate to a point at which an embryo can implant and initiate early pregnancy. The CL exists for a limited life span that in mammalian species depends on the fate of the oocyte released by the antecedent ovulatory follicle. For primate species of monkeys, great apes, and humans, a functional CL forms rapidly after ovulation and functions for an appreciable interval, a so-called "long luteal phase" (Rothchild, 1981), which is sufficient to permit timely movement of the gamete/early embryo through the oviduct and to prepare the embryo attachment in the uterus for implantation, even though conception does not occur (Knobil *et al.*, 1973; Buffer *et al.*, 1998). The functional lifespan of the CL, supported by the anterior pituitary hormone LH, lasts for approximately two weeks corresponding to the luteal phase. However, if conception and implantation occurs, regression of the CL is

delayed and luteal function is extended for a species-specific interval until its essential steroidogenic activities are assumed by the developing placenta. Pituitary LH secretion is required only until late in the lutal phase of the fecund cycle. Thereafter, an LH-like hormone, chorionic gonadotropin (CG), is secreted by developing placenta, which extends the functional life span of the CL in early pregnancy (Stouffer and Hearn, 1998). Once the placenta assumes the steroidogenic (progesterone producing) function of the CL, the ovaries become nonessential for pregnancy and cease cyclic function until after parturition.

2.1.4 Luteolysis in Primates

The CL typically regresses near the end of nonfecund ovarian cycle. The process of luteal regression consists of the cessation of progesterone production, or “functional regression”, and physical involution of the CL, or “structural regression”. This loss of luteal structure-function is often referred to as luteolysis (Davis and Rueda, 2002). In ruminant species and rodents, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is accepted to be the major luteolytic signal (Niswender *et al.*, 2000). $PGF_{2\alpha}$ produced by the nonpregnant uterus induces luteal regression. In contrast, luteolysis in primates does not depend on the uterus nor is mediated by uterine $PGF_{2\alpha}$ (Beling *et al.*, 1970). Researchers have proposed "self-destruct" mechanisms near the end of the menstrual cycle whereby local factors, e.g. estrogen or $PGF_{2\alpha}$, that are synthesized in the ovary or CL lead to loss of luteal structure-function (Knobil, 1973). Likewise, oxytocin was identified in the primate CL and ovary that could act on ovarian oxytocin receptors to generate low levels of intraovarian $PGF_{2\alpha}$ that may cause luteolysis (Fuchs, 1987). There is also evidence for less LH support (i.e. reduced LH pulsatility) (Ellinwood *et al.*, 1984) and reduced CL sensitivity to LH (Duffy *et al.*, 1999) in the late luteal phase of the menstrual cycle, and luteolysis may be due to less exposure to luteotropic factors. Nevertheless, luteolytic signals in primates remain controversial.

The CL of primates ceases function approximately three days before menstruation at the end of the cycle (Knobil, 1973). The hallmark of early luteal regression is declining progesterone production, clearly demarcated by the fall of circulating progesterone levels

below concentrations (< 1ng/ml) required to sustain its action on the reproductive tract (Niswender *et al.,* 1994a). However, residual luteal mass is evident into the next follicular phase and can even respond in a limited manner to exogenous gonadotropin treatment (Knobil, 1973). In primates, notably the humans, luteolysis appears to occur first in the granulosa-derived luteal tissue. Sasano and Szuki (Sasano *et al.,* 1997) noted that the number of steroidogenically active granulosa-lutein cells decreased by the late luteal phase, whereas the paraluteal region of presumed theca-lutein cells remain as prominent and steroidogenically active. Cell death in the CL during structural involution may occur by the process of apoptosis which results in a reduction in luteal weight (Shikone *et al.*, 1996; Young *et al.,* 1998). Whether or not apoptosis is responsible for early disruption of steroidogenesis as well as structural involution in later regression remains to be clarified.

2.2 Luteinizing Hormone and the Primate CL during the Menstrual Cycle

Pituitary gonadotropins, notably LH, play essential roles in the events leading to ovulation of the mature follicle and development of the subsequent CL in primates. LH can act directly on the ovary through its receptor or indirectly via local factors, such as ovarian steroids (e.g., progesterone). There are gonadotropin-stimulated, progesterone-dependent processes that promote luteotrophic and suppress luteolytic pathways in the primate CL.

2.2.1 Luteinizing Hormone (LH) in Primates

LH is a glycoprotein hormone secreted by the anterior pituitary that stimulates the gonads (Pierce and Parsons, 1981). It was suggested that higher primates developed a much simpler solution to regulation of the CL than other species, in that the luteotrophic process does not include a principal role for pituitary prolactin, which is essential in rodents, and the luteolytic process does not involve a uterine signal, which is in many non-primate species (Zeleznik and Benyo, 1994). It is now generally accepted that the predominant luteotropic hormonal regulator of the CL in macaques and women is LH-

like gonadotropin, such that: (1) the strength-duration of the midcycle surge of gonadotropins, notably the LH, secreted by the anterior pituitary is the signal for follicle rupture, i.e. ovulation, and for conversion of the mature follicle into the CL, i.e. luteinization; (2) the tonic, pulsatile release of LH by the pituitary during the luteal phase is essential for continued development and maintenance of luteal structure-function of the primate CL during the menstrual cycle (Stouffer, 2003); and (3) the exponential secretion of an LH-related hormone, CG, by the implanting embryo and developing placenta extends the CL function and lifespan in early pregnancy.

Elegant studies conducted from the 1970s to 1980s, using a variety of techniques from gonadotropin ablation by hypophysectomy (Denamur *et al.*, 1973) or administration of LH antisera (Moudgal *et al.*, 1972) to pulsatile gonadotropin releasing hormone (GnRH) infusion to control LH availability, support the critical role of LH-like gonadotropins in regulating the development, function, and lifespan of the CL in primates (Moudgal *et al.*, 1984; Zeleznik and Hutchison, 1987). More recent investigations performed over the past decade, using GnRH antagonists (Reissmann *et al.*, 1995; Duffy *et al.*, 1999) and pure recombinant human LH (Chandrasekher *et al.*, 1994; Duffy *et al.*, 1999) have strengthened this concept that suppression of endogenous LH secretion inhibits the CL, whereas coadministration of LH with such treatments can restore the normal functional lifespan of the CL during the nonfecund ovarian cycle (Farin *et al.*, 1990). Potent GnRH antagonists can interrupt the ongoing LH surge and cause the LH levels to fall even after the midcycle rise has begun (Fluker *et al.*, 1991; Leroy *et al.*, 1994). The antagonists also can reduce circulating LH levels when administered in the early, as well as mid or late luteal phase (Fraser *et al.*, 1987; Dubourdieu *et al.*, 1991). LH depletion/neutralization beginning within one to two days of ovulation can prevent the normal development of the CL, whereas treatment at mid luteal phase causes premature regression of the developed CL (Stouffer, 2003a). The data clarify three points: (1) a GnRH-induced LH surge of substantial length is needed for ovulation and development of normal luteal function. The duration of the LH surge in the ovarian cycle is much longer in monkeys and women compared with that in many non-primate species. Initial studies on monkeys and women employing GnRH-induced LH surges or

administering exogenous LH suggest that gonadotropin surges of lesser duration and amplitude are sufficient to reinitiate oocyte maturation and early luteinization of granulosa cells. However, surges of longer duration and higher amplitude optimize oocyte recovery, fertilization and CL development (Zelinski-Wooten *et al.*, 2000). (2) The developing CL in the early luteal phase is critically dependent on circulating LH for continued function (Dubourdieu *et al.*, 1991), just like the developed CL of the mid luteal phase (Hall *et al.*, 1991). Several reports indicated that suppression of LH support for 72 hours results in irreversible loss of luteal structure-function (Fraser *et al.*, 1987; Dubourdieu *et al.*, 1991). However, exogenous LH replacement initiated at the time of GnRH antagonist treatment will sustain luteal structure-function (Collins *et al.*, 1986; Duffy *et al.*, 1999). In an effort to titrate the amount of LH needed to maintain the normal functional lifespan of the CL in GnRH antagonist-treated monkeys, it was noted that repeated injections of a constant amount of LH were unsuccessful, but that increasing the dose from mid to late luteal phase was required (Duffy *et al.*, 1999). These, and other studies employing constant infusion of low-dose LH (Zeleznik, 1998; Duffy *et al.*, 1999), support the concept that the primate CL becomes less sensitive to LH as its lifespan progresses, and that emerging gonadotropin insensitivity could be a critical factor in the timely onset of luteolysis near the end of the menstrual cycle (Zeleznik and Little-Ihrig, 1990). (3) Exponential increases in LH bioactivity will extend the functional lifespan of the primate CL in a pattern consistent with that observed in the fertile menstrual cycle (Zeleznik, 1998). A more intense gonadotropin stimulus, e.g., in the form of rising levels of CG secreted by the trophoblast of the implanting blastocyst, is required to extend the functional lifespan of the primate CL.

2.2.2 LH-Induced Actions

Cellular and molecular studies are beginning to identify processes that are LH-regulated in the ovulatory follicle and CL in primates. But detailed studies are lacking on the regulation and vital roles of the processes, particularly in the ovulatory, luteinizing follicle, and CL of the natural ovarian cycle. Since the 1970s, investigators have recognized that addition of LH to luteal tissue/cells in vitro causes a rapid stimulation of

progesterone production which is associated with a rise in cyclic adenosine monophosphate (cAMP) levels (Marsh *et al.*, 1974; Stouffer *et al.*, 1978). More recent studies led to an emerging view on how gonadotropins stimulate steroidogenesis via the following pathways:

(1) LH/CG receptor binding and stimulatory G-protein α activation of adenylate cyclase.

(2) The cAMP activation of protein kinase A (PKA).

(3) Transcriptional and post-transcriptional changes that promote cholesterol availability and progression through existing steroidogenic pathways, e.g. promoting cholesterol transport into the mitochondria by phosphorylation of steroidogenic acute regulatory protein (StAR) (Devoto *et al.*, 2002) and converting cholesterol to progesterone by cholesterol side chain cleavage cytochrome P450 (CYP11A) and 3 beta-hydroxysteroid dehydrogenase (HSD3B) (Ravindranath *et al.*, 1992).

(4) Subsequent transcriptional events, e.g. increased StAR expression, resulting in greater steroid production (Niswender *et al.*, 2002; Wood *et al.*, 2002).

Existing reports of basal and agonist (LH/cAMP)-sitmulated PKA activity in the primate CL are consistent with this model for LH-stimulated steroidogenesis (Benyo *et al.*, 1997). Likewise, pulses of secreted LH are entrained to increased progesterone secretion by mid-to-late luteal phase of the menstrual cycle in primates, with steroid levels at baseline in the lengthening intervals between LH pulses by mid-to-late luteal phase (Ellinwood *et al.*, 1984), which demonstrates that (1) the continued absolute requirement of primate luteal cells for LH exposure to maintain and stimulate progesterone secretion; and hence (2) the absence of any luteal cell type that constitutively produces progesterone.

Although studies continue to increase current understanding of the cellular and molecular actions of LH to stimulate steroidogenesis, the extent to which the LH-stimulated pathways that acutely promote steroidogenesis are involved in the longer term processes that control luteal structure-function during the lifespan of the CL remains limited. Evidence suggests that LH stimulates the production of additional factors that act as “local luteotropins” which are not only essential for ovulation and luteinization of the

mature follicle, but also for maintenance of the structure-function and/or lifespan of the CL during the menstrual cycle (Stouffer, 1999; 2003). One such local luteotropin is the primary steroid hormone progesterone produced by the ovulatory follicle and CL. The LH-induced early rise in progesterone synthesis appears essential for a number of hormonal as well as local actions of progesterone, ranging from controlling the strength and duration of the LH surge (Chappell *et al.*, 1997) to ovulation (Chaffin *et al.*; 2002), respectively. In addition, progesterone regulates luteal tissue remodeling via control of protease expression, luteal health via potent anti-apoptotic effects, and sensitivity of luteal tissue to other local factors, e.g. estrogen receptor expression. Elimination of endogenous LH support using a GnRH antagonist causes a complete and rapid suppression of circulating progesterone levels in primates, including monkeys and women, and early regression of the CL at all stages of the luteal phase (Reissmann *et al.*, 1995; Duffy *et al.*, 1999), which can be prevented by exogenous replacement LH (Duffy *et al.*, 1999). Mid-cycle LH surge also promotes vascular endothelial growth factor (VEGF) and angiopoietin (ANGPT1) production through either transcriptional or post-transcriptional actions in the developing CL to regulate luteal angiogenesis and function (Hazzard *et al.*, 1999; Stouffer *et al.*, 2001).

LH may also inhibit production or action of local luteolytic factors to prevent luteal regression until the late luteal phase. LH suppresses matrix metalloproteinases (MMPs), proteases implicated in luteal remodeling, and $PGF_{2\alpha}$ via progesterone in the CL (Young and Stouffer, 2004; Pate, 1988). There are also reports that LH inhibited Fas-induced apoptosis in human ovarian surface epithelial cell lines (Slot *et al.*, 2006). Fas ligand and its receptor Fas are associated with luteolysis in several species (Sakamaki *et al.*, 1997; Roughton *et al.*, 1999; Taniguchi *et al.*, 2002). LH suppresses estrogen receptor (ERβ) expression which may suppress luteal sensitivity to estrogens until the late luteal phase of the menstrual cycle (Duffy *et al.*, 2000). Estrogen was proposed to be a local luteolytic factor in primates (Knobil, 1973).

To date, research groups have used techniques, including northern blotting, RT-PCR, and differential mRNA display, to study individual genes or members of families that are regulated by LH in the ovulatory follicle or CL. There is growing evidence that

the primary luteotropic hormone LH exert its actions, at least in part, through LH-regulated synthesis and secretion of local factors, including steroid and peptide/proteins (Espey *et al.*, 2002). However, the mechanisms by which LH regulates luteinization and luteolysis remain unclear and controversial. The following dissertation research will introduce a novel regulatory system, the corticotropin releasing hormone/urocortin-receptor-binding protein system in the primate CL, that is LH-regulated and possibly functions in luteal development.

2.3 Corticotropin Releasing Hormone/Urocortin-Receptor-Binding Protein (CRH/UCN-R-BP) System

Components of the CRH/UCN-R-BP system, differentally expressed in the central nervous system (CNS) and anterior pituitary, were initially discovered to function in neurotransmission and neuroendocrine regulation of the stress response (Bittencourt *et al.*, 2000). Today, a considerable body of evidence suggests that the CRH/UCN-R-BP system is also widely distributed in a variety of peripheral tissues outside of the CNS in mammals, including in the immune, cardiovascular, digestive, and reproductive systems, skin, as well as in some types of human tumors (Grammatopoulos *et al.*, 2002). The ubiquitous distribution of CRH/UCN-R-BP system, with its ability to activate diverse signaling mechanisms in different tissues, gives this system enormous versatility and plasticity.

2.3.1 CRH/UCN-R-BP System Ligands

Current data supports the existence of four paralogous genes in mammals that encode the CRH-like peptide ligands, CRH, UCN, UCN2, and UCN3 (Dautzenberg *et al.*, 2001).

1. CRH

For more than a century, researchers and clinicians have been interested in how the body adapts to stressful stimuli. In 1981, a 41-amino acid polypeptide corticotropin-releasing factor (CRF), later called CRH, was isolated and characterized from ovine

hypothalamus and yielded a major breakthrough in understanding animals' stress response (Vale *et al.*, 1981). This peptide exists and is highly conserved in a large variety of mammalian species, including primates. Human and rat CRH are identical. CRH, generated by cleavage of the C-terminus of the 196-amino acid precursor pre-proCRH (Vale *et al.*, 1997), is the major hypothalamic releasing factor for adrenocorticotropin hormone (ACTH) and hence the primary neuroendocrine regulator of the stress response. Physical or emotional stress increases the synthesis and release of CRH from cells originating in the paraventricular nucleus of the hypothalamus. Following its release in the hypothalamic portal circulation, it is carried to the pituitary, where CRH binds to CRHR1 on corticotroph cells in the anterior lobe of pituitary to increase production and secretion of ACTH. ACTH, in turn, promotes adrenal cortical structure-function, and stimulates adrenal glucocorticoid hormone biosysthesis and secretion. Glucocorticoids mediate many metabolic changes, including glucose, protein, and fat metabolism, associated with the stress response (Sapolsky *et al.*, 2000). In the mammalian brain, CRH is also expressed in the cortex and brainstem and acts as a neurotransmitter or a neuromodulator to induce anxiety-like behaviors, decrease food intake, increase arousal, alter blood pressure, and diminish sexual behavior (Koob *et al.*, 1999; Smagin *et al.*, 2001). Thus, CRH not only controls the hypothalamo-pituitary-adrenal (HPA) axis, but also integrates autonomic and behavioral responses to stress through its actions within the CNS (Bale *et al.*, 2004).

Many neuropeptides and hormones once thought to be restricted to the brain and pituitary, respectively, are now known to be widely expressed in peripheral tissues. Shortly after CRH was isolated, CRH immunoreactivity was detected in peripheral tissues outside the brain including lung, liver, ovary, and skin (Suda *et al.*, 1984; Petrusz *et al.*, 1985; Asakura *et al.*, 1997; Vale *et al.*, 1997). CRH applied directly to isolated heart preparations resulted in increased coronary output and the rapid release of atrial natriuretic peptide, suggesting that CRH could act directly on tissues such as the heart, perhaps functioning in either a paracrine or autocrine manner (Grunt *et al.*, 1992; 1993).

2. UCNs

While CRH was receiving considerable attention as a stress-related neurohomone,

15 years passed before a second CRH-related peptide was discovered in the rat brain and named urocortin (Vaughan *et al.,* 1995). This 40-amino acid peptide, with 40-50% identity to CRH, established that mammals possess additional members of the CRH peptide family. To date, UCN has been isolated from humans, rodents, and other species (Zhao *et al.,* 1998; Cepoi *et al.,* 1999). UCN is more highly conserved than CRH across species. The interest in UCN in primates began in 1996, when Donaldson et al. (Donaldson *et al.,* 1996), using a placental genomic library, cloned the human counterpart of rat UCN. The human gene encodes a 123 amino acid precursor polypeptide. The putative 40 amino acid mature polypeptides of rat and human UCN share 95% identity. Synthetic human UCN was found to bind with high affinity to CRHR1 and act in vitro to release ACTH from rat anterior pituitary cells (Donaldson *et al.*, 1996). The expression of UCN mRNA and protein is identified in certain areas of the brain (Bittencourt *et al.*, 1999), and is also found in peripheral tissues including heart, liver, ovary, and skin (Kageyama *et al.*, 1999; Muramatsu *et al.*; Slominski *et al.*, 2000).

In addition to binding to CRHR1, UCN was hypothesized to be the endogenous ligand of CRHR2 (Vale *et al.*, 1997; Dautzenberg *et al.*, 2001). However, the limited proximity between UCN-containing neurons and those neurons expressing CRHR2 in the rat CNS suggested that additional endogenous CRHR2 ligands exist in mammals (Bittencourt *et al.*, 1999). Further search for CRH-like peptides culminated in the identification of stresscopin and stresscopin-related peptide by genomic analysis and their subsequent molecular cloning from the human and mouse tissues (Hsu and Hsueh, 2001; Reyes *et al.,* 2001; Lewis *et al.,* 2001). The accepted nomenclature for stresscopin-related peptide and stresscopin is now UCN2 and UCN3, respectively (Hauger *et al.*, 2003). UCN2 and UCN3 are exclusive CRHR2 ligands, although UCN3 has lower potency. The UCN2 and UCN3 precursors predict 38-amino acid mature peptides (Reyes *et al.*, 2001; Lewis *et al.*, 2001). While the amino acid sequences of human and mouse UCN3 are 90% identical, only 76% sequence conservation was deduced for UCN2 homologs. UCN2 shows moderate homology to CRH at 34%, UCN at 43%, and UCN3 at 37-40%. UCN3 is more distant from other members of the CRH-like peptide family at 18-32% identity. Secondary structure, rather than linear sequence, appears to determine biological activity.

UCN2 mRNA is highly expressed in brain and also detected in peripheral tissues including heart, lung, testis, and skin (Hsu and Hsueh, 2001; Chen *et al.*, 2004). Although UCN3 mRNA is considerably less abundant than UCN2 mRNA in brain, high levels of UCN3 mRNA are detected in peripheral tissues, including heart, liver, testis, and skin (Hsu and Hsueh, 2001; Lewis *et al.*, 2001).

2.3.2 CRH/UCN-R-BP System Receptors

CRH/UCN ligand actions are mediated through activation of two distinct members of G-protein-coupled receptors, CRHR1 and CRHR2, encoded by separate genes (Hillhouse *et al.*, 2002; Hauger *et al.*, 2003). The cDNAs encoding these two receptors were cloned from mammals including humans. The CRHR1 and CRHR2 receptors are remarkably similar sharing high sequence homology with about 70% amino acid identity. The lowest degree of homology exists in their hormone-binding extracellular domains, particularly in the N-termini (Dautzenberg *et al.*, 2001; Arai *et al.*, 2001). These receptors are widely expressed in the CNS and peripheral tissues, where they overlap with the tissue distribution of CRH-like peptides (Chalmers *et al.*, 1996; Potter *et al.*, 1994; Dautzenberg *et al.*, 2001a). Therefore, it is likely that signaling pathways selectively activated when endogenous ligands bind to these receptors mediate the physiological responses to CRH/UCNs in brain, pituitary and peripheral tissues. CRHRs modulate a plethora of intracellular protein kinases as well as other important signaling intermediates, including prostaglandins (PGs) and Fas Ligand (FasL), in a tissue-specific manner. It is well established that the binding of CRHR agonists to extracellular domains of the CRHR1 or CRHR2 (Fig. 2.3) transforms the membrane conformation of these receptors into an active state, thereby increasing their affinity for G protein. Both CRHR1 and CRHR2 are seven-transmembrane receptors that signal by coupling to G_s protein in most brain/ peripheral tissues and brain-derived/peripheral cell lines. Signals from CRH and CRH-like peptides transduced across cell membranes via the receptors leads to the activation of adenylate cyclase (AC) with a resultant increase in intracellular cyclic adenosine monophosphate (cAMP) and subsequent activation of protein kinase A (PKA)-dependent pathways (Hauger *et al.*, 1999; Dautzenberg *et al.*, 2000). However, it has also

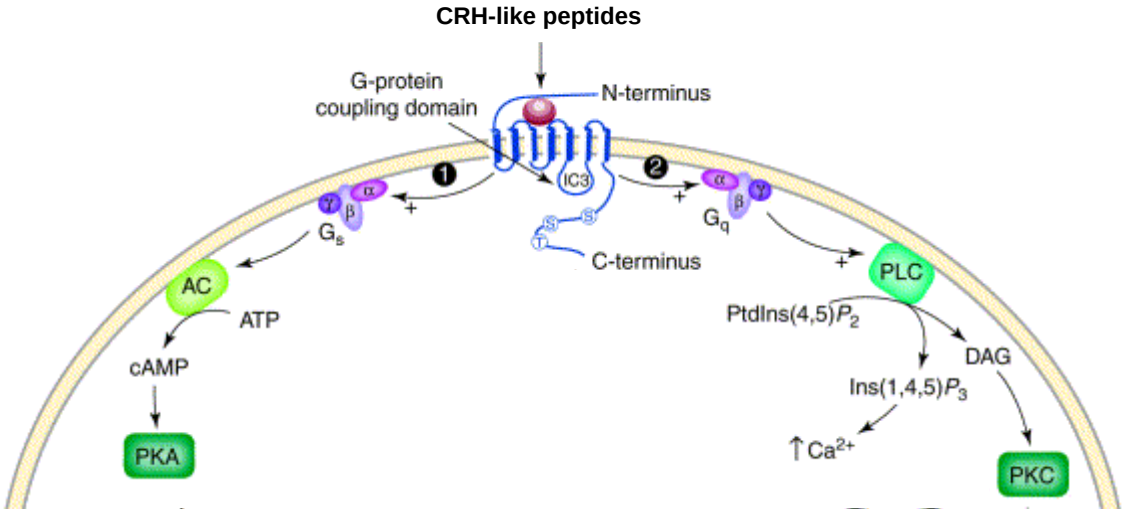

Fig. 2.3 Corticotropin releasing hormone (CRH) receptor signaling pathways (adapted from Dautzenberg *et al.*, 2002)

been reported that CRH stimulates alternative signaling cascades, such as stimulation of phosphoinositol hydrolysis in certain tissues (e.g., testis and placenta) where this ligand is unable to activate the AC pathway. Phospholipase C (PLC) is activated with the production of diacylglycerol (DAG) and inositol triphosphate (IP3), which in turn activates protein kinase C (PKC)-dependent and calcium-activated pathways, respectively (Grammatopoulos *et al.*, 2001; Dautzenberg *et al.*, 2002; Hillhouse *et al.*, 2002). Such differential outcomes of CRHR activation is attributed to activation of multiple endogenous G-proteins, e.g. G_s and G_q, a finding that predicts activation of several second messenger signals and suggests that CRH and CRH-like peptides can generate various responses in different target tissues (Dautzenberg *et al.*, 2002) (Fig. 2.3).

1. CRHR1

CRHR1 is a 415-420-amino acid polypeptide, containing seven hydrophobic helices that are predicted to span the plasma membrane (Vale *et al.*, 1997; Dautzenberg *et al.*, 2001). CRHR1 is widely expressed in mammalian brain and pituitary (Chalmers *et al.*, 1995; Sanchez *et al.*, 1999). A lower level of CRHR1 expression also occurs in several peripheral tissues including heart, lung, ovary, and skin (Hillhouse *et al.*, 2006; Tantisira *et al.*, 2004). Both CRH and UCN bind with high affinities to the mammalian CRHR1 in a nonselective manner (Perrin *et al.*, 1999; Dautzenberg *et al.*, 2001a). It is likely that CRH and UCN represent the natural agonists for CRHR1. UCN2 and UCN3 fail to bind and activate CRHR1, thus providing evidence that CRHR1 possesses ligand selectivity (Hsu and Hsueh, 2001; Reyes TM, 2001; Lewis K, 2001). CRHR1 has been implicated in mediating normal responses to stress.

The fact that thirteen mRNA spliced variants of the CRHR1, termed α, β, c, d, e, f, g, h, v_1, j, k, m, and n, were identified in mammals, the first nine in humans (Fig. 2.4), increases the complexity of the CRH/UCN-R-BP system (Pisarchik and Slominski, 2001; Johnson *et al.*, 2003; Pisarchik and Slominski, 2002; Hillhouse and Grammatopoulos, 2006). Researchers have proposed a division of all CRHR1 variants into four groups according to their potential impact on agonist signaling (Pisarchik and Slominski, 2002; Hillhouse and Grammatopoulos, 2006):

(1) variants without a frame-shift (e.g., CRHR1β, c, d, g, and n) but with a variable

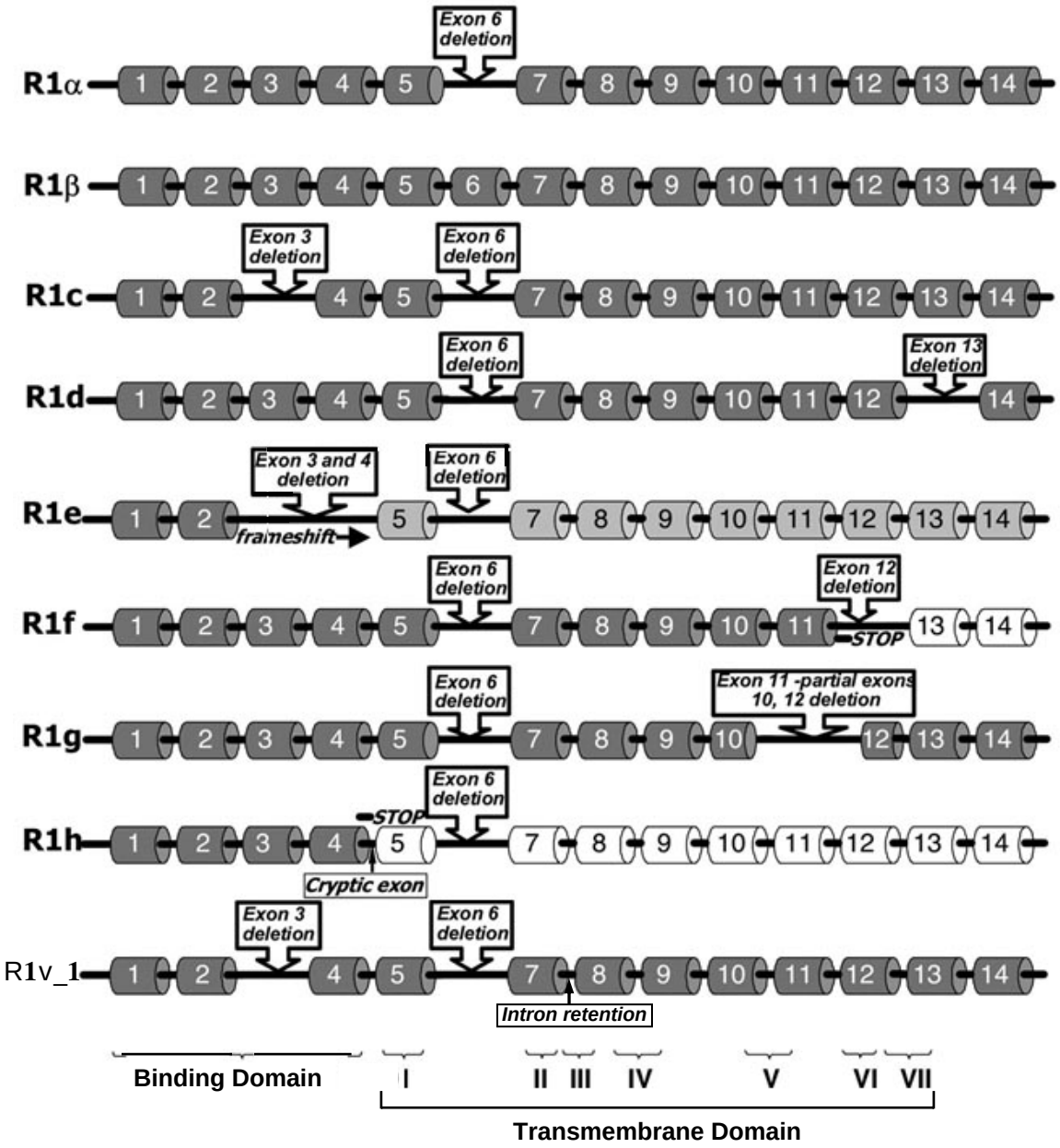

Fig. 2.4 Schematic diagram of alternatively spliced isoforms of human CRHR1 (adapted from Hillhouse and Grammatopoulos, 2006).

number of transmembrane domains (TMDs) and intra- or extracellular- C terminus that differ in their agonist binding and G protein-coupling characteristics.

(2) variants missing exons 3 and 4, which are important for agonist binding and therefore appear functionally inert (e.g., CRHR1e). Although it is difficult to attribute any direct role to individual members of this group, they might have important functional significance by modulating CRHR1α activity.

(3) variants that conserve the original reading frame of CRHR1 until the fifth exon, thus retaining an intact CRH-binding domain, but have no TMDs (e.g., CRHR1j and h). CRHR1j has exon 5 deleted, whereas CRHR1h has an insertion of a cryptic exon between exons 4 and 5. If translated, these isoforms could potentially serve as soluble binding proteins or modulate agonist binding.

(4) variants with an intact CRH-binding domain and a variable number of TMDs, e.g. CRHR1f, k, and m. Each of these transcripts contains a frame-shift that potentially alters their C terminus, raising questions about their potential for participation in signal transduction.

The physiological relevance of the CRHR1 variants has been dismissed as being a result of aberrant post-transcriptional splicing. Characterization of the biological activity and signaling properties of some of these variant proteins is based on heterologous overexpression systems using recombinant protein expression and not native cells (Hillhouse and Grammatopoulos, 2006). This is due to a lack of suitable methods to demonstrate endogenous CRHR1 variant expression and to study their physiological roles in mammalian tissues. CRHR1α appears to be the main functional variant which primarily mediates CRH actions and is widely expressed throughout the body. CRHR1β can be regarded as a “pro-CRHR1” with unknown physiological function. CRHR1c has a decreased CRH binding capacity because of missing binding regions from the N terminus (Hillhouse and Grammatopoulos, 2006). The CRHR1 alternative splicing appears to be species, tissue, and physiological process specific, and affected by environmental stimuli. For example, CRHR1β was expressed in humans but not rodents (Tsai-Morris *et al.*, 1996); CRHR1d expression was only detected in human myometrium during pregnancy (Grammatopoulos, 1999); CRHR1g was only expressed in skin cell lines after UV

irradiation (Pisarchik and Slominski, 2001). The aberrant variants might act as "decoy receptors" capable of competing with the full-length receptors for regulating CRH and CRH-like peptide bioactivity and therefore, change the efficiency of hormonal stimulation. For example, in coexpression experiments, CRHR1e attenuated, whereas CRHR1h amplified, CRHR1α signaling (Pisarchik and Slominski, 2004). However, our current knowledge of the mechanisms regulating CRHR1 splicing and the specific function of distinct CRHR1 variants is limited.

2. CRHR2

CRHR2, a 397-438-amino acid protein, has three functional splice variants, α, β, and γ. Although their N-terminal sequences and tissue distribution differ, these splice variants show no major pharmacological differences (Vale *et al.*, 1997; Dautzenberg *et al.*, 2001). CRHR2 shows a clear preference in the affinity for UCNs and is involved in maintaining and restoring homeostasis following a stress response. Because CRHR2 binds UCN with considerably higher affinity, >100-fold, than it binds CRH, UCN was hypothesized to be the endogenous ligand of this receptor (Vaughan *et al.*, 1995; Vale *et al.*, 1997; Dautzenberg *et al.*, 2001a). UCN2 and UCN3 were observed to bind exclusively to the CRHR2, although the K_d of UCN3 for CRHR2 is more than 10-fold lower than K_d values for UCN and UCN2 (Hsu and Hsueh, 2001; Lewis *et al.*, 2001). Thus, the UCNs are likely to be the natural ligands for CRHR2. CRHR2 is expressed in a discrete pattern in the brain, but widely expressed with high levels in several peripheral tissues, particularly in heart, liver, skin, and ovary (Hillhouse *et al.*, 2006; Yokohama *et al.*, 2001; Muramatsu *et al.*, 2001).

CRHR2α is the dominant CRHR2 splice variant expressed in the brain and peripheral tissue of primates. However, the CRHR2β is the major splice variant peripherally expressed in rodents (Sanchez *et al.*, 1999; Lovenberg *et al.*, 1995; Palchaudhuri *et al.*, 1999; Dautzenberg *et al.*, 2001). CRHR2γ has so far been found only in the limbic regions of the human CNS (Kostich *et al.*, 1998). CRHR1 appears to be more crucial in regulating brain and pituitary functions whereas the CRHR2 might be more important in the periphery. Interestingly, the rat pituitary and neural cortex express CRHR1 exclusively, whereas in primates these tissues express substantial levels of both

CRHR1 and CRHR2 (Sanchez *et al.*, 1999). Thus, CRHR2 expression appears species specific and may possess additional functions in primates.

2.3.3 CRHBP

The interactions of CRH/UCN ligands with their receptors in target tissues may be modulated by the binding protein, CRHBP (Seacholtz *et al.*, 2001; 2002). CRHBP was originally isolated and characterized from human liver and rat brain (Potter E, 1991). While highest expression is observed in brain and pituitary (Cortright *et al.*, 1995; Potter *et al.*, 1991), CRHBP is also expressed in peripheral tissues including liver and placenta in primates (Behan *et al.*, 1995; Kemp *et al.*, 1998). CRHBP is a 37kDa secreted glycoprotein of 322 amino acids that is structurally unrelated to the CRHRs. It forms a dimer complex with CRH, 2 CRH:2 BP, that neutralizes circulating or local ligand action. It inhibits CRH-induced ACTH secretion from pituitary corticotropes and may exert similar actions at central sites of CRH release (Potter *et al.*, 1991). CRHBP is smaller than the CRHRs, but binds both CRH and UCN with an affinity equal to or greater than that of the receptors, and blocks both CRH- and UCN-stimulated ACTH secretion in vitro (Donaldson *et al.*, 1996). CRHBP might also modulate the stress response by limiting CRHR activation (Kemp *et al.*, 1998). While the function of CRHBP is thought to be primarily inhibitory, recent studies indicate that CRHBP may exhibit diverse extra- and intracellular roles in a cell-specific fashion and at specific times in development. For example, administration of a synthetic CRHBP-specific ligand, which does not activate CRHRs, specifically increases Fos expression in CRHBP-expressing neurons in rat (Chan *et al.*, 2000). This suggests an active role for the binding protein in signaling by CRH-related peptides. CRHBP was identified not only in mammals, but also in non-mammalian vertebrates, suggesting that it is a phylogenetically ancient protein with extensive structural and functional conservation (Seasholtz *et al.*, 2002).

Recently, a soluble splice variant of CRHR2α was identified in mouse brain. It binds CRH and UCN with nanomolar affinities, whereas the affinities for UCN2 and UCN3 are much lower. It was proposed that this binding protein may be a possible biological modulator, analogous to CRHBP, of CRH family ligands (Chen *et al.*, 2004a).

2.3.4 CRH/UCN-R-BP System Function in Peripheral Tissues

In addition to effects in the CNS, CRH produced in peripheral tissues under stress conditions is involved in many biological functions through CRHR1-mediated signaling, such as energy balance, metabolism, and regulation of the immune response (Richard *et al.*, 2000; Baigent, 2001) (Fig. 2.5). UCNs appear to signal through CRHR2 and modulate an array of physiological processes, such as ingestive behavior, inflammation, steroidogenesis, and vascularization, which mediates stresscoping responses during the recovery phase of stress. However, studies on their mechanisms of action indicate that UCNs display diverse functions, and have various effects and targets that may be divergent from CRH actions (Hillhouse *et al.*, 2002) (Fig. 2.5). CRH/UCN ligands are known to be vasoactive (Chen *et al.*, 2005; Lederis *et al.*, 1982). These peptides have also been found to stimulate proliferation (Emanuel *et al.*, 2000; Ikeda *et al.*, 2002) and to protect diverse cell types from environmental insults, a so-called cytoprotective function in mammals (Brar *et al.*, 2000; Fox *et al.*, 1993; Pedersen *et al.*, 2002). Thus, CRH and CRH-like peptides, their receptors and binding protein form an important physiological system, influencing a wide spectrum of nervous, endocrine, vascular, cardiovascular, skeletomuscular, behavioral, metabolic, autonomic, immune and reproductive mechanisms that allow mammals to coordinate the adaptive behavioral and physical changes that occur during acute and chronic stress and recovery conditions (Vale *et al.*, 1997; Dautzenberg *et al.*, 2001).

2.4 Corticotropin Releasing Hormone/Urocortin-Receptor-Binding Protein (CRH/UCN-R-BP) System in the Ovary

2.4.1 CRH and CRHR1 Expression in the Ovary

The hypothalamic neuropeptide CRH, as well as its receptor and binding protein, have been identified in several female reproductive tissues, including the placenta, uterus, and ovary in primates (Saeed *et al.*, 1997; DiBlasio *et al.*, 1997; Asakura *et al.*, 1997). It was proposed that “reproductive” CRH, as a local paracrine factor in peripheral tissues, participates in various reproductive functions. Placental CRH may participate in the

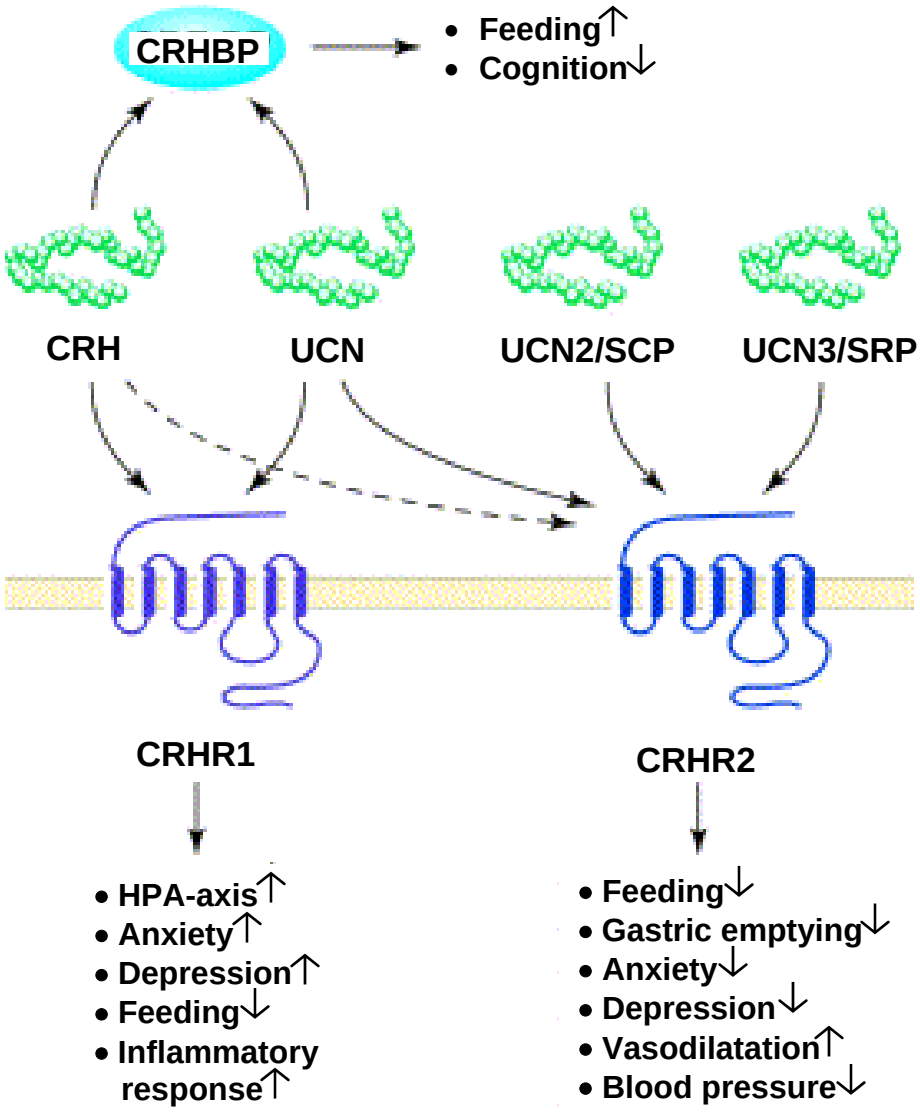

Fig. 2.5 Interactions between mammalian corticotropin releasing hormone (CRH) and CRH-like peptides, their receptors (CRHRs) and the binding protein (CRHBP) (adapted from Dautzenberg *et al.*, 2001).

physiology of pregnancy, fetal maturation, and the onset of parturition (McLean *et al.*, 2001); uterine CRH may play a role in decidualization, blastocyst implantation, and early maternal tolerance (Makrigiannalis *et al.*, 2003); and ovarian CRH may help regulate follicular or luteal activity (Mastorakos *et al.*, 1994), including sex steroid hormone production (Calogero *et al.*, 1996; Roguski *et al.*, 2000).

There are very limited data regarding the presence of CRH and its receptor, CRHR1, in ovaries. CRH was detected in the cytoplasm of theca cells and follicular fluid within antral follicles, stroma cells, and luteal cells in the developing CL in rodents and human. CRHR1 was also detected in the stroma and theca of the developing antral follicle (Mastorakos *et al.*, 1993; Mastorakos *et al.*, 1994). In normal human ovaries, there was no detectable CRH in primordial follicles, but abundant expression of the CRH and CRHR1 in growing antral follicles, suggesting that CRH plays a local role(s) in follicular maturation (Mastorakos *et al.*, 1994; Asakura *et al.*, 1997). In women, the concentration of CRH is higher in pre-menopausal than post-menopausal ovaries, suggesting that ovarian CRH is related to normal ovarian function during the reproductive life span (Zoumakis *et al.*, 2001). Nevertheless, no studies have yet been performed on CRH or CRHR1 in the mature preovulatory follicle around the midcycle LH surge and there is limited evidence for any biological action or function of CRH in the ovary, especially the CL.

2.4.2 UCNs and CRHR2 Expression in the Ovary

Data on the localization and possible roles of CRH triggered recent studies on the expression of UCNs and their function in reproductive tissues. The placenta, uterus, and ovary are also sources of UCN and they express the UCN receptor, CRHR2, thus suggesting that these tissues are also targets of UCN (Fadalti *et al.*, 2000; Grammatopoulos *et al.*, 2000; Muramatsu *et al.*, 2001). UCN may affect the physiology of reproduction, including pregnancy and parturition, through paracrine/autocrine/ intracrine actions. In particular, in vitro data suggest that UCN plays a major role in the human placenta: the addition of UCN significantly increased ACTH secretion by cultured human trophoblast cells and PG release in explants of human placental tissue at term

(Petraglia *et al.*, 1999), and also stimulated activin A secretion by cultured human placental cells (Reis *et al.*, 2002). Furthermore, when incubated with myometrial strips, UCN stimulated uterine contractility, by activating specific intracellular pathways (Florio *et al.*, 2004). UCN2 and UCN3 were recently identified in human placenta. They were proposed as regulators of placental vascular endothelial tone without stimulating ACTH secretion (Imperatore *et al.*, 2006). UCN2 effects on human pregnant myometrial cells suggest that it acts to control uterine contractility during human pregnancy (Karteris *et al.*, 2004).

There are few reported studies on UCN expression or action in the ovary. Using immunohistochemistry techniques and human ovaries, weak UCN staining was detected in both granulosa and theca interna cells of dominant antral follicles, as well as in theca interna cells of non-dominant and atretic follicles during both the follicular and luteal phase. After ovulation, immunoreactive UCN was detected in granulosa- and theca-lutein cells in the CL during the luteal phase. Immunolocalization patterns for CRHR2 in dominant, non-dominant, and atretic follicles, and in the CL, were similar to those for UCN (Muramatsu *et al.*, 2001). These data suggest that UCN is locally synthesized in steroidogenic luteal cells, and acts via CRHR2 as a paracrime/autocrine/intracrine regulator of ovarian steroidogenesis (refer to Chapter 6). To date, there is only one publication reporting UCN2 and UCN3 mRNA expression in the human ovary (Hsu and Hsueh, 2001) and UCN2 mRNA expression in the rat ovary (Yamauchi *et al.*, 2005), without protein data and functional analysis. Thus, using the same (CRHR1) or different (CRHR2) signaling machinery as CRH, UCNs may act in the reproductive tissues to counterbalance, mimic or synergize CRH effects. The high binding affinity for CRHR1 suggests that UCN may be involved in activation of CRHR1 pathway. For example, UCN mediated by CRHR1 was 10-fold more potent than CRH in protecting rat hippocampal neurons from insult in vitro (Pedersen *et al.*, 2002). However, UCN2 and UCN3, as well as UCN, may act via the CRHR2 pathway, resulting in heretofore unknown ovarian actions.

2.4.3 CRHBP Expression in the Ovary

CRHBP protein, but not mRNA, was detected in theca cells and lumen of capillary vessels of the thecal-stromal compartment of mature follicles in human ovary (Asakura *et al.*, 1997). It was proposed that the CRHBP originated in the liver and was sequestered in the theca layer from the blood circulation. Regardless of source, the presence of CRHBP could modulate the functionality of the ovarian CRH/UCN-R system by virtue of its ability to compete with ligands for their receptors.

Studies on the expression, regulation, and physiological role of CRH/UCN-R-BP system in the primate ovary are only beginning. The molecular, cellular, and tissue actions of the ligands and their receptors, as well as their interactions with other hormonal systems regulating ovarian function, await evaluation.

CHAPTER 3
DISCOVERY OF LUTEINIZING HORMONE (LH)-REGULATED GENES IN THE PRIMATE CORPUS LUTEUM *

3.1 Abstract

Circulating luteinizing hormone (LH) is essential for the development and function of the primate corpus luteum during the menstrual cycle. However, the cellular and molecular processes whereby LH controls luteal structure and function are poorly understood. Therefore, studies were initiated to identify gene products that are regulated by gonadotropin in the monkey corpus luteum. Rhesus monkeys were either untreated (controls, CTRL; n = 3) or received the GnRH antagonist Antide (ANT; 3 mg/kg body weight, n = 3) to inhibit pituitary LH secretion at mid luteal phase (day 6 post-LH surge) in spontaneous menstrual cycles. The corpus luteum was removed 24 h later. RNA was extracted and converted to cDNA. The CTRL and ANT cDNAs were differentially labeled with fluorescent dyes (Cy3-CTRL and Cy5-ANT) and hybridized to spotted microarrays containing 11,600 human cDNAs. Selected cDNAs were analyzed further via semi-quantitative RT-PCR (a) to validate the microarray results, and (b) to determine if their expression varies in the corpus luteum (n = 3/stage) between mid (day 6-8), late (day 14-16), or very late (day 18-19, menses) luteal phase of the natural cycle. After normalization of fluorescence data, 206 cDNAs (1.8% of total) exhibited ≥2-fold change in expression after ANT. Of the 25 cDNAs exhibiting a ≥6-fold change, 6 were up-regulated and 19 were down-regulated. Twenty-two of these 25 cDNAs were validated

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by RT-PCR as differentially expressed in the ANT group relative to the CTRL group, and 11 of 25 changed ($P < 0.05$) correspondingly in the late-to-very late luteal phase. Thus, we have identified gene products that are regulated by gonadotropin in the primate corpus luteum that may be important in luteal regression during the menstrual cycle.

3.2 Introduction

The development and maintenance of the functional corpus luteum (CL) in primates during the menstrual cycle requires the actions of the pituitary-derived gonadotropin, luteinizing hormone (LH) (Zeleznik and Benyo, 1994; Niswender *et al.*, 2000; Stouffer, 2003). Several lines of investigation indicate a critical role for LH in supporting the proper structure and function of the primate CL. The inhibition of LH synthesis and secretion (by lesioning the arcuate nucleus in the hypothalamus or administering a GnRH antagonist) (Hutchison and Zeleznik, 1984; Collins *et al.*, 1986; Dubourdieu *et al.*, 1991; Duffy *et al.*, 1999) or action (by administering a neutralizing LH antibody) (Asch *et al.*, 1981; Groff *et al.*, 1984) reduces progesterone production to a level that results in premature luteal regression as evidenced by menstruation. Replacement of LH in such protocols (Hutchison and Zeleznik, 1984), notably with an escalating dose-regimen (Duffy *et al.*, 1999), leads to maintenance of luteal function as determined by continued progesterone production, as well as normal luteal phase length. From these studies, it is clear that LH is an essential "luteotropin" required for the primate CL during the menstrual cycle. Nevertheless, the intracellular processes and mechanisms whereby LH acts to develop and maintain primate CL structure and function are not well understood.

Previous investigations identified individual, LH-regulated genes in the primate CL of the menstrual cycle that are involved in steroid synthesis or action, such as steroidogenic enzymes (Ravindranath *et al.*, 1992), steroidogenic acute regulatory protein (StAR) (Devoto *et al.*, 2004) and estrogen receptor (ERβ) (Duffy *et al.*, 2000), or tissue remodeling, e.g., members of the matrix metalloproteinase family (MMPs) (Young and Stouffer, 2004) and angiogenic factors (Ravindranath *et al.*, 1992a). Differential mRNA display was also used to identify novel genes that are regulated by LH in the ovulatory,

luteinizing follicle in the rat (Espey LL and Richards, 2002) and the CL of the nonhuman primate (Yadav *et al.*, 2004). However, relatively little information exists regarding the specific genes that are under the regulatory control of gonadotropin in the primate CL.

The present study was initiated, therefore, to systematically investigate those genes that are acutely dependent on gonadotropin for expression in the monkey CL following its development in the natural menstrual cycle (Xu *et al.*, 2003a). Using spotted (human cDNA) microarrays, the mRNAs for 11,600 genes were compared in CL from control and GnRH antagonist-treated monkeys. Twenty-five cDNAs exhibiting a ≥6-fold change between groups were further analyzed by semi-quantitative RT-PCR. In addition, since there is evidence for less LH support (fewer LH pulses in the circulation) (Ellinwood *et al.*, 1984) and reduced CL sensitivity to LH (Duffy *et al.*, 1999) in the late luteal phase of the menstrual cycle, we tested the hypothesis that LH-regulated genes will display a similar change of expression during spontaneous luteal regression as during GnRH antagonist-induced luteolysis.

3.3 Materials and Methods

3.3.1 Animal Treatment and Hormone Assays

Adult, female rhesus monkeys (*Macaca mulatta*) were individually caged at 24°C with a daily light cycle of 0700 to 1900 in the Division of Animal Resources of the Oregon National Primate Research Center (ONPRC, Beaverton, OR). Animals received a diet of Purina monkey chow (Ralston Purina Company, St. Louis, MO) and water ad libitum, supplemented with fresh fruit and Iron Dextran Complex (100 mg/2 weeks, im) (Chemical Sources International, Inc., Clemson, SC). Animals were checked daily for menses and records were kept of each monkey's menstrual pattern. Monkeys exhibiting menstrual cycles of approximately 28 days were selected for these studies (Molskness *et al.*, 1987). Beginning six days after onset of menses (day one of the menstrual cycle), daily blood samples were collected by saphenous venipuncture during the follicular and luteal phase up to the day of luteectomy. Serum was separated and assayed for estradiol and progesterone concentrations by a specific electrochemoluminescent assay using a

Roche Elecsys 2010 analyzer (Roche Diagnostics Corporation, Indianapolis, IN) in the Endocrine Services Laboratory, ONPRC (Young *et al.*, 2003). Hormone concentration values were validated against previous radioimmunoassays in this laboratory (Hess *et al*., 1981; Zelinski-Wooten *et al*., 1995). The first day of low (<100 pg/ml) serum estradiol following the midcycle estradiol peak typically corresponds with the day after the LH surge, and is therefore termed day 1 of the luteal phase (Duffy *et al.*, 1999). All protocols were approved by the ONPRC Animal Care and Use Committee, and conducted in accordance with NIH Guidelines for the Care and Use of Laboratory Animals.

To block pituitary LH release, the GnRH antagonist Antide (ANT) (Leal *et al.*, 1988) was administered by sc injection in a vehicle of 50% propylene glycol and 50% water. ANT was synthesized at the Salk Institute for Biological Studies (San Diego, CA) and made available by the Contraceptive Development Branch, Center for Population Research, NICHD (Rockville, MD). Monkeys (n = 3) were injected with ANT (3 mg/kg body weight) at 0800 hours on day 6 of the luteal phase. CL were isolated and dissected 24 h later (day 7 of the luteal phase) from anesthetized monkeys during an aseptic ventral midline laparotomy (Duffy *et al.*, 2000). Control (CTRL) animals (n = 3) received no ANT injection prior to CL removal on day 7. The samples were immediately flash frozen in liquid nitrogen and stored at -80°C until processed.

3.3.2 RNA Extraction and Spotted cDNA Microarray Technique

RNA was extracted from each CL using TRIzol (Invitrogen Corporation, Carlsbad, CA) according to standard protocols. RNA was further purified using a RNeasy column (QIAGEN Inc., Valencia, CA) according to the manufacturer's directions. RNA (10 μg pool, formed from the 3 CL comprising either the CTRL or ANT group) was converted to cDNA with the appropriately labeled nucleotide mixture (biotin-11-dCTP for the CTRL cDNA and fluorescein-12-dCTP for the ANT cDNA) (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA). The CTRL and ANT cDNA was then hybridized to two sets of DNA array slides (Human Spotted Array Set A and Set B) at the Spotted Microarray Core of the OHSU Gene Microarray Shared Resource, each corresponding to 5,800 independent human Integrated Molecular Analysis of Genomes and their Expression

(IMAGE) clones (The American Type Culture Collection, ATCC, Manassas, VA) spotted in duplicate. A total of 11,600 human IMAGE clones were available for analysis.

The slides were first incubated with a streptavidin-horseradish peroxidase (HRP) conjugate, followed by tyramide-Cy5. The HRP catalyses the tyramide reaction and deposits the fluorescent dye Cy5 to adjacent probe, generating a fluorescent signal corresponding to the mRNA expression level within the CTRL CL. The slides were then washed and an inactivater of HRP (HRP Inactivation Reagent) was applied to destroy the streptavidin-HRP activity. The slides were then incubated with an anti-fluorescein antibody that is also covalently conjugated to HRP, washed, and incubated with tyramide-Cy3. The fluorescent signal generated from the deposition of the Cy3 was subsequently determined. The fluorescent signal generated from Cy3 corresponds to the mRNA expression level in the CL of ANT-treated animals. Cy3 and Cy5 fluorescence intensity was determined using a ScanArray 4000 XL scanner. Each slide was scanned at two laser intensities to obtain the greatest possible dynamic spread. Images were stored as 16-bit TIFF files and analyzed using ImaGene software (BioDiscovery, Inc., El Segundo, CA). The chemicals and scanner for microarray analysis were from PerkinElmer Life and Analytical Sciences, Inc. (Boston, MA).

A generic grid was generated for each batch of slides using a Syto61 dye (Molecular Probes, Inc. Eugene, OR) stained array. The generic grid was overlaid on the experimental microarray image and the location and size of the circles were adjusted by ImaGene to accommodate slide-to-slide variation. Signals derived from contaminants were removed and the intensity of each spot was calculated as the mean intensity of the pixels in the image. The background intensity surrounding each spot was averaged and subtracted from the mean fluorescent value. The data were normalized according to the method of Yang et al. (Yang *et al.*, 2002): (1) intensity-dependent normalization used the robust scatter plot smoother lowess, implemented in the statistical software package R (Ihaka R and Gentleman R, 1996), to perform a local *A*-dependent normalization

$$\log_2 R/G \rightarrow \log_2 R/G - c(A) = \log_2 R/[k(A)G] \ .$$

R = red for Cy5 and G = green for Cy3 fluorescence intensity pairs for each gene on each array, $A = \log_2\sqrt{(R \times G)}$, and $c(A)$ is the lowess fit to the MA-plot as described by Dudoit *et al*. (Dudoit S, Yang YH, 2002). The lowess scatter plot smoother performs robust locally linear fits; (2) within-print tip group normalization is simply a (print tip + A)-dependent normalization, i.e.

$$\log_2 R/G \rightarrow \log_2 R/G - c_i(A) = \log_2 R/[k_i(A)G]$$

where $c_i(A)$ is the lowess fit to the MA-plot for the ith grid only, i.e. for the ith print tip group, $i = 1, ..., I$, and I denotes the number of print tips.

3.3.3 Clone Sequence Validation

From the microarray database, genes displaying a ≥6-fold difference in mRNA levels between CTRL and ANT groups were chosen for further analysis. To confirm the sequence of the corresponding spotted cDNAs, individual IMAGE clones were ordered from Invitrogen Corporation (Carlsbad, CA) and streaked onto LB/agar plates containing ampicillin (50 μg/ml). Individual clones were isolated and grown overnight in 2 ml LB broth containing ampicillin (50 μg/ml). Plasmids were isolated using the Wizard Mini-Prep Kit (Promega Biosciences, Inc., San Luis Obispo, CA), quantitated by UV spectroscopy, and sequenced in the SCCPRR's (U54) Molecular and Cell Biology Core at the ONPRC using an ABI 3100 automated sequencer (Applied Biosystems, Foster City, CA). Sequence data derived from the plasmid inserts were compared against the corresponding IMAGE cDNAs spotted on the array using Vector NTI Suite software (version 7.0, Invitrogen Corporation, Carlsbad, CA). In 24 out 25 cases, the validation result matched the microarray report, except for the cDNA spots corresponding to the gene "tumor suppressor deleted in oral cancer-related 1" (*DOC-1R*), which was listed as "*S-100L*" on the cDNA array.

3.3.4 Semi-quantitative RT-PCR Analysis

Reverse transcription (RT) was carried out on 1 μg DNase-treated RNA using

Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen Corporation, Carlsbad, CA) for 2 h at 37°C according to the supplier's protocol. For the validation of differential mRNA expression, the sequence obtained from the individual IMAGE clone was employed to generate primers using Vector NTI software (Table 3.1). Primers were used in a polymerase chain reaction (PCR) to analyze cDNA generated from CTRL and ANT RNA. To serve as an internal control, a parallel PCR was performed using primers specific for the macaque cyclophilin A gene (forward primer: 5'-GCTGGACCCAACACAAATG-3'; reverse primer: 5'-TCTTCTTGCTGGTCTTGCC-3'). The PCR was terminated at different cycle numbers (every 3 cycles) to ensure that product generation was in the linear phase of amplification. The parameters for the PCR were as follows: Initial Denaturation 94°C/1.5 min, Denaturation 94°C/30 s, Annealing 56-66°C/45 s, and Extension 72°C/1 min. The Advantage 2 polymerase, reaction buffer, and nucleotides were purchased from Clontech, BD Biosciences (Palo Alto, CA). Densitometry analysis was performed using a gel documentation system and Quantity One software (Bio-Rad Laboratories, Philadelphia, PA). The resultant PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, CA) and sequenced by the ONPRC Molecular and Cell Biology Core to verify their identity.

3.3.5 Gene Expression Analysis Throughout the Menstrual Cycle

To analyze gene expression throughout the luteal phase of the natural cycle, RNA isolated from CL collected during the early (day 3-5 post LH surge), mid (day 6-8), mid-late (day 10-12), late (day 14-16) and very late (day 18-19; menses) luteal phase was converted to cDNA (Young *et al.*, 2002). PCR was conducted on samples from individual CL (n = 3/stage) as described above, with cyclophilin A again serving as the internal control. Cycle number was chosen from the linear portion of the amplification curve for all genes analyzed. After densitometry, the data were standardized to the internal control.

3.3.6 Statistical Analysis

Statistical evaluation of mean differences among experimental groups was performed by one-way ANOVA with significance level set at 0.05 using the SigmaStat

Table 3.1 Forward and reverse primers used for PCR amplification of selected micorarray genes

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Aminolevulinate, delta-, synthase 1	GAGGAGCGTTTCGTTTGGA	CACTCTTTCTGGTCTTTGC
Aminolevulinate, delta-, synthase 2	CCGTAACAGTGGAGCAGCC	GCAGGGGATGACAGGAAGG
3-Beta-hydroxysteroid dehydrogenase/isomerase 1	CTTGTGCCTTACGACCCAT	TTCTGCTTGGCTTCCTCCC
Complement component 4A	ACCCCTGTCCAGTGTTAGAC	TCTGCTTTGCCCTCGTGAA
Corticotropin releasing hormone binding protein	CCAGCATGTCGCCCAACTT	CCTATTCCCTCGCAACCTG
Cysteine dioxygenase, type I	GGTGGGTCTCTTGCTGTCT	GGTTCCGTATGGCTGATGT
Glutathione S-transferase A2	AAAAATCGCTACTTCCCTGC	GTTCTTGACCTCTATGGCTGG
Helix-loop-helix protein	CCATCTGCTACATCTCCTATCT	TCTGTGCCTTCCATCTCTA
Hemoglobin gamma G	GAGGAGGACAAGGCTACTATC	GAGATCATCCAGGTGCTTTA
Hemoglobin zeta	GGACAAGTTCCTATCGGTCGTAT	CTTCGTTCATTGGTTTATTGGC
Inhibin, beta A	TCAAGGACATCGGCTGGAA	CAAGGGGGGAAAGGACAAT
Lectin, galactoside-binding, soluble, 7	ATGTAAACCTGCTGTGCGGG	CCTTGAAGCCGTCGTCTGA
LIM domain only 7	GGTGTTCTGCTGTGTGATTTG	TTCTCTCTTATGGTGCCTTGG
Metallothionein 1A	CACTGGCTCCTGCAAATG	TGGGTCAGGGTTGTATGG
Metallothionein 1B	TGTACCTCCTGCAAGAAGT	TCTCCCAACATCAGGCACA
Metallothionein 1F	GTCCACCACGCCTTCCACC	GCACTTCTCTGACGCCCCT
Metallothionein 1L	AACTCCTGCTTCTCCTTGC	CCTGGGCACACTTGGCACA
Nucleolin	CCGTCGCTTGGCTTCTTCT	GCTGCTTTCATCGCTGCTG
Open reading frame 1	ACAACAGGCAGCAGCGTCA	GCCAAAGTCTCCCACAAGG
Phosphatidic acid selective phospholipase A1	CGCACAAACCATCAACTCC	TCAGCATAATAGCCCAGAAGG
Phosphoinositide-3-kinase	CCCCGCCTCTTCTTATCAA	TCCCGTCTGCTGTATCTCG
Polyposis locus protein 1-like 1	GCCTGTATCTGCTGTTCGG	TGCTTCACTTGTCCTTCGG
Sorbitol dehydrogenase	GGCTCTGAGATGACCACCGTA	GGAACCAGGAAACCCAAGG
Sterol-C4-methyl oxidase-like	TGGAATCGTGCTTTTGTGTG	GCTACTTGGGAGGCTGAGGC
Tumor suppressor deleted in oral cancer-related 1	CACCTACACGGACCTGCTG	ATGGGGACCTCGCTGCTAA

software package (SPSS Inc., Chicago, IL). To identify significant differences between groups, the Student-Newman-Keuls post-hoc test was used for pairwise multiple comparisons. A Student's t-test was used to compare parameters between CTRL and ANT-treated animals.

3.4 Results

3.4.1 Effect of ANT Treatment on Serum Progesterone Levels

Prior to treatment, serum progesterone concentrations (Fig. 3.1) increased in both CTRL and ANT-treated monkeys during the early luteal phase (day 1-4 post LH surge). ANT administration on day 6 of the luteal phase significantly ($P < 0.05$) decreased progesterone levels within 24 h, whereas levels remained unchanged in CTRL animals. On the day of lutectomy (day 7), progesterone levels (1.3 ± 0.8 vs. 3.0 ± 0.04 ng/ml) were significantly ($P < 0.05$) lower in ANT versus CTRL animals.

3.4.2 Microarray Identification of mRNA Levels in the CL of ANT-treated Monkeys Relative to the CTRL

A spotted array containing 11,600 individual human genes was used in this study as the longer PCR-generated DNA probes would likely cross-hybridize more effectively with rhesus macaque cDNA than shorter oligo DNA probes (i.e. Affymetrix^{TM} arrays). In preliminary validation studies (not shown), RNA was extracted from rhesus fetal fibroblasts, converted to Cy5 and Cy3 labeled cDNA, and hybridized to the spotted arrays to determine the level of cross-species hybridization. When maximal background levels were subtracted, approximately 60% of the rhesus fetal fibroblasts cDNAs exhibited specific hybridization to the spotted human cDNAs (data not shown). This level of hybridization is comparable to our previous experience with same-species (human) hybridization of tissues.

A total of 206 mRNAs were detected with ≥2-fold difference in expression between CL of ANT-treated and CTRL animals (Table 3.2). Using a cut-off of either a 4-fold increase or decrease in mRNA levels, 51 genes were identified that were differentially

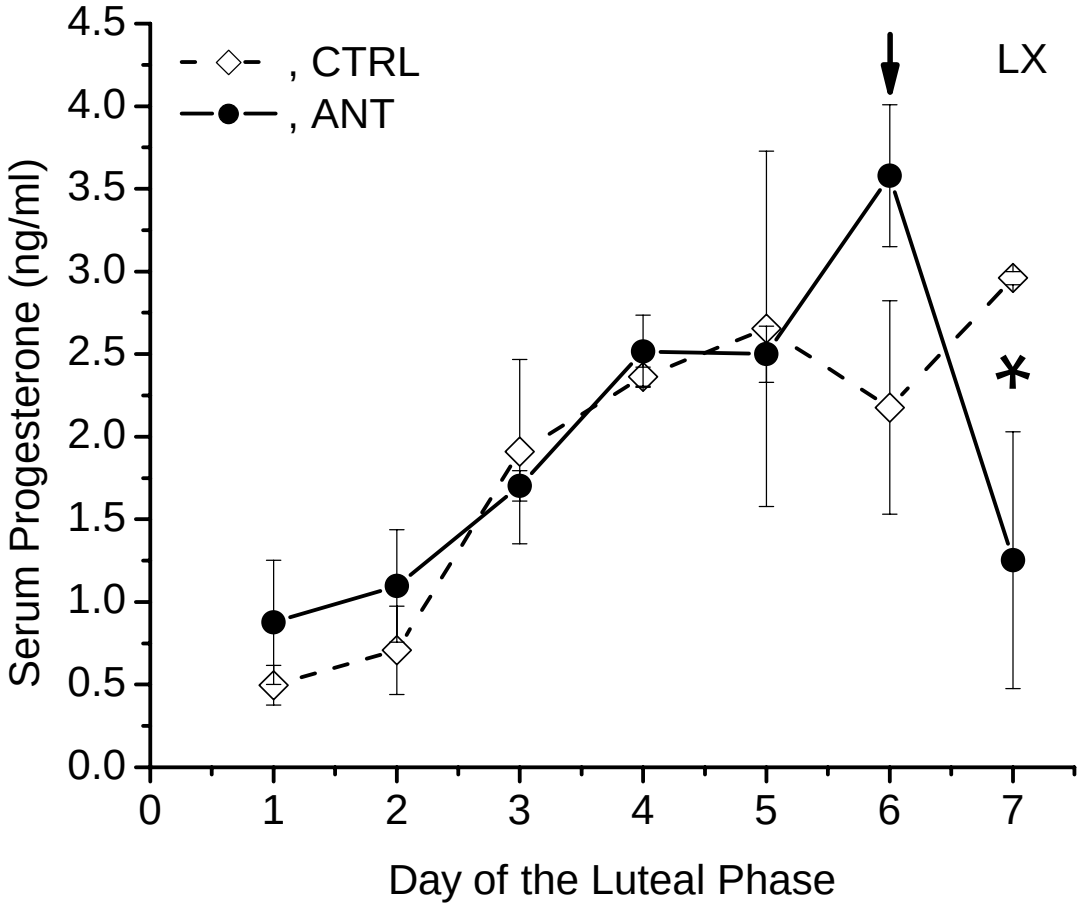

Fig. 3.1 Serum progesterone concentrations (mean ± SEM) during the luteal phase in rhesus monkeys (n = 3/group) prior to (day 1-6) and 24 h (day 7) after ANT treatment (arrow), compared to CTRL. Progesterone levels at lutectomy (LX, day 7) were significantly different (*; $P < 0.05$) between groups.

Table 3.2 Summary of microarray results identifying the number of mRNAs whose levels increased or decreased (≥2-, 4- and 6-fold) in the macaque corpus luteum by 24 h after Antide (ANT) treatment, compared to untreated controls (CTRL)

ANT vs CTRL	A Chip*	B Chip*	A+B (% of total genes)*
# Genes (≥2-fold increase)	29	35	64 (0.55%)
# Genes (≥2-fold decrease)	63	79	142 (1.22%)
Total	92	114	206 (1.78%)
# Genes (≥4-fold increase)	5	2	7 (0.06%)
# Genes (≥4-fold decrease)	14	30	44 (0.38%)
Total	19	32	51 (0.44%)
# Genes (≥6-fold increase)	4	2	6 (0.05%)
# Genes (≥6-fold decrease)	9	10	19 (0.16%)
Total	13	12	25 (0.22%)

* Each chip included 5800 human IMAGE clones, for a total of 11,600 genes

expressed in the monkey CL within 24 h after ANT administration. The expression of 7 of the 51 genes was up-regulated following ANT treatment. In contrast, 44 genes were down-regulated after ANT administration. Most of these genes are associated with signal transduction pathways, endocrine/paracrine regulation, cell growth/proliferation, immune response, and lipid/cholesterol/steroid metabolism (Table 3.3).

3.4.3 Semi-quantitative (sq) RT-PCR Validation of Microarray Results

Twenty-five mRNAs whose expression changed ≥6-fold (Table 3.2) were analyzed further by sq RT-PCR. Twenty-two of 25 cDNAs exhibited similar changes in expression with microarray and RT-PCR analysis of ANT relative CTRL tissues (Table 3.4). Only one change was opposite to the microarray results (*DOC-1R*) and two did not change (*C4A* and *ORF1*). One example of an up-regulated gene is corticotropin releasing hormone binding protein, *CRHBP*, whose mRNA expression increased 10-fold in the CL of ANT-treated animals according to the microarray results. There is a 5.5-cycle difference at the linear part of the PCR curves, which corresponds to a 45-fold difference in gene expression between the CTRL and the ANT group (Fig. 3.2A). Cyclophilin A mRNA showed the same level of expression in these (Fig. 3.2A) and all of the CTRL and ANT cDNA samples tested (data not shown), thereby re-affirming (Young *et al.*, 2002) the utility of this gene as an internal standard.

3.4.4 Gene Expression in the Monkey CL During the Natural Menstrual Cycle

Eleven of these 25 genes that were analyzed by RT-PCR changed correspondingly in the late-to-very late luteal phase during luteal regression in the natural menstrual cycle (Table 3.4). For example, *CRHBP* expression in the CL increased significantly ($P < 0.05$) in the late luteal phase during luteolysis, and then declined in the very late luteal phase at menses. Expression increased 10-fold from the mid-late to late luteal phase (Fig. 3.2B).

There were three predominant patterns of mRNA expression throughout the natural luteal phase for the LH-responsive genes that were up-regulated by GnRH antagonist treatment (LH withdrawal). For one pattern of expression, mRNA levels were elevated in the early luteal phase, declined at mid luteal phase, and rose again at late luteal phase

Table 3.3 Functional classification of 51 genes whose expression increased or decreased (≥4-fold) in the macaque corpus luteum by 24 h after Antide (ANT) treatment*

Function	Up-regulated genes	Down-regulated genes
Signal transduction	LIM domain only 7 (*LMO7*)	
	Membrane-bound phosphatidic acid selective phospholipase A1 (*PASPA1*)	
	Phosphoinositide-3-kinase, regulatory subunit, polypeptide 1 (p85 alpha) (*PIK3R1*)	
Endocrine/paracrine factors	Corticotropin releasing hormone binding protein (*CRHBP*)	Synaptosomal-associated protein, 25kDa (*SNAP25*)
	Inhibin, beta A (activin A, activin AB alpha polypeptide) (*INHBA*)	
Cell growth/proliferation	Tumor suppressor deleted in oral cancer-related 1 (*DOC-1R*)	Lectin, galactoside-binding, soluble, 7 (galectin 7) (*LGALS7*)
		Retinoic acid receptor responder (tazarotene induced) 1 (*RARRES1*)
		Tumor protein p53-binding protein (*TP53BPL*)
Transcription regulation		Helix-loop-helix protein (*HEN2*)
		Metastasis associated 1 (*MTA1*)
Heavy metal response		Metallothionein 1A (functional) (*MT1A*)
		Metallothionein 1B (functional) (*MT1B*)
		Metallothionein 1F (functional) (*MT1F*)
		Metallothionein 1L (*MT1L*)
Immune response		Complement component 4A (*C4A*)
		HLA-A2 class I antigen (*HLA-A2*)
		Interferon, alpha-inducible protein 27 (*IFI27*)
		Major histocompatibility complex, class I, C (*HLA-C*)
		MHC class I region (*MIC*)
Lipid/cholesterol /steroid metabolism		Apolipoprotein A-I (*APOA1*)
		3-beta-hydroxysteroid dehydrogenase/isomerase 1 (*HSD3B1*)
		Cytochrome P450 aromatase (*CYP19A*)

Table 3.3 *Continued*

Function	Up-regulated genes	Down-regulated genes
		Emopamil binding protein (sterol isomerase) (*EBP*)
		Niemann-Pick disease, type C1 (*NPC1*)
		Scavenger receptor class B, member 1 (*SCARB1*)
Lipid/cholesterol		Stearoyl-CoA desaturase (delta-9- desaturase) (*SCD*)
/steroid metabolism		Sterol-C4-methyl oxidase-like (*SC4MOL*)
Protein metabolism		Cathepsin D (lysosomal aspartyl protease) (*CTSD*)
		Pepsinogen 5, group I (pepsinogen A) (*PGA5*)
Heme biosynthesis		Aminolevulinate, delta-, synthase 1 (*ALAS1*)
		Aminolevulinate, delta-, synthase 2 (sideroblastic/ hypochromic anemia) (*ALAS2*)
Transporter		Cysteine dioxygenase, type I (*CDO1*)
		Erythrocyte membrane protein band 4.1 like 4B (*EPB41L4B*)
		GDP-fucose transporter 1 (*GFT1*)
		Hemoglobin gamma G (*HBG2*)
		Hemoglobin zeta (*HBZ*)
Other metabolism		Glutathione S-transferase A2 (*GSTA2*)
		Glutathione S-transferase A3 (*GSTA3*)
		Sorbitol dehydrogenase (*SORD*)
Others		Angiopoietin-related protein 4 (*ARP4*)
		Nucleolin (*NCL*)
Unknown	EST (AK094603)	Open reading frame 1 (*ORF1*)
		Polyposis locus protein 1-like 1 (*DP1L1*)
		Transmembrane protein 164 (*TMEM164*)
		EST (AC011374)
		EST (AL834346)
		EST (AP000437)
		EST (U79280)

* Approved gene names and symbols were from HUGO Gene Nomenclature Committee at www.gene.ucl.ac.uk/nomenclature. Functional classification through www.ensembl.org according to the accession number of each IMAGE Clone

Table 3.4 Summary comparing microarray results for selected (≥6-fold change) genes with semi-quantitative RT-PCR analysis of gene expression in the same pools of luteal tissue from Antide-treated vs. control animals, plus the pattern of gene expression in the corpus luteum during regression in the natural menstrual cycle

	Gene	Microarray (fold change)	RT-PCR (fold change)	Luteal phase
Up-regulated	Corticotropin releasing hormone binding protein	↑ (10.0)	↑ (45)	LLP ↑ *
genes	Phosphoinositide-3-kinase	↑ (9.9)	↑ (2)	LLP ↑ *
	LIM domain only 7	↑ (5.9)	↑ (2)	LLP ↑ *
	Phosphatidic acid selective phospholipase A1	↑ (3.6)	↑ (2)	LLP ↑ *
	Inhibin, beta A	↑ (5.8)	↑ (4)	NC
	Tumor suppressor deleted in oral cancer-related 1	↑ (58.5)	↓ (2)	LLP ↑ *
Down-regulated	Polyposis locus protein 1-like 1	↓ (9.4)	↓ (4)	LLP ↓ *
genes	3-Beta-hydroxysteroid dehydrogenase/isomerase 1	↓ (7.3)	↓ (32)	LLP ↓ *
	Sterol-C4-methyl oxidase-like	↓ (6.8)	↓ (4)	LLP ↓ *
	Aminolevulinate, delta-, synthase 1	↓ (6.1)	↓ (2)	LLP ↓ *
	Cysteine dioxygenase, type I	↓ (8.7)	↓ (8)	VLLP ↓ *
	Lectin, galactoside-binding, soluble, 7	↓ (6.7)	↓ (8)	VLLP ↓ *
	Sorbitol dehydrogenase	↓ (6.1)	↓ (4)	VLLP ↓ *
	Hemoglobin zeta	↓ (12.7)	↓ (4)	NC
	Hemoglobin gamma G	↓ (11.9)	↓ (16)	NC
	Metallothionein 1L	↓ (8.2)	↓ (2)	NC
	Aminolevulinate, delta-, synthase 2	↓ (8.1)	↓ (8)	NC
	Helix-loop-helix protein	↓ (7.8)	↓ (8)	NC
	Metallothionein 1B	↓ (6.9)	↓ (4)	NC
	Glutathione S-transferase A2	↓ (6.8)	↓ (32)	NC
	Metallothionein 1A	↓ (6.8)	↓ (4)	NC
	Nucleolin	↓ (6.1)	↓ (2)	NC
	Metallothionein 1F	↓ (9.1)	↓ (2)	LLP ↑ *
	Complement component 4A	↓ (7.6)	NC	NC
	Open reading frame 1	↓ (6.5)	NC	LLP ↑ *

*, Significant ($P < 0.05$) difference in CL from the L, late or VL, very late luteal phase (LP), compared to mid luteal phase. NC, no Change between mid to LLP or VLLP. Dotted line separates gene products whose microarray results either correlated (above line) or did not correlated (below line) with RT-PCR or luteal phase analyses

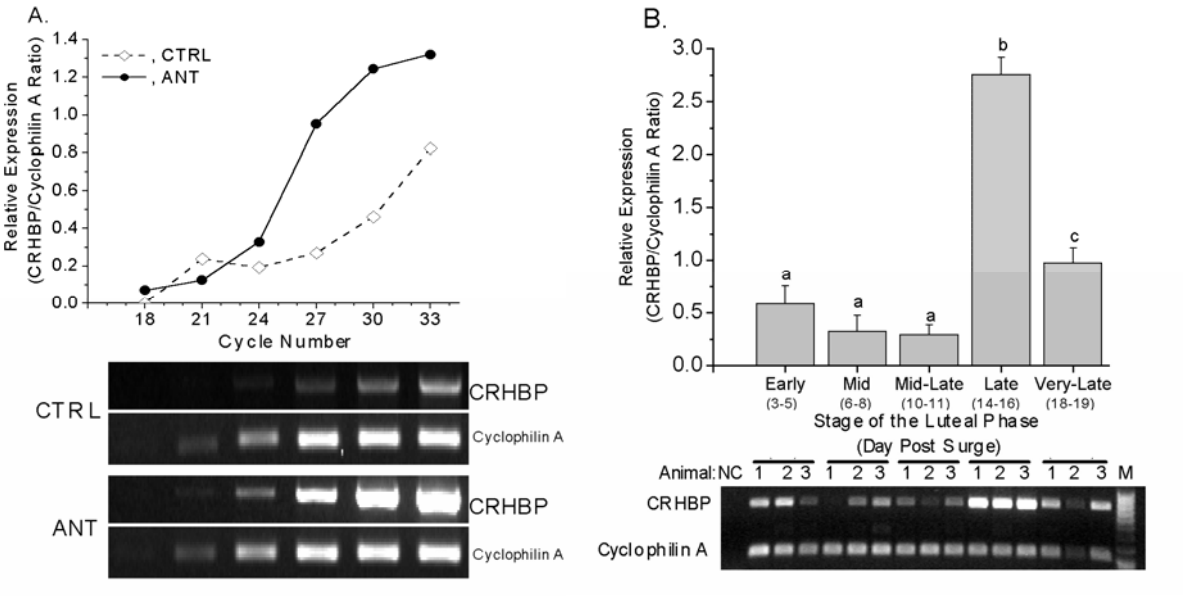

Fig. 3.2 (A) The differential mRNA expression for corticotropin releasing hormone binding protein (*CRHBP*) in the rhesus macaque CL following the acute withdrawal of gonadotropin support. The cDNA template for PCR was generated from CTRL and ANT RNA. The PCR was terminated every 3 cycles from cycle 18 to 33. Results were from densitometric analysis of PCR products in ethidium bromide-stained agarose gels. (B) The mRNA levels for *CRHBP* in CL throughout the luteal phase of the natural menstrual cycle. The cDNA template for PCR was converted from CL RNA collected during the early (day 3-5), mid (day 6-8), mid-late (day 10-11), late (day 14-16) and very late (day 18-19; mense) luteal phase. Results were from densitometric analysis of PCR products in ethidium bromide-stained agarose gels. Values are mean ± SEM for triplicates. Columns with different letters are significantly ($P < 0.05$) different.

(Fig 3.3A); this pattern included the genes for *LMO7* and *DOC-1R*. In a second pattern, mRNA levels increased progressively from early to late luteal phase, and then declined in the very late stage (Fig. 3.3B); examples of genes displaying this pattern were *PASPA1* and *PIK3R1*. Lastly, mRNA levels were detectable throughout, but increased abruptly in the late luteal phase (Fig. 3.3C); a gene that displayed this pattern was *CRHBP*.

There were two patterns of mRNA expression during the luteal phase for genes that were down-regulated following ANT treatment (LH withdrawal). Most of the down-regulated genes displayed mRNA levels that were elevated throughout the luteal phase, until declining in the late luteal stage (Fig. 3.4A); examples included *HSD3B1*, *CDO1*, *ALAS1*, *DP1L1*, and *SC4MOL*. The others had low mRNA levels in the early luteal phase, which increased in the mid-to-late stages, and then declined by the very late luteal phase (Fig. 3.4B); genes that displayed this pattern included *SORD* and *LGALS7*.

3.5 Discussion

Recent studies established that the overall high degree of sequence simililarity (~97%) between humans and rhesus macaques genes allows for the effective use of monkey material with human arrays (Wang *et al.*, 2004). We set out, therefore, to identify genes in the monkey CL that are under the regulatory control of LH utilizing a human spotted cDNA microarray system. To ensure that the genes identified using such a microarray technique are in fact differentially expressed, emphasis was placed on post-array validation studies that included semi-quantitative RT-PCR. To further focus our efforts, genes differentially expressed following ANT suppression of gonadotropin secretion (≥6-fold change) were analyzed for corresponding changes in expression during luteal regression in spontaneously occurring menstrual cycles.

After background subtraction and normalization of the array data according to the method outlined by Yang et al. (Yang *et al.*, 2002), 206 cDNAs were identified that show a ≥2-fold change in expression in the monkey CL after ANT treatment. At the level of a 4-fold change in expression, 51 cDNAs were differentially expressed. Those cDNAs exhibiting a ≥4-fold change in expression after ANT treatment were subsequently

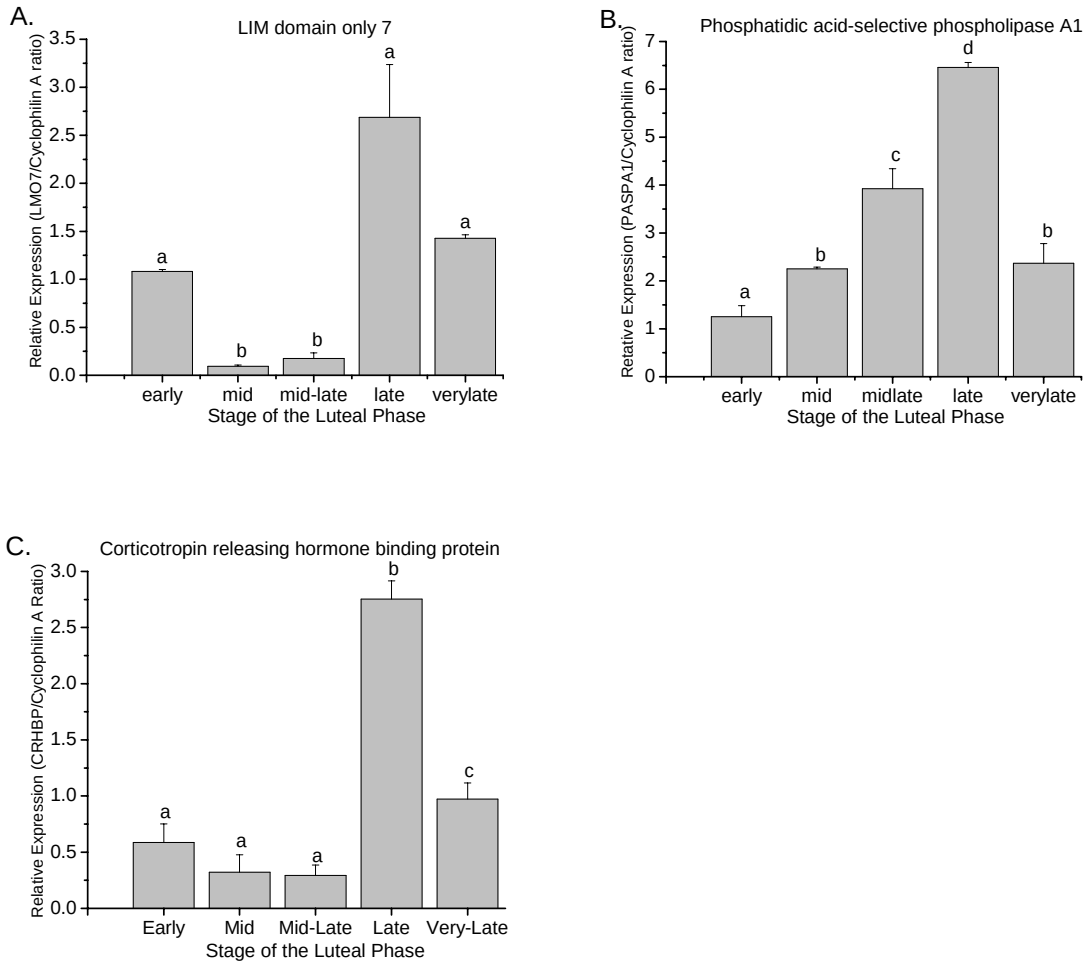

Fig. 3.3 Three different patterns of mRNA levels in CL throughout the natural luteal phase for gonadotropin-responsive genes that were up-regulated by LH withdrawal according to the microarray analysis. Values are mean ± SEM for triplicates. Columns with different letters are significantly ($P < 0.05$) different. (A) Levels (eg., for *LMO7*) are elevated in the early luteal phase, decline at mid luteal phase, and rise again at late luteal phase. (B) Levels (eg., for *PASPA1*) increase progressively from early to late luteal phase, and then decline in the very late stage. (C) Levels (eg., for *CRHBP*) are detectable throughout, but increase abruptly in the late luteal phase.

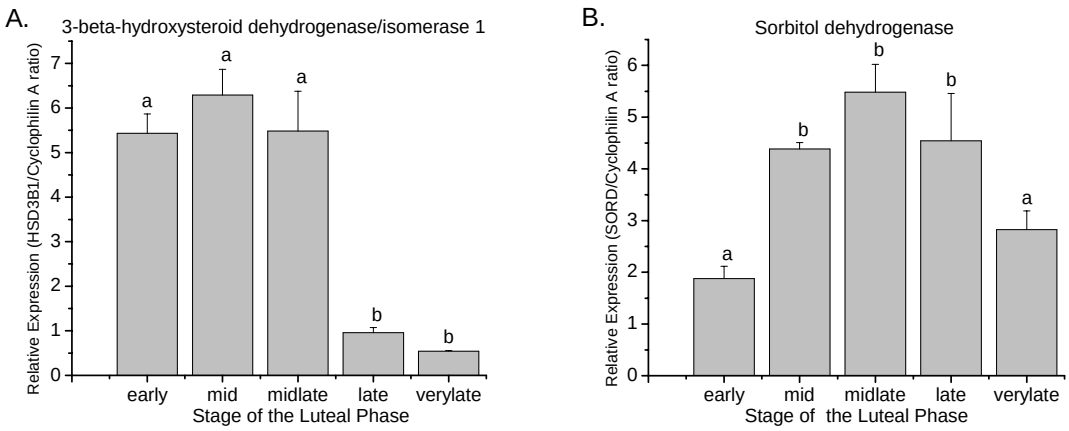

Fig. 3.4 Two different patterns of mRNA levels in CL throughout the natural luteal phase for gonadotropin-responsive genes that were down-regulated by LH-withdrawal according to the microarray analyses. Values are mean ± SEM for triplicates. Columns with different letters are significantly ($P < 0.05$) different. (A) Levels (eg., for *HSD3B1*) are elevated throughout the luteal phase, until declining in the late luteal stage. (B) Levels (eg., for *SORD*) are low in the early luteal phase, elevate in the mid-to-late stages, and then decline by the very late luteal phase.

classified according to functional domains and published information. Genes encoding proteins involved in signal transduction, transcriptional regulation, cell growth and proliferation, as well as immune function were identified as differentially expressed in the monkey CL after LH withdrawal. The majority of the differentially expressed cDNAs identified were down-regulated following ANT administration (86.3%), which suggests that LH serves to predominantly stimulate rather than inhibit gene expression in the monkey CL. The fewer (13.7%) cDNAs up-regulated following ANT treatment are endocrine and paracrine factors or signal transduction intermediates. As such, the proteins encoded by these up-regulated genes following LH withdrawal may serve as key initiators or intermediates required for luteal regression. In contrast, those genes down-regulated following the loss of LH support may be required for promoting luteal activities.

LH is critical for luteal cell progesterone production by maintaining the expression of several genes that encode critical steroidogenic enzymes (Duncan *et al.*, 1999; Niswender *et al.*, 2000). For example, 3β-hydroxysteroid dehydrogenase 1 (*HSD3B1*) expression is elevated following LH addition to human granulosa cell cultures (Sasson *et al.*, 2004). Also, *HSD3B1* mRNA levels decrease during luteal regression in the monkey CL during natural menstrual cycles (Young *et al.*, 2003a). Accordingly, our microarray data demonstrated that *HSD3B1* mRNA levels decreased in the monkey CL after ANT administration. In contrast, certain well-studied LH-regulated genes encoding proteins involved in steroidogenesis that are expected to change following ANT treatment were not identified in the present microarray study. Such examples include the steroidogenic acute regulatory protein (*StAR*) (Christenson and Strauss, 2000; Devoto *et al.*, 2004) and cytochrome P450 subfamily XIA (cholesterol side chain cleavage, *CYP11A*) genes (Gizard *et al.*, 2002). Analysis of the list of human genes spotted on the array revealed that *CYP11A* was absent. *StAR* cDNA was spotted on the array but did not yield a hybridization signal above background. This is likely caused by a lack of hybridization between the monkey cDNA and the spotted human gene due to species-related sequence differences.

Those cDNAs that changed by 6-fold or greater in the monkey CL after ANT treatment were subsequently selected as targets for additional analysis. Of the 25 cDNAs

observed to change by ≥6-fold, 6 were up-regulated (0.05% of total) and 19 were down-regulated (0.16% of total). Out of these 25 cDNAs, 22 were validated as differentially expressed by RT-PCR analysis, suggesting efficient cross-species hybridization. Subsequent studies were performed to determine whether these 25 cDNAs exhibit a corresponding change in mRNA expression during luteal regression in naturally occurring menstrual cycles. Of the 22 validated cDNA target genes, 11 changed correspondingly in the late-to-very late luteal phase, the period when luteal regression is well underway. These results suggest that, in some cases, mid luteal phase loss of LH support reflects the changes in gene expression that occurs during natural luteal regression. Several of the genes differentially regulated following ANT treatment exhibited similar patterns of mRNA expression throughout the natural luteal phase. For example, the mRNA expression profile for *HSD3B1* was similar to the mRNA expression profile observed for *CDO1*, *ALAS1*, *DP1L1*, and *SC4MOL* in the monkey CL through the course of a natural luteal phase. In this particular pattern, peak expression occurred during luteal development and optimal progesterone production (i.e. through the early and mid-late stages). When regression is well underway (late stage CL) or complete (very late stage CL), however, the expression of these genes drops dramatically. The mRNA expression profile for *SORD* was similar to that observed for *LGALS7* mRNA. In this pattern, mRNA levels were low in the early luteal phase, peaked at the mid-to-late stages, and then declined again during luteal regression (late to very late stage).

Previous studies utilizing various differential analysis methodologies have been conducted with the goal of identifying LH regulated genes in the ovary (Espey and Richards, 2002; Sasson *et al.*, 2004; Yadav *et al.*, 2004). For example, Espey and Richards used differential display to identify genes that were up-regulated in rodents following an ovulatory stimulus (Espey and Richards, 2002; Espey *et al.*, 2003). In their study, the expression of the *ALAS*, *GST*, and *MT1* genes increased through the periovulatory and luteal phases of stimulated estrous cycles. The orthologous monkey genes were also found to be expressed in the macaque CL by microarray analysis as LH-regulated (i.e. down-regulated following ANT treatment). Metallothioneins bind to and are transcriptionally regulated by heavy metals, but have not been previously implicated

in the LH-dependent regulation of luteal function. In another study, Yadav and co-workers set out to identify genes regulated by LH in the rhesus macaque CL using differential display (Yadav *et al.*, 2004). Following GnRH antagonist treatment, 7 genes were determined to be differentially expressed, including aldose reductase and low density lipoprotein receptor. Both genes were spotted on the human array but did not yield a signal above background, again potentially due to cross-species hybridization failure. Lastly, using a microarray approach, Sasson et al. identified genes that were differentially expressed following LH addition to human granulosa cell cultures (Sasson *et al.*, 2004). Some of the LH-regulated genes identified in our studies, such as *SC4MOL* and *CYP19A*, were also found to be regulated in a similar manner in human granulosa cell cultures by LH.

Several genes not previously associated with LH-dependent regulation of CL function were also identified in the present study. For example, corticotropin releasing hormone binding protein (*CRHBP*) mRNA expression was up-regulated after ANT treatment. The expression of *CRHBP* mRNA also increased significantly at the late stage relative to all others stages of a natural luteal phase. Thus, LH may suppress the expression of *CRHBP* mRNA when the CL is at its peak with regard to progesterone production. The suppression of *CRHBP* may be lost, however, when the CL begins to undergo regression. *CRH* was discovered originally in the hypothalamus-pituitary axis (Vale *et al.*, 1981), and later in the placenta (Shibasaki *et al.*, 1982; Ni *et al.*, 2004). It is now known that the CRH system is composed of four ligands (corticotropin, urocortin, urocortin II, and urocortin III), one binding protein (CRHBP), and two receptors (CRHR1 and CRHR2). CRHBP is known to bind CRH and urocortin, thus preventing activation of their receptors (Dautzenberg and Hauger, 2002). Some of the CRH system components, including CRH, CRHR1, and CRHBP, are also expressed in the human ovary (Asakura *et al.*, 1997). Recently, CRH, urocortin, CRHR1, and CRHR2α have been observed specifically within the human CL (Muramatsu *et al.*, 2001). Since the cellular and molecular mechanisms of luteolysis in primates remain obscure, the CRH system may be a key component in the initiation or execution of CL regression.

Thus, we have identified a number of genes under the regulatory control of LH that

have not previously been implicated in primate luteal function. Genes stimulated by LH, and thus suppressed following LH removal, may serve to maintain CL structure and function. Genes inhibited by LH, and thus increase in expression following LH removal, may play a role in luteal regression. Studies are now underway to evaluate the role such novel systems play in LH-mediated maintenance of luteal function and regression. Specifically, the expression of all of the CRH ligands and receptors are being evaluated in the primate CL (Xu *et al.*, 2003b). An added level of complexity in the CRH system stems from the observations that each receptor type exists as multiple protein forms due to the generation of alternatively spliced mRNA (Catalano *et al.*, 2003; Johnson *et al.*, 2003; Pisarchik and Slominski, 2001). Studies are also underway, therefore, to determine which receptor isoforms are expressed, and if so, whether they are regulated by LH.

CHAPTER 4
DYNAMIC EXPRESSION AND REGULATION OF THE CORTICOTROPIN RELEASING HORMONE/UROCORTIN-RECEPTOR-BINDING PROTEIN (CRH/UCN-R-BP) SYSTEM IN THE PRIMATE OVARY DURING THE MENSTRUAL CYCLE *

4.1 Abstract

Microarray analysis discovered that mRNA for CRH-binding protein (*CRHBP*) increased significantly in the primate corpus luteum (CL) following LH withdrawal. Therefore, studies were designed to determine if other components of the CRH/urocortin (UCN)-receptor (R)-BP system are expressed in the CL during the menstrual cycle and regulated by LH. CL were collected from monkeys during the early (day 3-5 post LH surge) to very late (day 18-19) luteal phase and from controls or animals receiving GnRH antagonist (Antide, 3 mg/kg BW). CRH/UCN-R-BP system components were quantitated for mRNA levels by real-time PCR and analyzed for protein localization by immunohistochemistry. All genes encoding the CRH/UCN-R-BP system, except *UCN3*, were expressed in the CL. *CRH* mRNA levels did not change during the luteal phase, whereas expression of *UCN*, *UCN2*, *CRHR1* and *CRHR2* was maximal at early or mid luteal phase before declining ($P < 0.05$) at the later stages. *CRHBP* mRNA levels were lowest at mid and increased ($P < 0.05$) in the late luteal phase. Suppressing gonadotropin secretion reduced *UCN2* ($P < 0.05$) and increased *CRHBP* ($P < 0.05$) mRNA levels,

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Xu, J., Hennebold, J. D., and Stouffer, R. L. (2006) Dynamic expression and regulation of the corticotropin-releasing hormone/urocortin-receptor-binding protein system in the primate ovary during the menstrual cycle. *J. Clin. Endocrinol. Metab.* **91,** 1544-1553.

without altering the expression of other ligands or receptors. CRH, UCN, UCN2 and their receptors were localized to the granulosa-lutein cells of the CL, whereas CRHBP was limited to the theca and theca-lutein cells of the preovulatory follicle and CL.

Thus, a local CRH/UCN-R-BP system exists in the macaque CL that is dynamically expressed and LH-regulated during the luteal phase of the menstrual cycle. Ligand-receptor activity may regulate luteal structure-function, at this point in an unknown manner, in primates.

4.2 Introduction

The development and maintenance of the functional corpus luteum (CL) in primates during the menstrual cycle requires the actions of the pituitary-derived gonadotropin, LH (Zeleznik and Benyo, 1994; Niswender *et al.*, 2000; Stouffer, 2003). Nevertheless, the processes whereby LH acts to develop and maintain primate CL structure and function are not well understood. Microarray analysis, to systematically investigate genes that are acutely dependent on LH for expression in the monkey CL, revealed that mRNA levels of *CRH*-binding protein (*CRHBP*) increased significantly following withdrawal of LH support (Xu *et al.*, 2005b). CRHBP is known to bind CRH and urocortin (UCN), thus preventing activation of their receptors (Kemp *et al.*, 1998). CRH was discovered originally in the hypothalamus-pituitary axis (Vale *et al.*, 1981), and later in the placenta (Shibasaki *et al.*, 1982; Ni *et al.*, 2004). It is currently known that the CRH/UCN-receptor (R)-BP system is composed of four ligands (CRH, UCN, UCN2, and UCN3), two receptors (CRHR1 and CRHR2), and one binding protein in mammals (Dautzenberg and Hauger, 2002). An added level of complexity in the CRH/UCN-R-BP system stems from observations that each receptor type exists as multiple forms due to the generation of alternatively spliced mRNAs. The *CRHR1* gene expresses nine subtypes (*α*, *β*, *c*, *d*, *e*, *f*, *g*, *h*, *v_1*) in human produced by differential splicing of exons 3-6 and 10-13. The *CRHR2* gene expresses three known subtypes (*α*, *β*, *γ*) that are produced by the use of alternate 5' exons (Catalano *et al.*, 2003; Johnson *et al.*, 2003; Pisarchik and Slominski, 2001). Some of the CRH/UCN-R-BP system components, including CRH, UCN, CRHR1, CRHR2α,

and CRHBP, were recently detected in the human ovary (Asakura *et al.*, 1997) or CL (Muramatsu *et al.*, 2001). However, detailed studies on the dynamics of their expression during the ovarian cycle, or their regulation by luteotropic hormones, have not been reported in any species.

Therefore, the present study was initiated to test the hypotheses that: (a) various components of the CRH/UCN-R-BP system were expressed in the macaque CL during the menstrual cycle, and (b) their expression was regulated by LH. The mRNAs for the CRH/UCN-R-BP components were detected initially using RT-PCR. Subsequently, real-time PCR was performed to quantitate mRNA levels in the CL at each stage of the luteal phase and after acute LH deprivation. Finally, CRH/UCN-R-BP proteins were localized to specific cell types within the primate ovary and CL by immunohistochemistry.

4.3 Materials and Methods

4.3.1 Animal Treatment and Hormone Assays

The general care and housing of rhesus monkeys (*Macaca mulatta*) at the Oregon National Primate Research Center (ONPRC) were described previously in chapter 3 section 3.3.1. All protocols were approved by the ONPRC Animal Care and Use Committee, and conducted in accordance with NIH Guidelines for the Care and Use of Laboratory Animals. Beginning six days after onset of menses, daily blood samples were collected by saphenous venipuncture during the follicular and luteal phase up to the day of lutectomy. Serum was assayed for estradiol and progesterone (P) concentrations by specific electrochemoluminescent assays, as previously described in chapter 3 section 3.3.1. The first day of low (<100 pg/ml) serum estradiol following the midcycle estradiol peak typically corresponds with the day after the LH surge, and is therefore termed day 1 of the luteal phase (Duffy *et al.*, 1999).

4.3.2 Tissue Collection

To analyze luteal tissues throughout the luteal phase of the natural cycle, CL (n = 5/stage) were collected during the early (ECL, day 3-5 post LH surge), mid (MCL, day 6-8), mid-late (MLCL, day 10-12), late (LCL, day 14-16) and very late (VLCL, day 18-19;

menses) luteal phase. These intervals provided tissues representing the developing, developed functional, on the verge of regressing, regressing and regressed CL, respectively (Young *et al.*, 2002).

To analyze gene expression in the CL after acute LH withdrawal, the GnRH antagonist Antide (ANT) was administered by sc injection in a vehicle of 50% propylene glycol and 50% water, as previously described in chapter 3 section 3.3.1. Monkeys (n = 3) were injected with ANT (3 mg/kg body weight) at 0800 h on day 6 of the luteal phase. CL were isolated 24 h later (day 7 of the luteal phase) from anesthetized monkeys during an aseptic ventral midline laparotomy (Duffy *et al.*, 2000). Control (CTRL) animals (n = 3) received no ANT injection prior to CL removal on day 7. ANT administration on day 6 of the luteal phase significantly ($P < 0.05$) decreased LH (Duffy *et al.*, 1999) and P (Xu *et al.*, 2005b) levels within 24 h, whereas levels remained unchanged in CTRL animals. CL samples collected previously from CTRL and ANT-treated monkeys to evaluate LH-regulated genes (chapter 3 section 3.3.1) were used in the study.

To localize protein expression in the individual cell types, ovaries (n = 3/stage) were surgically removed during the preovulatory follicular phase (day 0-1 prior to LH surge) and the early to late luteal phases (stages described previously in this section) of the natural menstrual cycle, fixed overnight in 10% neutral buffered formalin, dehydrated in 70% ethanol solutions, and paraffin-embedded. The 5 μm sections were prepared in the SCCPRR's (U54) Imaging and Morphology Core at the ONPRC (Hazzard *et al.*, 2000).

4.3.3 RNA Extraction and RT-PCR Analysis

RNA was extracted from each CL using TRIzol Reagent (Invitrogen Corporation, Carlsbad, CA) according to standard protocols. Reverse transcription was performed using 1 μg DNase-treated RNA and Moloney Murine Leukemia Virus reverse transcriptase for 1 h at 37 °C according to the supplier's protocol. The individual components of the rhesus macaque CRH/UCN-R-BP system (*CRH*, *UCN*, *UCN2*, *UCN3*, *CRHR1*, *CRHR2*, and *CRHBP*) were PCR amplified with specific primers generated from human sequences (www.ncbi.nlm.nih.gov) (Table 4.1). To serve as an internal control, a

Table 4.1 Primer and probe sequences used for PCR amplification and real-time PCR quantitation.

Gene	NCBI accession number	Primer sequences and annealing temperature for RT-PCR	Primer and probe sequences for real-time PCR
CRH	NM_000756	5'-CGCTGCTCTTATGCCATTT-3' 5'-AACACCTGGAAACGGAAACT-3' 56 °C	5'-CGTAGACAAAACGAATAACATTGTGTT-3' 5'-AGAGAGCCTATACCCCTTACTTAGCA-3' 5'-6FAM-CTGCACATGAATACAC-MGBNFQ-3'
UCN	NM_003353	5'-GACCTCACCTTTCACCTGCT-3' 5'-TGCCCCGCATCCCAACTCT-3' 62 °C	5'-GCCCCGCATCCCAACT-3' 5'-GCAGAACCGCATCATATTCGA-3' 5'-6FAM-CCGTCACTTGCCC-MGBNFQ-3'
UCN2	NM_033199	5'-GCTCGCGCATTGTCCTATC-3' 5'-CTCCAGGTCTTCCCATCCAG-3' 66 °C	5'-GCTTGCTCCAGTAAGATCTGCAA-3' 5'-CCCCACCCGCCACTCT-3' 5'-6FAM-CATCCAGCGATAGGAC-MGBNFQ-3'
UCN3	NM_053049	5'-GGACCGTTTCCATAGAGAG-3' 5'-AGTGGACTTCCCTCCGCA-3' 64 °C	N/A
CRHR1	NM_004382	5'-GTGCCTGCGAAACATCATC-3' 5'-CGGGATTGACGAAGAACAG-3' 64 °C	5'-GAGGTCCACCAGAGCAACGT-3' 5'-TAGCAGCCCTCACCGAACAT-3' 5'-6FAM-GGCGGTCACCAACCTGCACCA-MGBNFQ-3'
1α	L23332	5'-CGGGCATTCAGGACGGTAG-3' 5'-GGAAGTAGTTGTAGGCGGC-3' 60 °C	N/A
1c	U16273	5'-GCCAGCAACATCTCAGACA-3' 5'-GCCAAACCAGCACTTCTCA-3' 64 °C	N/A
1β,d, f, g, v_1	L23333, AF180301, AF369652, AF369653, AY429529	5'-GTCGCAGTCATCATCAACTACC-3' 5'-CGGATGGCAGAACGGACCT-3' 56 °C	N/A
1e, h	AF3696151, AF374231	5'-TGAACCCCGTCTCTGCCTC-3' 5'-TCACCAACCTGCACCAGC-3' 56 °C	N/A
CRHR2	NM_001883	5'-AGTGCCTCTTCCTCTTCATCGG-3' 5'-TCTCCCCTCCCATCTCCTG-3' 60 °C	5'-GGATCAGCTTCCACAGCATCA-3' 5'-GGAGAACCCAGAGGAAGAAAGTG-3' 5'-6FAM-CACCGTGTGACCCC-MGBNFQ-3'
2α	U34587	5'-GTCCATCCCTACATCACCCA-3' 5'-CTGCTATTCCCATCCCACG-3' 58 °C	N/A
2β	AF011406	5'-CTAACCCCCAGCCACTACT-3' 5'-GCCTTCCACAAACATCCA-3' 66 °C	N/A
2γ	AF019381	5'-TGGGCTTTCCTCAGCTCTTCTG-3' 5'-CCATTCTCCAAGCATTCTCGATAG-3' (21) 56 °C	N/A
CRHBP	NM_001882	5'-CCAGCATGTCGCCCAACTT-3' 5'-CCTATTCCCTCGCAACCTG-3' 56 °C	5'-GACAGACCCCAACCTCTTTCC-3' 5'-TGGAGAAGCTGCAGTTTCGA-3' 5'-6FAM-CCCTGGTCGTTCCAC-MGBNFQ-3'

N/A, not applicable.

parallel PCR was performed using primers specific for the macaque cyclophilin A gene (forward primer: 5'-GCTGGACCCAACACAAATG-3'; reverse primer: 5'-TCTTCTTGCTGGTCTTGCC-3'). The parameters for the PCR were as follows: initial denaturation 94 °C/1.5 min, denaturation 94 °C/30 s, annealing/45 s, and extension 72°C/1 min. The Advantage 2 polymerase, reaction buffer, and nucleotides were purchased from BD Biosciences (San Jose, CA). The resultant PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, CA) and sequenced at the SCCPRR's (U54) Molecular and Cell Biology Core in the ONPRC. Sequence data were compared against the corresponding human genes using the Vector NTI Suite software (Invitrogen).

4.3.4 Real-time PCR Assay

The partial sequences of the rhesus macaque CRH/UCN-R-BP system genes were subsequently used to design real-time PCR primer and TaqMan probe sets using Primer Express software from Applied Biosystems (Foster City, CA) (Table 4.1). Perkin-Elmer parameters were adhered to during probe design: sequences with clusters of identical nucleotides were avoided to prevent non-specific interactions, selected probes were <27 mer, contained less than three Gs or Cs at the 5' end, and had a melting temperature at least 10 °C higher than both forward and reverse primers to ensure sufficient hybridization stability of probes during primer extension (Young *et al.*, 2002). Primers were synthesized by Invitrogen and TaqMan probes were synthesized by Applied Biosystems. Probes were labeled with the 5' reporter dye 6FAM and the 3' quencher dye MGBNFQ. A matrix of varying primer concentrations was employed to determine optimal concentrations of assay components.

The CRH/UCN-R-BP system mRNA expression were analysed using the TaqMan PCR Core Reagent Kit with the Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems). To control for the amount of total RNA added to each RT reaction and to normalize the target signal, *18S* rRNA was used as an active endogenous control in each well. Amplifications were conducted in a 10 µl final volume containing: 250 nmol/l TaqMan probe, 300-900 nmol/l forward and reverse primers, 250 nmol/l

TaqMan *18S* rRNA probe labelled with the 5' reporter dye VIC, 80 nmol/l forward and reverse *18S* rRNA primers, 20 ng cDNA, and 5 µl TaqMan Universal PCR master mix containing ROX dye as a passive reference (Applied Biosystems). The PCR reactions were conducted in sealed 96-well optical plates with thermal cycler conditions of: 2 min at 50 °C, 10 min at 95 °C and 45 cycles of 15 s at 95 °C (denaturation) and 1 min at 60 °C (primer annealing/extension). If the cDNA sample contained the target sequence, the TaqMan probe was cleaved with every PCR amplification cycle, resulting in increased fluorescent emission of the reporter dye. During the amplification cycles, the Real-Time PCR System monitored the amplification by quantitatively analysing changes in fluorescence emissions in each well. The number of amplification cycles for the fluorescence to reach a determined threshold level (C_T) was recorded for every unknown and an internal standard curve. The internal standard curve, used for relative mRNA quantification, was generated from five 10-fold dilutions of pooled CL samples. C_T values for unknown samples were used to extrapolate the amount of RNA equivalents from the internal standard curve. The RNA equivalent values were then divided by complimentary 18S rRNA equivalent values also derived from the same internal standard curve (Young *et al.*, 2002).

4.3.5 Immunohistochemistry (IHC)

The ovarian sections were deparaffinized and hydrated through CitriSolv Clearing Agent (Fisher Scientific, Pittsburgh, PA) and a graded series of ethanol. Sections were re-hydrated in PBS, followed by incubation in 3% hydrogen peroxide/60% methanol to quench any endogenous peroxidases activity. The sections were incubated for 1 h at room temperature with the primary anti-human antibodies (1:200 for CRH, sc-1759 and UCN, sc-1825; 1:100 for CRHR1, sc-12381 and CRHR2, sc-20550; 1:400 for CRHBP, sc-1822, Santa Cruz Biotechnology, Inc., Santa Cruz, CA; and 1:400 for UCN2, H-019-24, Phoenix Pharmaceuticals, Inc., Belmont, CA). As negative controls, antibodies pre-absorbed with blocking peptides (Santa Cruz Biotechnology, Inc. and Phoenix Pharmaceuticals, Inc.) at 4 °C overnight were incubated on adjacent tissue sections. The slides were then incubated with the appropriated secondary antibodies and processed

using an ECTASTAIN Elite ABC Kit from Vector Laboratories, Inc. (Burlingame, CA). The antigen-antibody complex was visualized by incubation with 3,3'-diaminobenzidine. The tissue was counterstained using hematoxylin and viewed via Zeiss Axioplan microscopy. A CoolSNAP CCD Camera (Photometrics Inc., Tucson, AZ) was used for image capture.

4.3.6 Statistical Analysis

Statistical evaluation of mean differences among stages of the luteal phase was performed by one-way ANOVA with a significance level set at 0.05 using the SigmaStat software package (SPSS Inc., Chicago, IL). To identify significant differences between stages, the Student-Newman-Keuls post-hoc test was used for pairwise multiple comparisons. A Student's t-test was used to compare parameters between CTRL and ANT-treated animals.

4.4 Results

4.4.1 CRH/UCN-R-BP mRNA Expression in the Macaque CL

RT-PCR analysis demonstrated that the genes encoding *CRH*, *UCN* and *UCN2*, but not *UCN3* (which was identified in monkey testis), were expressed in the macaque CL (Fig. 4.1, summarized in Table 4.2). Likewise, RT-PCR products for the α and c isoforms of *CRHR1*, the *CRHR2α* isoform, as well as *CRHBP*, were identified in macaque CL. *CRHR1e*, *1f*, and *CRHR2β* were not detected in the CL, but were detected in positive control monkey tissues (e.g. testis and heart) (Table 4.2). The partial sequences of the gene products for the macaque CRH/UCN-R-BP system (not shown), obtained by RT-PCR, have a high degree of similarity (95-98%) to the corresponding sequences from human genes.

As determined by real-time PCR, *CRH* mRNA levels did not change significantly in the macaque CL during the course of the luteal phase in the natural menstrual cycle (Fig. 4.2A). However, *UCN* gene expression levels increased 4-fold ($P<0.05$) from ECL to peak at MCL before declining significantly ($P < 0.05$) at the later stages (Fig. 4.2B).

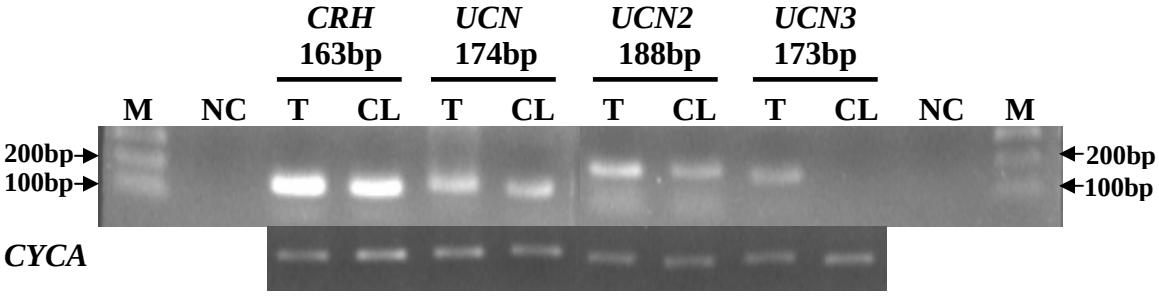


Fig. 4.1 The detection of cDNA products from RT-PCR for mRNAs of *CRH*, *UCN*, *UCN2*, and *UCN3*, as well as cyclophilin A (*CYCA*, positive control), in macaque CL. The cDNA templates for PCR were generated from RNA prepared from a positive control tissue (testes, T) and from pooled CL samples. The expression of control *CYCA* in corresponding tissue is shown in lower panel. M, DNA base pair (bp) markers of 100bp increments; NC, negative control.

Table 4.2 CRH/UCN-R-BP system expression in the primate CL

Gene	CL	Positive control (tissue)
CRH	+	+ (testis)
UCN	+	+ (testis)
UCN2	+	+ (testis)
UCN3	—	+ (testis)
CRHR1	+	+ (testis)
1α	+	+ (testis)
1c	+	+ (testis)
1e,f	—	+ (testis)
1β, d, g, h, v_1	—	— (brain, myometrium)
CRHR2	+	+ (brain)
2α	+	+ (brain)
2β	—	+ (heart)
2γ	—	— (brain)
CRHBP	+	+ (testis)

+/—, mRNA expression was detectable/ nondetectable by RT-PCR.

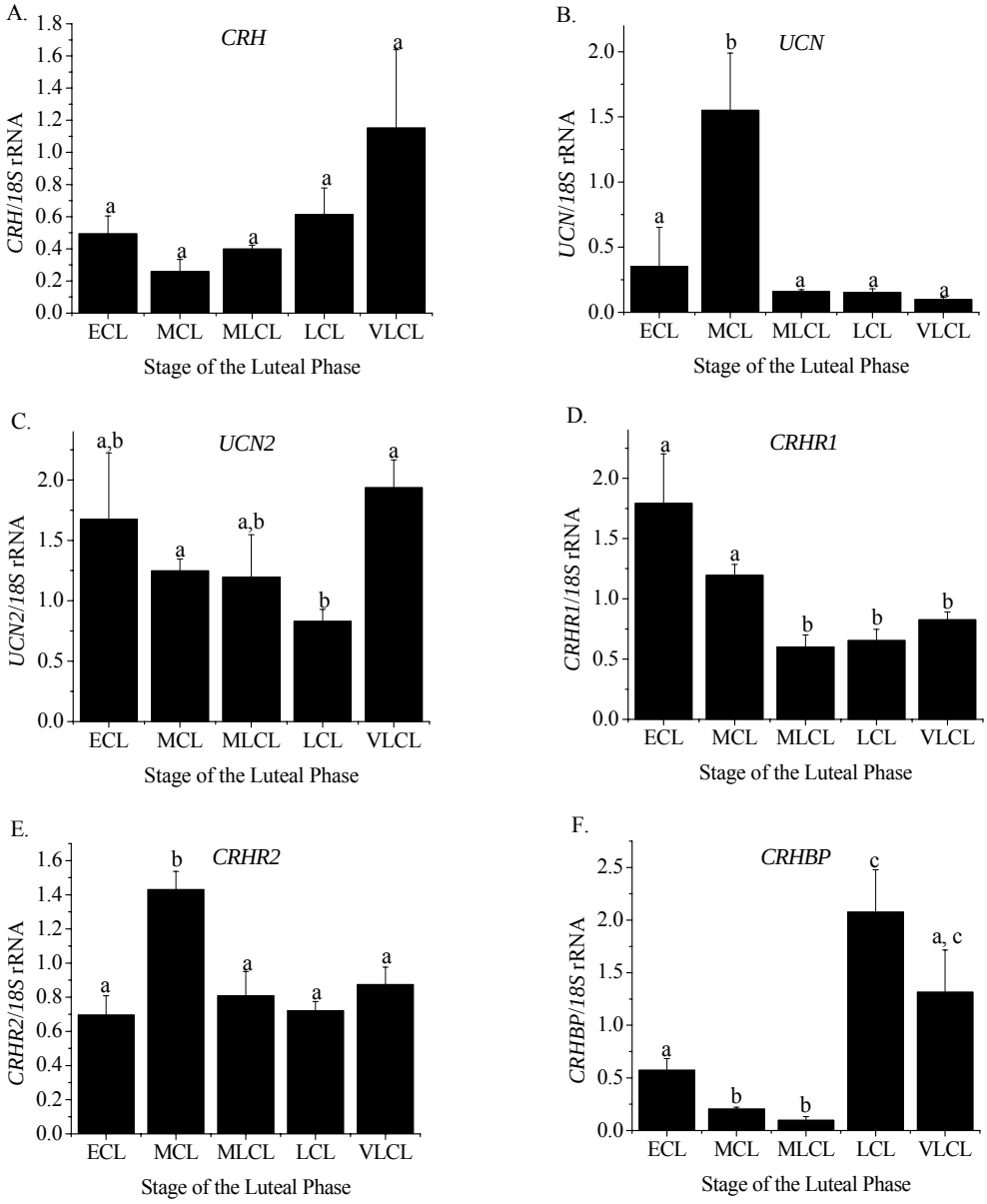


Fig. 4.2 Mean (± SEM) mRNA levels for CRH/UCN-R-BP components relative to *18S* rRNA in the rhesus macaque CL throughout the luteal phase of the natural menstrual cycle. The cDNA templates for real-time PCR was generated from RNA samples from individual CL (n = 5/stage) collected during the early (ECL; day 3-5 post LH surge), mid (MCL; day 6-8), mid-late (MLCL; day 10-12), late (LCL; day 14-16) and very late (VLCL; day 18-19; menses) luteal phase. Values were standardized to respective *18S* rRNA control values. Columns with different letters are significantly ($P < 0.05$) different.

UCN2 mRNA levels were detectable throughout, but decreased ($P < 0.05$) in the LCL compared to MCL and rose ($P < 0.05$) again in the VLCL (Fig. 4.2C). *CRHR1* mRNA levels were highest in the ECL and MCL relative to the remaining stages of the luteal phase (Fig. 4.2D). *CRHR2* mRNA increased ($P < 0.05$) in the MCL relative to its expression level in any of the other stages analyzed (Fig. 4.2E). In contrast, *CRHBP* mRNA levels were low at MCL, and then increased 10-fold ($P < 0.05$) from MLCL to LCL (Fig. 4.2F).

4.4.2 CRH/UCN-R-BP mRNA Levels in the CL of Gonadotropin-Depleted Monkeys

Real-time PCR analysis determined that *UCN2* mRNA levels in CL decreased 2.4-fold ($P < 0.05$) following ANT treatment, whereas those for *CRH* and *UCN* ($P = 0.13$) did not significantly change. There were no significant changes in mRNA levels for either *CRHR1* or *CRHR2* ($P = 0.09$) following LH withdrawal. In contrast, *CRHBP* mRNA levels increased 5.1-fold ($P < 0.05$) after ANT treatment relative to controls. (Table 4.3)

4.4.3 CRH/UCN-R-BP Protein Localization in the Monkey Ovary During the Menstrual Cycle

In the late follicular phase, there was minimal specific IHC staining (compared to negative controls; not shown) for CRH or UCN in the preovulatory follicle (Fig. 4.3A, B), atretic antral follicles or smaller preantral follicles (Fig. 4.3A). However, specific staining for both CRH and UCN was observed in the CL (Fig. 4.3C; Fig. 4.4A-C). The staining appeared to concentrate in granulosa-lutein cells of the CL, as well as in some of the interstitial cells in the ovarian stroma (Fig. 4.3C), whereas theca-lutein and endothelial cells in the CL did not label (Fig. 4.4A). In CL removed from the different stages of the luteal phase, intense staining for both ligands were most evident in the ECL (Fig. 4.4A) before decreasing in the MCL (CRH; Fig. 4.4B) and MLCL (UCN; Fig. 4.4C). In contrast, appreciable staining for UCN2 was observed in the granulosa, but not theca, cells of the preovulatory follicle (Fig. 4.3B). UCN2 immunostaining was also evident in the granulosa cells of smaller antral and growing preantral follicles (Fig. 4.3A, C).

Table 4.3 Analysis of CRH/UCN-R-BP system mRNA expression in luteal tissue obtained from untreated (CTRL) and ANT-treated animals (n = 3/group)

Gene	CTRL	ANT
CRH	0.7 ± 0.1[a]	0.5 ± 0.2
UCN	1.1 ± 0.1	0.6 ± 0.2 [b]
UCN2	0.7 ± 0.1	0.3 ± 0.03 [c]
CRHR1	1.3 ± 0.2	1.1 ± 0.3
CRHR2	0.7 ± 0.1	1.3 ± 0.2 [d]
CRHBP	0.5 ± 0.2	2.6 ± 0.7 [c]

[a] Ratio of target gene expression relative to *18S* rRNA levels (mean ± SEM) as determined by real-time PCR.

[b] $P = 0.13$.

[c] Significant ($P < 0.05$) difference in CL between CTRL and ANT-treated animals.

[d] $P = 0.09$.

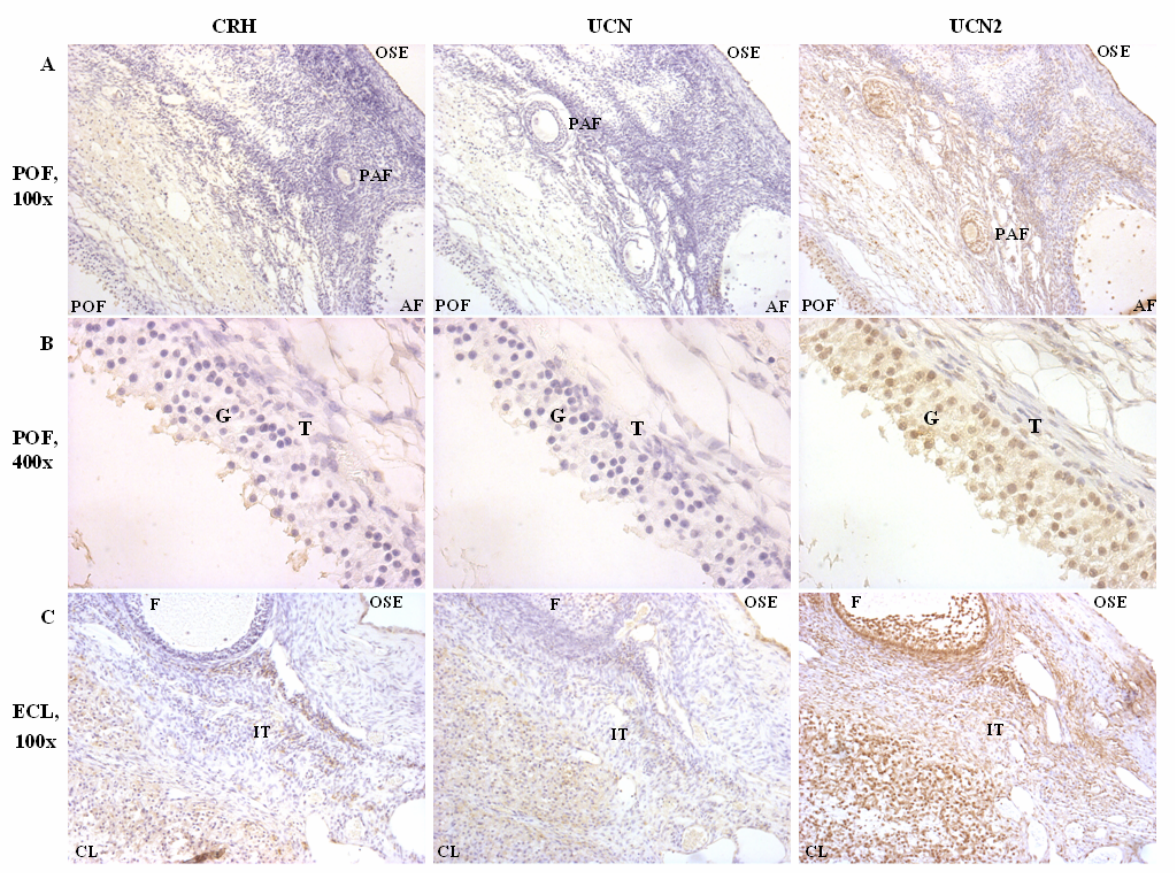


Fig. 4.3 Immunohistochemical staining for the CRH/UCN ligands in the macaque ovary containing the preovulatory follicle (POF) at original magnification ×100 (A) or ×400 (B) and the early CL (C; ECL, original magnification ×100). POF, preovulatory follicle; PAF, preantral follicle; F, antral follicle; AF, atretic follicle; OSE, ovarian surface epithelium; G, granulosa cells; T, theca cells; CL, corpus luteum; IT, interstitial tissue.

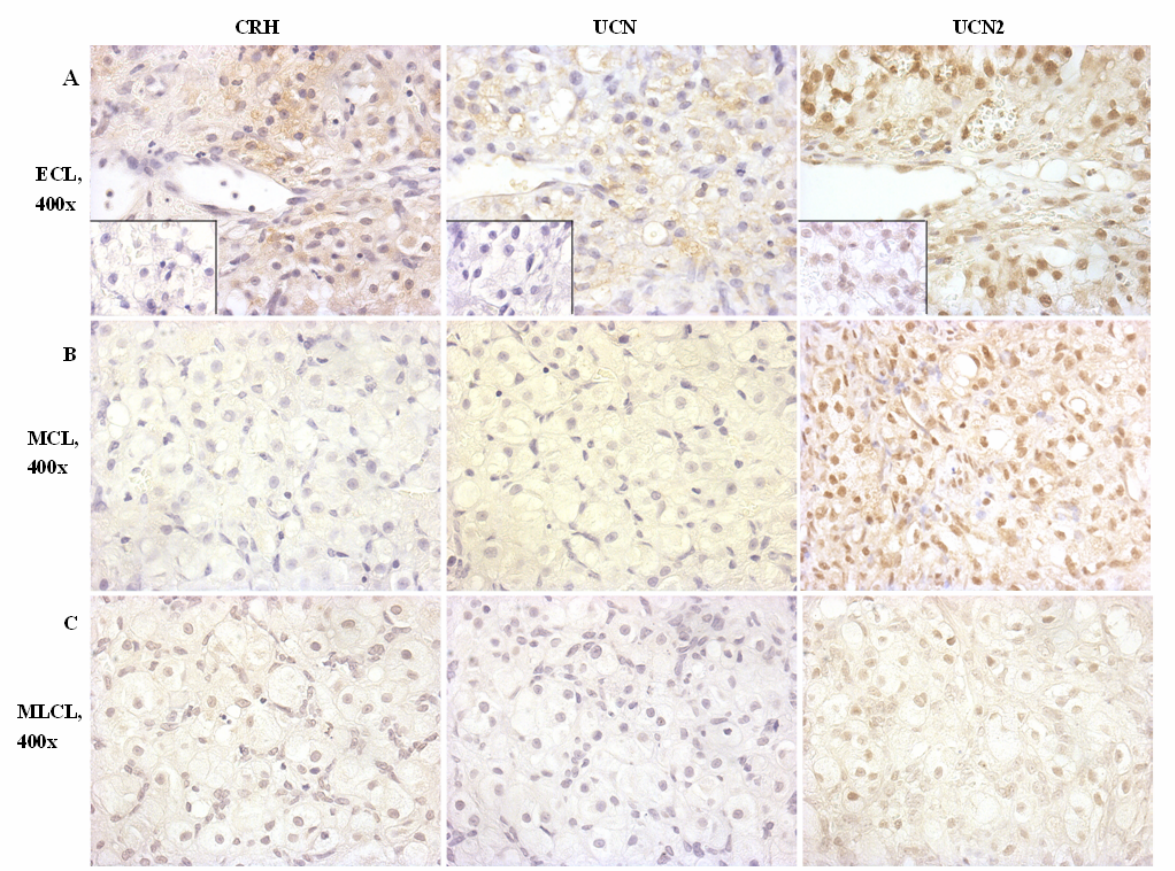


Fig. 4.4 Immunohistochemical staining for the CRH/UCN ligands in the macaque CL during the early (A; ECL, original magnification ×400), mid (B; MCL, ×400), and mid-late (C; MLCL, ×400) stage of the luteal phase. The non-specific staining associated with the pre-absorbed primary antibody is illustrated in the inset of panel A.

Specific immunostaining for UCN2 was particularly evident in the CL (Fig. 4.4A-C). In the ECL (Fig. 4.4A), both steroidogenic and endothelial cells displayed strong UCN2 immunolabeling, which subsequently declined in the MLCL (Fig. 4.4C). No significant staining was evident in control sections processed with pre-absorbed primary antibody (bottom left of Fig. 4.4A; representative ECL section shown).

Immunolabeling of CRHR1 and CRHR2 was not obvious in any follicles during the late follicular phase (Fig. 4.5A, B). In contrast, specific staining was evident in CL isolated from the different stages of the luteal phase (Fig. 4.5C; Fig. 4.6A-C). Expression of CRHR1 was detected at high levels in granulosa-lutein cells of the CL, as well as interstitial cells in the ovarian stroma, but not noted in theca-lutein or endothelial cells of ECL tissue (Fig. 4.5C; Fig. 4.6A). CRHR2 staining was also present but at modest levels. However, luteal cell CRHR staining was diminished in the MCL (CRHR1; Fig. 4.6B) and LCL (CRHR2; Fig. 4.6C). Immunohistochemical staining for CRHBP was not evident in preantral follicles, but was detectable only in the theca cells of preovulatory (Fig. 4.5B) and atretic follicles (Fig. 4.5A). CRHBP immunostaining was also observed in the interstitial tissue and CL during the luteal phase (Fig. 4.5C; Fig. 4.6A-C). The theca-lutein, but not the granulosa-lutein or endothelial cells of the CL were immunolabeled (Fig. 4.6A, C). Although positively stained cells were detectable early in the CL lifespan (Fig. 4.6A), few cells were positive in the MCL for CRHBP expression (Fig. 4.6B). The most intense level of CRHBP staining occurred in CL obtained from the late luteal phase (Fig. 4.6C). No receptor or binding protein staining was evident in control sections processed with pre-absorbed primary antibody (bottom left of Fig. 4.6A; representative ECL section shown).

4.5 Discussion

The present data strongly suggest that a complete CRH/UCN-R-BP system, with the ligands CRH, UCN, and UCN2, exists in the primate ovary. CRH was first discovered as the hypothalamic neuropeptide that promotes anterior pituitary ACTH release (Vale *et al.*, 1981), and is now believed to play a major role in the body's response to stress (Bale and Vale, 2004). More recently, UCN and two other family members (UCN2 and UCN3,

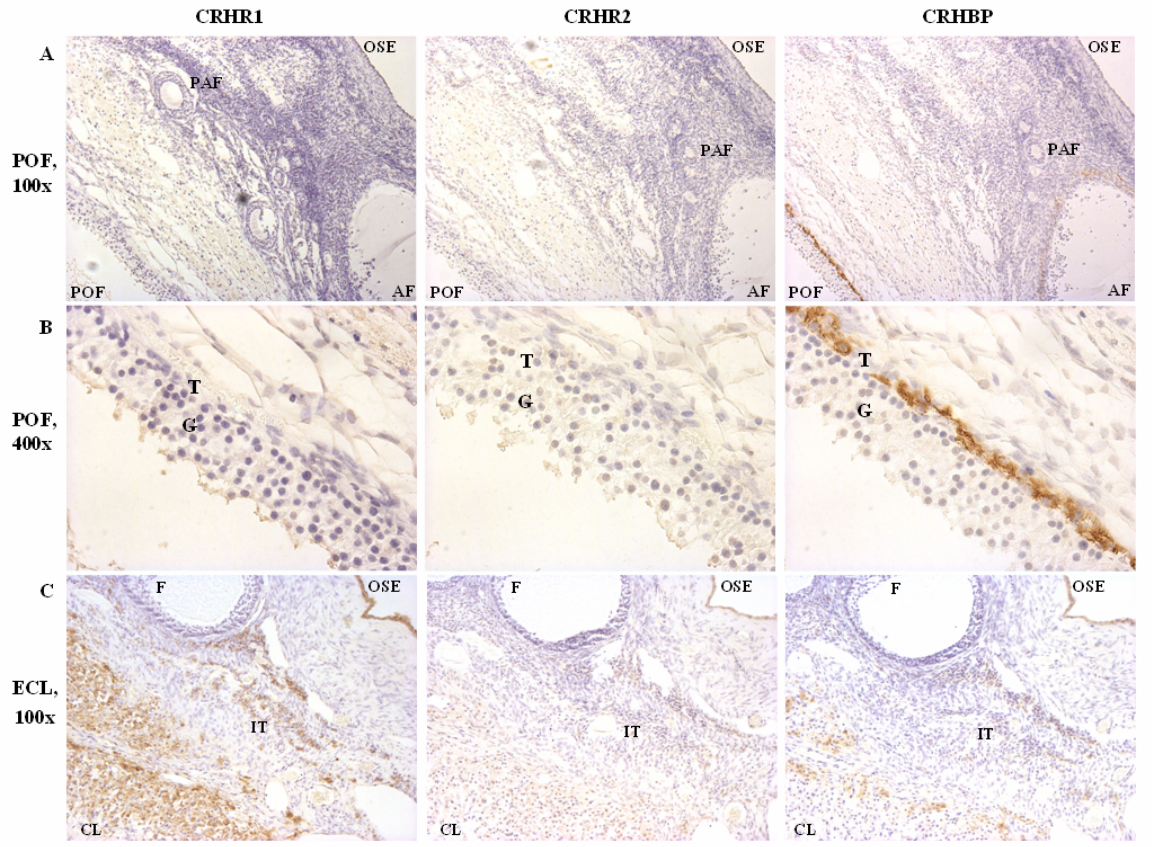


Fig. 4.5 Immunohistochemical staining for the CRH/UCN receptors and binding protein in the macaque ovary containing the preovulatory follicle (POF) at original magnification ×100 (A) or ×400 (B) and the early CL (C; ECL, original magnification ×100). POF, preovulatory follicle; PAF, preantral follicle; F, antral follicle; AF, atretic follicle; OSE, ovarian surface epithelium; G, granulosa cells; T, theca cells; CL, corpus luteum; IT, interstitial tissue.

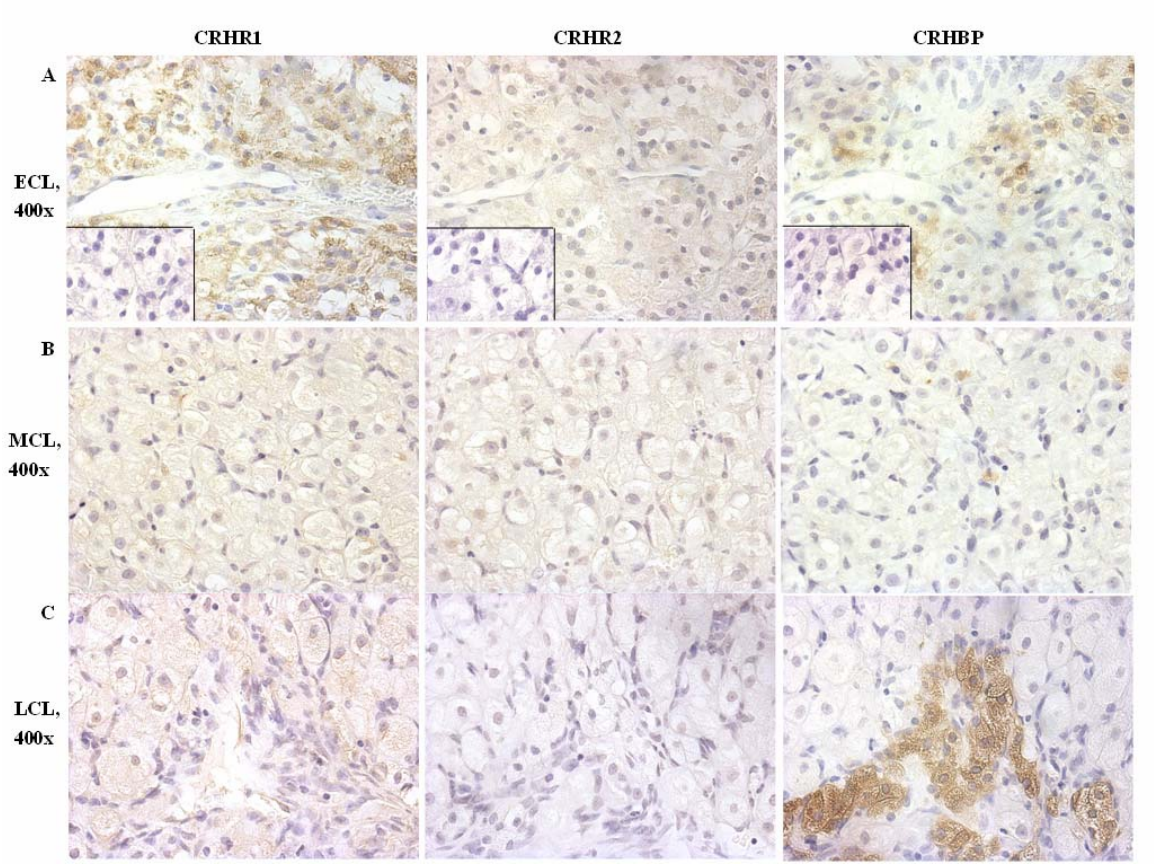


Fig. 4.6 Immunohistochemical staining for the CRH/UCN receptors and binding protein in the macaque CL during the early (A; ECL, original magnification ×400), mid (B; MCL, ×400), and late (C; LCL, ×400) stage of the luteal phase. The non-specific staining associated with the pre-absorbed primary antibody is illustrated in the inset of panel A.

originally called stresscopin-related peptide and stresscopin, respectively) were discovered and studied with regard to their apparent roles in adaptive responses during recovery from stress (Hsu and Hsueh, 2001). CRH has a high affinity for CRHR1, but a low affinity for CRHR2. UCN has a high affinity for both receptors, whereas UCN2 and 3 only have a high affinity for CRHR2 (Dautzenberg and Hauger, 2002). It was proposed that ligand binding to CRHR1 stimulates ACTH secretion during a "fight-or flight" response and also regulate energy balance, metabolism, and regulation of the immune response directly in peripheral tissues under stress conditions (Smith *et al.*, 1998), whereas ligand-CRHR2 signaling mediates the stress-coping responses during the recovery phase of stress without evoking ACTH release (Bale *et al.*, 2000). The CRHBP forms a dimer complex with CRH or UCN that neutralizes circulating or local ligand action. Whether the other stress-coping ligands, UCN2 and 3, bind to CRHBP is unknown (Dautzenberg and Hauger, 2002). Previously, the possible presence and actions of a CRH/UCN-R-BP system in the primate ovary has received little consideration (Florio *et al.*, 2004). The current study provides the first detailed information regarding mRNA expression and protein localization of the CRH/UCN ligands, receptors, and binding protein in the primate preovulatory follicle and the CL during the menstrual cycle.

The mRNA expression of all ligands, except *UCN3*, was detected in the rhesus macaque CL. *CRH* is abundantly distributed in the central nervous system (Bittencourt and Sawchenko, 2000), as well as in certain peripheral tissues, such as adrenal gland, testis, and placenta (Dautzenberg and Hauger, 2002). *UCN* expression is limited to specific regions of the brain (Bittencourt *et al.*, 1999), but is also found in a number of organ systems that include the pituitary, thymus, and testis (Kageyama *et al.*, 1999). *UCN2*, highly expressed in brain, is also present in adrenal gland, heart, and peripheral blood cells (Hsu and Hsueh, 2001). Our evidence that *CRH* and *UCN* are also expressed in the macaque ovary is consistent with previous reports describing their presence in the human ovary (Asakura *et al.*, 1997; Muramatsu *et al.*, 2001). The present study also provides the first evidence that *UCN2*, but not *UCN3*, exists in the primate CL.

The mRNA expression of both receptors and the binding protein was also detected in the rhesus macaque ovary. *CRHR1* expression was previously documented in the brain

and pituitary (Chalmers *et al.*, 1995) with low levels also being reported in the testis and ovary (Vita *et al.*, 1993; Nappi *et al.*, 1995; Asakura *et al.*, 1997; Muramatsu *et al.*, 2001). The present study identified two isoforms, α and c, for *CRHR1* in the monkey CL. The identification of these two isoforms in the ovary may be of significance, as the amino acid differences between the various *CRHR1* isoforms alters binding affinity for CRH (Ross *et al.*, 1994). *CRHR2α* was the only type 2 receptor isoform identified in the monkey ovary, which is consistent with a previous analysis of *CRHR* expression in the human ovary (Muramatsu *et al.*, 2001). *CRHR2* mRNA was undetectable in rat ovary (Nappi *et al.*, 1995), however, suggesting that *CRHR2* gene subtype expression is species-specific (Catalano *et al.*, 2003; Valdenaire *et al.*, 1997). Previously, *CRHR2α* was mainly localized to the subcortical structures of the brain (Lovenberg *et al.*, 1995), but also detected in muscle (Valdenaire *et al.*, 1997), including myometrium (Grammatopoulos *et al.*, 1998). Other receptor variants were undetectable in the ovary, although some, such as *CRHR1e*, *1f* and *CRHR2β*, were identified in other macaque tissues considered putative positive controls based on human studies (Pisarchik and Slominski, 2001; Valdenaire *et al.*, 1997). Prior analysis of tissue distribution indicated that CRHBP is expressed in human liver (Potter *et al.*, 1991), placenta (Linton *et al.*, 1990), and brain (Potter *et al.*, 1992). Asakara et al. (Asakura *et al.*, 1997) could not detect *CRHBP* mRNA expression in the human follicle, and proposed that CRHBP protein in the ovary originated from the circulation. However, based on our current findings and published studies (Xu *et al.*, 2005b), *CRHBP* is also expressed in the macaque antral follicle and CL.

Many components of the CRH/UCN-R-BP system are dynamically expressed in the macaque CL during the luteal lifespan in the menstrual cycle. Although *CRH* mRNA levels did not change during the luteal phase, *UCN*, *CRHR1*, and *CRHR2* expression peaked during either CL development or optimal P production (i.e. through the early and mid stages). When CL regression was underway or complete (i.e. late to very late luteal phase), however, the expression of these genes dropped dramatically. In contrast, the real-time PCR data confirmed our previous semi-quantitative results (Xu *et al.*, 2005b), that *CRHBP* mRNA expression increased significantly in the CL during luteal regression.

Our findings differ from those of Muramatsu et al. (Muramatsu *et al.*, 2001), who reported that *CRH*, *UCN*, and *CRHR* gene expression was higher during the regression of the human CL than at earlier stages of the luteal phase. This discrepancy may due to the differences between species, methods for classifying stages of the luteal phase, and/or techniques employed (e.g., RT-PCR versus quantitative real-time PCR).

Our present study indicates that luteal *UCN2* mRNA expression is up-regulated and *CRHBP* was down regulated by LH, whereas other ligand and *CRHR* mRNAs appeared to be mostly unaffected by this luteotropic hormone. Since there is evidence for less LH support (i.e. reduced LH pulsatility) (Ellinwood *et al.*, 1984) and reduced CL sensitivity to LH (Duffy *et al.*, 1999) in the late luteal phase of the menstrual cycle, it is noteworthy that *UCN2* and *CRHBP* displayed similar changes in mRNA expression during spontaneous luteal regression as during GnRH antagonist-induced LH withdrawal. Thus, LH may promote the expression of *UCN2* and suppress *CRHBP* when the CL is at its peak function with regard to P production. This regulation may be lost, however, when the CL begins to undergo regression. The factors regulating the changes in mRNA expression for other ligands and *CRHR* during the natural luteal phase remain unknown, although a role for LH cannot be ruled out. For genes exhibiting a tendency to change after LH withdrawal, such as *UCN* ($P = 0.13$) and *CRHR2* ($P = 0.09$), increasing the sample size or altering the time interval of collection may yield significant changes in mRNA levels.

This is the first report detailing the expression and localization of CRH/UCN-R-BP proteins in the ovary during the preovulatory stage of the ovarian cycle. There was no obvious immunoreactivity for CRH, UCN, and CRHR in the macaque ovary during the preovulatory follicular phase. However, UCN2 was detected in the granulosa cells of the preantral, preovulatory, and atretic follicles, as well as the interstitial cells in the ovarian stroma. In contrast, intense CRHBP staining was exclusively localized to the theca cells of the preovulatory and atretic antral follicles. Previous studies focused on selected components of the CRH/UCN-R-BP system in developing follicles. For example, CRH was reported to localize in theca cells of growing antral (7-8 mm in diameter) follicles (Asakura *et al.*, 1997) and the dominant follicle (Muramatsu *et al.*, 2001) in the human

ovary during the follicular phase. Weak immunoreactive UCN and CRHR were detected in granulosa and theca interna cells in dominant follicles (Muramatsu *et al.*, 2001). CRHBP signal was in theca cells of growing antral follicles in the human ovary (Asakura *et al.*, 1997), which is consistent with our observation.

Immunohistochemical results suggest that ligands (CRH, UCN, and UCN2) and receptor proteins are associated with the granulosa-lutein cells in the CL and interstitial cells in the ovarian stroma during luteal phase. A high level of staining intensity for ligands and receptors was observed in luteal cells in the early stage of the luteal phase, suggesting that processes causing (e.g., the mid cycle LH surge) or associated with (e.g., LH-induced local factors) ovulation or luteinization of the follicle wall influence CRH/UCN-R expression. Subsequently, ligand and receptor staining declined whereas binding protein staining increased in the CL during the later stages of the luteal phase. The results suggest that steroidogenic cells in the CL are the sites of CRH/UCN ligand synthesis and receptor-mediated action. In contrast, CRHBP localizes to the theca-lutein and interstitial cells particularly during the late luteal phase. Previous IHC studies demonstrated that CRH, UCN, and CRHR were present in luteinized granulosa and theca cells (Mastorakos *et al.*, 1994; Muramatsu *et al.*, 2001), and that significant CRH immunostaining was observed in developing CL and less prominent or totally absent in regressing CL of rodents and women (Mastorakos *et al.*, 1993; Mastorakos *et al.*, 1994). These findings are consistent with our observations. However, as noted earlier, Muramatsu et al. (Muramatsu *et al.*, 2001) reported higher levels of immunoreactive CRH and UCN mRNA, as well as protein, in the regressing CL relative to the developing CL in women.

In summary, this study provides the first detailed analysis of the CRH/UCN-R-BP system expression in the primate ovary during the menstrual cycle. According to the mRNA data, there is a dynamic regulation of *UCN*s, *CRHR*s or *CRHBP* gene expression in the CL during the menstrual cycle. The pattern of CRH/UCN-R-BP system mRNA expression during the natural luteal phase suggests that ligands regulate cellular processes in the CL primarily during the early luteal phase when there is greater expression of ligands/receptors and less expression of binding protein. In the late luteal phase, however,

their associated activities may be restricted as the expression of the ligands/receptors decreases and the binding protein increases. This is consistent with the hypothesis that ligand-receptor action promotes luteal structure-function, and its loss is associated with luteal regression. Nevertheless, one group (Calogero *et al.*, 2002) reports that CRH suppressed estrogen and IGF-1 production by human and rat granulosa cells in vitro, and as such, opposite "anti-gonadotropic" actions cannot be ruled out. Further studies are warranted to evaluate the endocrine (LH) and local control, plus the functional significance of the CRH/UCN-R-BP system in the preovulatory follicle, as well as in the maintenance/regression of the CL. Since the cellular and molecular mechanisms of luteolysis in primates remain obscure, the CRH/UCN-R-BP system may represent a previously unappreciated component in the initiation or execution of CL regression.

CHAPTER 5
EXPRESSION AND ROLE OF THE CRH/UROCORTIN-RECEPTOR-BINDING PROTEIN (CRH/UCN-R-BP) SYSTEM IN THE PRIMATE CORPUS LUTEUM DURING THE MENSTRUAL CYCLE *

5.1 Abstract

We recently reported that the mRNAs for components of corticotropin releasing hormone/urocortin-receptor-binding protein (CRH/UCN-R-BP) system are dynamically expressed in the primate ovary, particularly in the developing corpus luteum (CL) of the menstrual cycle. Therefore, studies were designed to (a) localize CRH/UCN-R-BP mRNAs to the cell types in the primate ovary; (b) quantitate protein expression during the CL lifespan; and (c) investigate the role of this system in the ovulatory, luteinizing follicle. First, ovaries (n = 3-4/stage) were removed from rhesus monkeys during the preovulatory phase (day 0-1 prior to LH surge) and from early (ECL, day 3-5 post LH surge) to very late (VLCL, day 18-19, menses) luteal phase of the menstrual cycle. In situ hybridization (ISH) was used to localize CRH/UCN-R-BP mRNAs in tissue sections. Also, total protein was extracted from individual CL to semi-quantitate CRH/UCN-R-BP proteins by western blotting. Second, either vehicle or a CRHR antagonist (5 μg of Astressin) was injected into the preovulatory follicle (day 0-1 prior to LH surge) of monkeys (n = 5/treatment) during spontaneous menstrual cycles. Ovulation was evaluated on day 3 post-injection, and daily serum samples were analyzed for levels of progesterone and estradiol. In subsequent treatment cycles, the ovary (n = 3/treatment) bearing the injected follicle was removed on day 9 of the luteal phase for histological

* Material in chapter 5 is in submission to *Endocrinology* of the Endocrine Society.

analysis to identify apoptotic cells. There was minimal ISH staining for ligand mRNAs, whereas *CRHR1*, *CRHR2*, and *CRHBP* staining was detected in the granulosa and theca cells of the preovulatory follicle. Intense mRNA staining for *CRH*, *UCN*, and *UCN2*, as well as for the receptors and *CRHBP* was evident in the CL, notably in the steroidogenic luteal cells. In situ hybridization staining for ligands and receptors was appreciable in the ECL and/or MCL, and diminished in the LCL. However, ISH staining for *CRHBP* was low in the ECL to MCL, and increased markedly in the LCL. Protein expression for ligands and receptors were high from ECL to MLCL but decreased ($P < 0.05$) by the LCL or VLCL. In contrast, CRHBP expression was highest ($P < 0.05$) at the LCL compared to other stages. Similar to controls, intrafollicular injection of Astressin did not disrupt timely ovulation. However, progesterone levels were significantly less in the Astressin-treated animals by the mid luteal phase compared to vehicle-injected controls. In addition, estradiol levels never increased above baseline (20-30 pg/ml) during the CL lifespan in Astressin-treated monkeys. Unlike in controls, a luteal "cavity" and many apoptotic (TUNEL-positive) cells were observed in the CL following Astressin injection. Thus, (1) CRH/UCN-R-BP components are expressed in the primate ovary in a cell-specific manner during the menstrual cycle, and (2) the pattern of CRH/UCN-R-BP protein expression and results of the in vivo study are consistent with the hypothesis that ligand-receptor activation promotes luteal development and/or structure-function in monkeys during the menstrual cycle.

5.2 Introduction

Peptides of the corticotropin releasing hormone/urocortin-receptor-binding protein (CRH/UCN-R-BP) system are expressed on brain neurons and pituitary corticotropes and play a key role in neurotransmission and neuroendocrine regulation of the stress response (Bittencourt and Sawchenko, 2000). However, recent evidence suggests that the CRH/UCN-R-BP system is also present in a variety of other tissues in mammals, within the immune, cardiovascular, digestive, and reproductive systems, skin, and also some types of human tumors (Dautzenberg *et al.*, 2002; Grammatopoulos and Chrousos, 2002).

The ubiquitous distribution of CRH/UCN-R-BP peptides, capable of activation diverse signaling mechanisms in different tissues, gives this system enormous versatility and plasticity. It is currently known that the primate CRH/UCN-R-BP system is composed of four ligands (CRH, UCN, UCN2, and UCN3) and two seven-transmembrane G-protein-coupled receptors (CRHR1 and CRHR2). The interactions of ligands with their receptors are modulated by the only binding protein, CRHBP, with affinity for CRH and CRH-like peptides (Dautzenberg *et al.*, 2002; Seacholtz *et al.*, 2002).

Recent studies indicated that a local CRH/UCN-R-BP system exists in the primate ovary, including human and rhesus monkey (Asakura *et al.*, 1997; Muramatsu *et al.*, 2001; Xu *et al.*, 2005b). The mRNAs for the CRH/UCN-R-BP components are dynamically expressed in the primate corpus luteum (CL) during the menstrual cycle, with *UCN* and *CRHBP* expression, up-regulated and down-regulated, respectively, by the primary luteotropin, LH (Xu *et al.*, 2006). *CRH*/*UCN-R* mRNA levels peaked in the macaque CL during either luteal development or optimal progesterone production (i.e. through the early and mid stages of the luteal phase). When CL regression was underway or complete (i.e. late to very late luteal phase), the expression of these components dropped whereas *CRHBP* mRNA levels increased significantly (Xu *et al.*, 2006).

However, detailed studies designed to localize CRH/UCN-R-BP mRNA expression to specific ovarian cell types, analyse the dynamics of protein expression during the mestrual cycle, and evaluate the role(s) of the CRH/UCN-R-BP system in ovary in vivo have not been reported in any species. Therefore, in the present study: (1) the CRH/UCN-R-BP mRNAs were localized to specific cell types within the macaque ovary by in situ hybridization; (2) western blotting was performed to semi-quantitate protein levels in the macaque CL at each stage of the luteal phase during the menstrual cycle; and (3) in vivo protocols were performed to test the hypothesis that a CRHR1 and 2 antagonist, Astressin, would disrupt luteal development and/or function when delivered directly into the periovulatory, luteinizing follicle of the ovary during the spontaneous menstrual cycle in the rhesus monkey.

5.3 Materials and Methods

5.3.1 Animal Treatment, Hormone Assays, and Ovarian Tissue Collection

The general care and housing of rhesus monkeys (*Macaca mulatta*) at the Oregon National Primate Research Center (ONPRC, Beaverton, OR) were described previously in chapter 3 section 3.3.1. Adult female monkeys exhibiting normal menstrual cycles of approximately 28 days were bled daily by saphenous venipuncture beginning 6 days after the onset of menses. Serum was separated and assayed for estradiol (E) and progesterone (P) concentrations by a specific electrochemoluminescent assay using a Roche Elecsys 2010 analyzer (Roche Diagnostics Corporation, Indianapolis, IN) in the Endocrine Services Laboratory, ONPRC (Young *et al.*, 2003). The interassay variations were 6.1% for E and 5.4% for P, and the limits of sensitivity were 5 pg/ml for E and 0.03 ng/ml for P. The first day of low (<100 pg/ml) serum E following the midcycle E peak (>200 pg/ml) typically corresponds with the day after the LH surge, and is therefore termed day 1 of the luteal phase (Duffy *et al.*, 1999). All protocols were approved by the ONPRC Animal Care and Use Committee, and conducted in accordance with NIH Guidelines for the Care and Use of Laboratory Animals.

CL (n = 4/stage) were collected from the early (ECL, day 3-5 post LH surge), mid (MCL, day 6-8), midlate (MLCL, day 10-12), late (LCL, day 14-16) and very late (VLCL, day 17-18; menses) luteal phase of the natural menstrual cycle and frozen in liquid nitrogen for extraction of total protein using T-PER Tissue Protein Extraction Reagent (Pierce Biotechnology, Inc., Rockford, IL). These intervals provided tissues representing the developing, developed functional, on the verge of regressing, regressing and regressed CL, respectively (Young *et al.*, 2002). In addition, ovaries (n = 3/stage) were surgically removed from rhesus monkeys during the preovulatory follicular phase (day 0-1 prior to LH surge) and the early to late luteal phase, fixed overnight in 10% neutral buffered formalin, dehydrated in 70% ethanol solutions, and paraffin-embedded. The 5 μm serial sections were prepared in the SCCPRR's (U54) Imaging and Morphology Core at the ONPRC using an American Optical (Southbridge, MA) microtome and mounted on Superfrost/Plus slides (Fisher, Santa Clara, CA) **(**Hazzard et al., 2000).

5.3.2 In Situ Hybridyzation (ISH)

To identify CRH/UCN-R-BP mRNA in the individual ovarian cell types, ISH was performed as described in the DIG Application Manual (DIG Application Manual for Nonradioactive In situ Hybridization, 3rd edition, Roche Applied Science, Indianapolis, IN) with some modifications. Primers for PCR amplification of CRH/UCN-R-BP cDNAs were synthesized by Invitrogen Corporation (Carlsbad, CA) (Table 5.1). PCR products for ISH probe synthesis were separately subcloned into pGEM-T Easy Vector (Promega Corporation, Madison, WI). Digoxygenin (DIG)-labeled sense riboprobes and complementary antisense (negative control) riboprobes were generated from 1 μg linearized plasmid DNA templates in a 20 μl reaction volume containing 1×transcription buffer, 1×DIG RNA labeling Mix, 20 U of RNase Inhibitor, and 40 U of T7 or SP6 RNA polymerase (Roche Applied Science). After 2 hrs of incubation at 37 ˚C, the DNA template was removed by treatment with 10 U of DNase I (Promega Corporation) for 10 min at 37 ˚C. RNA was precipitated with 1/10 volumes of 5 M LiCl, and 2.5 volumes of -20 ˚C 100% ethanol at -20 ˚C overnight, centrifuged at 16000 g for 15 min, washed with 100 μl -20 ˚C 70% ethanol, centrifuged at 16000 g for 5 min, dried in a vacuum, and resuspended in 100 μl of diethylpolycarbonate-treated water. Probe concentrations were determined empirically in side-by-side comparisons with a RNA standard (Roche Applied Science) on a 1% agarose gel.

Ovarian sections were deparaffinized, hydrated through CitriSolv Clearing Agent (Fisher Scientific, Pittsburgh, PA) and a graded series of ethanol, washed in PBS before and after post-fixation with 4% paraformaldehyde/PBS for 5 min, dehydrated with a graded series of ethanol, and dried in air for 10 min. Sections were then incubated 2-4 hrs with pre-hybridization buffer, 2×SSC containing 50% (v/v) deionized formamide, at room temperature. DIG-labeled RNA probe was diluted to 1:200 in hybridization buffer containing 50% deionized formamide, 10 mM Tris-HCl (pH7.5), 2×SSC, 1×Denhardt's solution, 2.5 mM DTT, 10% Dextran sulfate, 400 μg/ml yeast tRNA, 1 mg/ml denatured and sheared salmon sperm DNA, and 0.5% SDS. Each section was overlayed with 100 μl of diluted DIG-labeled RNA probe, covered with parafilm, and incubated in a humid box containing 2×SSC and 50% deionized formamide at 50°C for 16-20 hrs. Then the slides

Table 5.1 Primer sequences and annealing temperature used for PCR amplification

Gene	NCBI accession number	Primer sequences and annealing temperature
CRH	NM_000756	5'-CGCTGCTCTTATGCCATTT-3' 5'-AACACCTGGAAACGGAAACT-3' 56 °C
UCN	NM_003353	5'-GACCTCACCTTTCACCTGCT-3' 5'-TGCCCCGCATCCCAACTCT-3' 62 °C
UCN2	NM_033199	5'-GCTCGCGCATTGTCCTATC-3' 5'-CTCCAGGTCTTCCCATCCAG-3' 66 °C
CRHR1	NM_004382	5'-CGGGCATTCAGGACGGTA-3' 5'-GCCAGCAGGTGCCAATGA-3' 60 °C
CRHR2	NM_001883	5'-ACCCCCTGAGAAGAGCCA-3' 5'-CGTAACCCACCCAACTGC-3' 58 °C
CRHBP	NM_001882	5'-CATCTTCCTGACGGCTCT-3' 5'-AATGAACTCCTCGGGCTC-3' 66 °C

were washed in 4× and 2×SSC at room temperature, 1× and 0.1×SSC at 42 °C, and DIG Wash Buffer for 10 min, following incubation with Blocking Solution for 1 hr (Roche Applied Science). The slides were incubated with anti-DIG-alkaline phosphatase (Fab fragments, Roche Applied Science) in a humid box for 2 hrs. Then the slides were washed in DIG wash buffer and developed in NBT/BCIP solution (NBT/BCIP ready-to-use tablets, Roche Applied Science) for 30 min. Thereafter, the sections were washed in DIG wash buffer and H_2O, counterstained with nuclear fast red and viewed via Zeiss Axioplan microscopy. A CoolSNAP CCD Camera (Photometrics Inc., Tucson, AZ) was used for image capture.

5.3.3 Western Blotting

To analyze CRH/UCN-R-BP protein expression by luteal tissues throughout the luteal phase of the natural cycle, western blotting for CRH/UCN-R-BP proteins in macaque CL was performed as described previously (Duffy *et al.*, 1997) with some minor modifications. Briefly, CL or positive control proteins (PC-12 + NGF cell lysate for CRH, mouse heart extract for UCN3, U-87 MG cell lysate for CRHR2, mouse liver extract for CRHBP, Santa Cruz Biotechnology, Inc., Santa Cruz, CA; human stresscopin related peptide for UCN2, Phoenix Pharmaceuticals, Inc., Belmont, CA; mouse embryo cell lysate for UCN, CRHR1 recombinant protein, Abnova GmbH, Heidelberg, Germany) plus loading dye were heated at 95 °C for 5 min and loaded onto a 4-20% Tris-HCl Ready Gel using a Mini-Protean II apparatus (Bio-Rad Laboratories, Hercules, CA). After proteins were transferred from the gel to a nitrocellulose membrane (Bio-Rad Laboratories) and washed, the membrane was blocked in 5% powered nonfat milk (Nestle, Inc., Solon, OH)/TBS (Bio-Rad Laboratories) for 1 hr. The membrane then was incubated overnight with primary anti-human antibodies (1:800 for CRH, 1:10 for UCN, and 1:4000 for control β-actin, Abcam Inc., Cambridge, MA; 1:400 for UCN2, Phoenix Pharmaceuticals, Inc.; 1:100 for UCN3 and CRHBP, Santa Cruz Biotechnology, Inc.; 1:100 for CRHR1 and CRHR2, Advanced Targeting Systems, San Diego, CA) in blocking buffer while rocking at 4 °C. Secondary antibodies conjugated with horseradish peroxidase generated against rabbit IgG (Zymed Laboratories, Inc., South San Francisco,

CA) were used at dilutions of 1:4000. CRH/UCN-R-BP proteins were visualized using an ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ) and Kodak X-OMAT film (Eastman Kodak Co., Rochester, NY). Densitometry analysis was performed using a gel documentation system and Quantity One software (BioRad Laboratories).

5.3.4 In vivo Protocol: Intrafollicular Injection and Evaluation

Intrafollicular injection was performed on anesthetized monkeys one day before the midcycle E surge (day -1) as described previously during a laparotomy to expose the ovary bearing the dominant follicle (Hazzard *et al.*, 2002; Xu *et al.*, 2005). A 28 gauge needle on an insulin syringe containing 50 µl solution, either 10 µg CRHR antagonist Astressin (Sigma-Aldrich, St. Louis, MO) or vehicle (0.1% BSA/PBS), was inserted through the stroma of the ovary before penetrating the follicular wall. Then, 50 µl of follicular fluid was aspirated into the syringe, diluting the injectable material by half, before injecting 50 µl of this mixed solution into the follicle. The final delivery doses of Astressin were 5 µg/follicle. Although vehicle treatment has little effect on ovulation and luteal development historically (Hazzard *et al.*, 2002; Xu *et al.*, 2005), a sequential protocol was used typically with vehicle injected during the first experiment cycle (protocol 1), then after a cycle for rest and recovery, Astressin was injected during the following experimental cycle (protocol 2). Ovaries (n = 5/group) were evaluated and video tape recorded by laparoscopy at 3 days postinjection for evidence of ovulation (i.e. presence of a follicular stigmata). Blood samples were collected on a daily basis throughout the expected luteal phase interval for 18 days or until menses, and analyzed for serum E and P levels as described earlier.

In subsequent protocols, monkeys received an intrafollicular injection of either vehicle (n = 3) or the Astressin (5 µg/follicle, n = 3) on day -1. On day 9 of the luteal phase, the ovary bearing the injected follicle was removed by laparoscopy and fixed in formaldehyde overnight before being embedded in paraffin (Hazzard *et al.*, 2002; Xu *et al.*, 2005; Xu and Stouffer, 2005a). The paraffin-embedded tissues were serially sectioned at 5 µm and mounted on the slides. A standard hematoxylin-eosin (H & E) staining was performed for general histologic analyses (Xu and Stouffer, 2005a). Nuclear

DNA fragmentation in cells was detected using the DeadEnd Colorimetric TUNEL System (Promega Corporation) following the manufacturer's instructions with minor modifications as previously reported (Peluffo *et al.*, 2005). Slides were viewed via Zeiss Axioplan microscopy to evaluate luteal development of the injected follicle. A CoolSNAP CCD Camera (Photometrics Inc.) was used for image capture.

5.3.5 Statistical Analysis

Statistical evaluation of mean differences in protein levels (western blotting densitometric analysis) among stages of the luteal phase was performed by one-way ANOVA, with a significance level set at 0.05, using the SigmaStat statistical software package, version 2.0 (SPSS Inc., Chicago, IL). To identify significant differences between stages, the Student-Newman-Keuls post-hoc test was used for pairwise multiple comparisons. P and E levels in serum were analyzed by analysis of variance with repeated measures to identify differences between the controls and treatment group during the luteal phase (SigmaStat software). Differences were considered significant at $P < 0.05$ and values are presented as mean ± SEM. A Student's t-test was used to compare length of the luteal phase between control and Astressin-treated animals.

5.4 Results

5.4.1 CRH/UCN-R-BP mRNA Localization in the Monkey Ovary by ISH

In the late follicular phase, there was minimal specific blue ISH staining (compared to negative controls; not shown) for ligands in the preovulatory follicle, atretic antral follicles or smaller preantral follicles (Fig. 5.1A, B). However, specific staining for *CRH*, *UCN*, and *UCN2* mRNAs was observed in the developing CL during the early luteal phase (Fig. 5.1C; Fig. 5.2A). *CRH* staining was also appreciable in the small preantral follicles in the ovarian cortex in the early luteal phase (Fig. 5.1C). In CL removed from the different stages of the luteal phase, specific staining for *CRH* was apparent throughout the luteal phase (Fig. 5.2A-C). In contrast, intense staining for both *UCN* and *UCN2* were most evident in the CL at early and mid luteal phase (Fig. 5.2A, B), but then appeared to

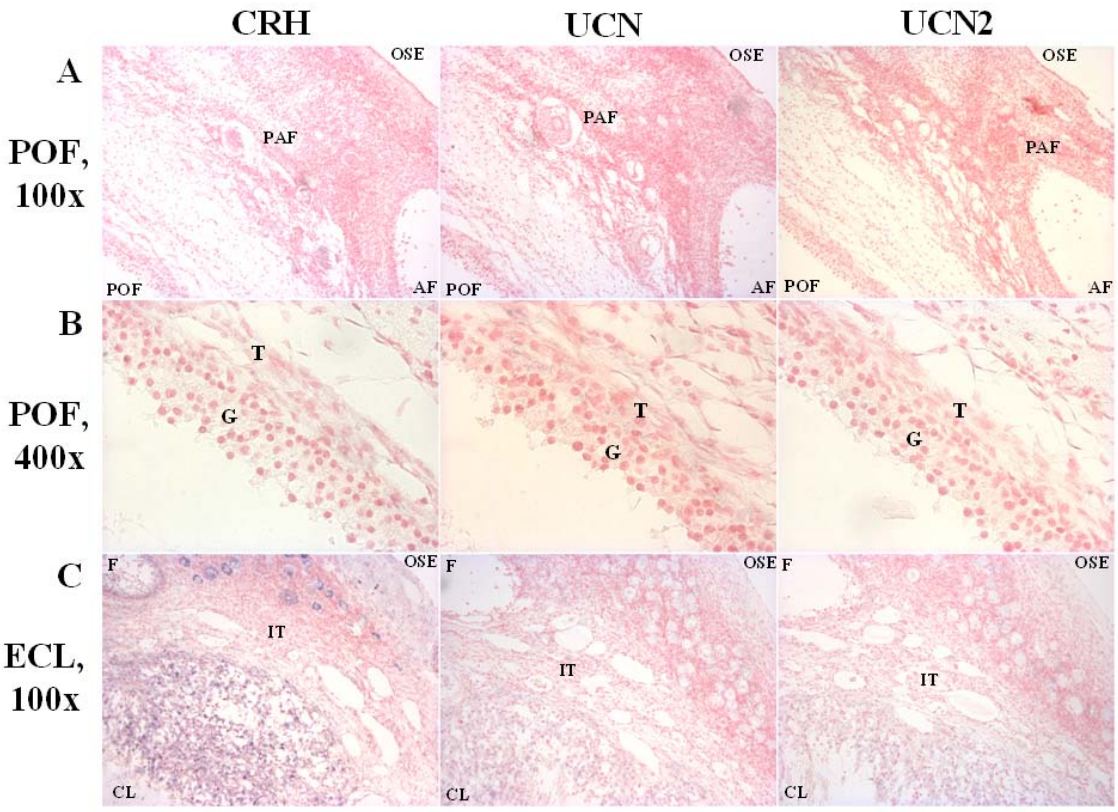


Fig. 5. 1 In situ localization of CRH/UCN ligand mRNAs in the rhesus macaque ovary containing the preovulatory follicle (POF) at original magnification ×100 (A) or ×400 (B) and the early CL (C; ECL, original magnification ×100). POF, preovulatory follicle; PAF, preantral follicle; F, antral follicle; AF, atretic follicle; OSE, ovarian surface epithelium; G, granulosa cells; T, theca cells; CL, corpus luteum; IT, interstitial tissue.

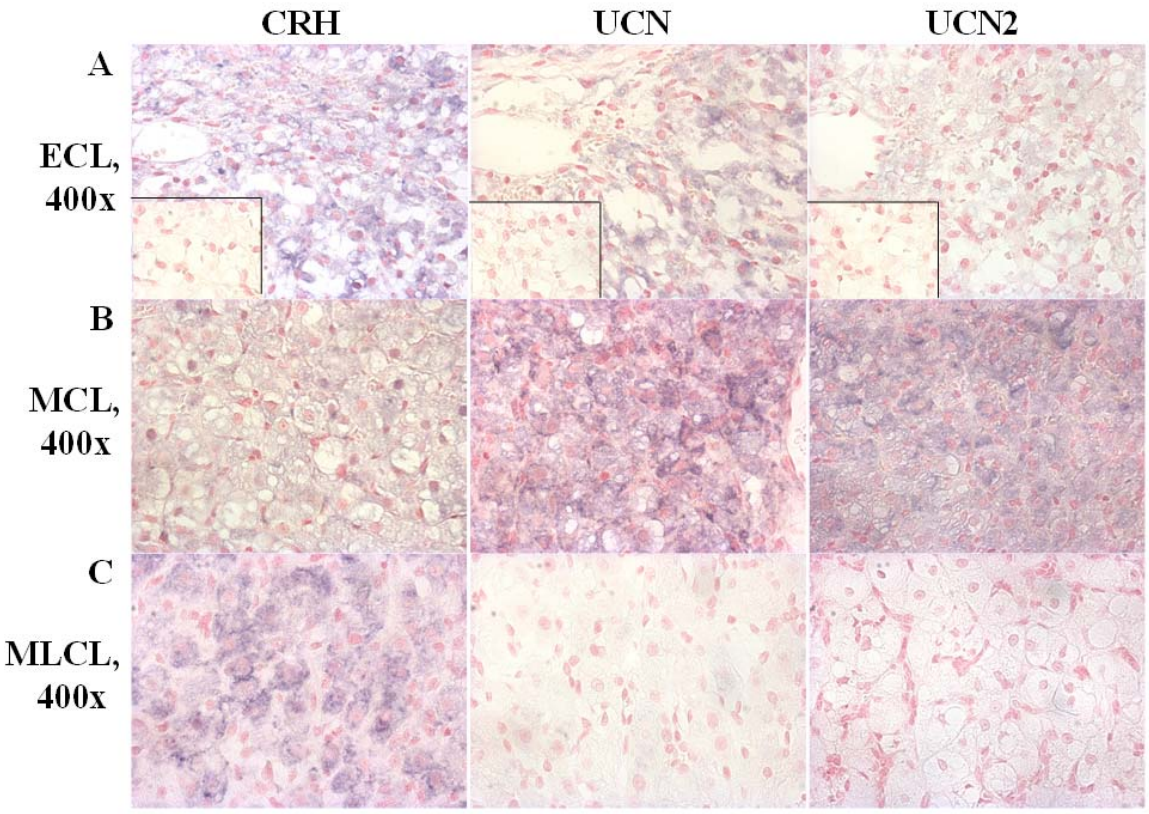


Fig. 5.2 In situ localization of CRH/UCN ligand mRNAs in the rhesus macaque CL during the early (A; ECL, original magnification ×400), mid (B; MCL, ×400), and midlate (C; MLCL, ×400) stage of the luteal phase. The non-specific staining associated with the pre-absorbed primary antibody is illustrated in the inset of panel A.

decrease as the CL approached regression in the midlate luteal phase (Fig. 5.2C). The positive mRNA staining appeared to concentrate in the cytoplasm of granulosa-lutein cells of the CL (Fig. 5.2A-C). Cell nuclei were counterstained red. No significant staining was evident in control sections processed with complement sequence probes (bottom left of Fig. 5.2A; representative ECL section shown).

During the late follicular phase, specific blue ISH staining for *CRHR1* and *CRHR2* mRNA was evident in theca cells of the preovulatory follicle and the surrounding stromal layer, as well as modest staining (notably for *CRHR2*) in the granulosa layer of the preovulatory follicle (Fig. 5.3A, B). Appreciable staining for *CRHR1* and *CRHR2* was detected in the developing CL, as well as interstitial cells in the ovarian stroma (Fig. 5.3C; Fig. 5.4A). Receptor staining was also observed in small preantral follicles during the early luteal phase, but not preovulatory follicular phase (Fig. 5.3A, C). In contrast, ISH staining for *CRHBP* was not evident in small preantral and atretic follicles or interstitial tissue, while relatively modest staining was observed in both the granulosa and theca cells of the preovulatory follicle (Fig. 5.3A, B) and in the developing CL during the early luteal phase (Fig. 5.3C; Fig. 5.4A). In CL isolated from the different stages of the luteal phase, the mRNA expression of *CRHR*s was detected at high levels from ECL to MCL (Fig. 5.4A, B), before diminishing in the LCL (Fig. 5.4C). Although modest in situ staining for *CRHBP* mRNA was detectable early in the CL lifespan (Fig. 5.4A), minimal staining was observed in the MCL (Fig. 5.B). The most intense *CRHBP* staining occurred in CL from the late luteal phase (Fig. 5.4C). Receptor specific staining appeared to be concentrated in granulosa-lutein cells, while the *CRHBP* presented in both theca-lutein and granulosa-lutein cells (Fig. 5.4A-C). Cell nuclei were counterstained red. No receptor or binding protein staining was evident in the endothelial cells or in control sections processed with complement sequence probes (bottom left of Fig. 5.4A; representative ECL section shown).

5.4.2 CRH/UCN-R-BP Protein Levels in the Macaque CL by Western Blotting

As depicted in representative western blotting (Fig. 5.5), the proteins for CRH, UCN and UCN2, but not UCN3 (which was identified in monkey testis; data not shown),

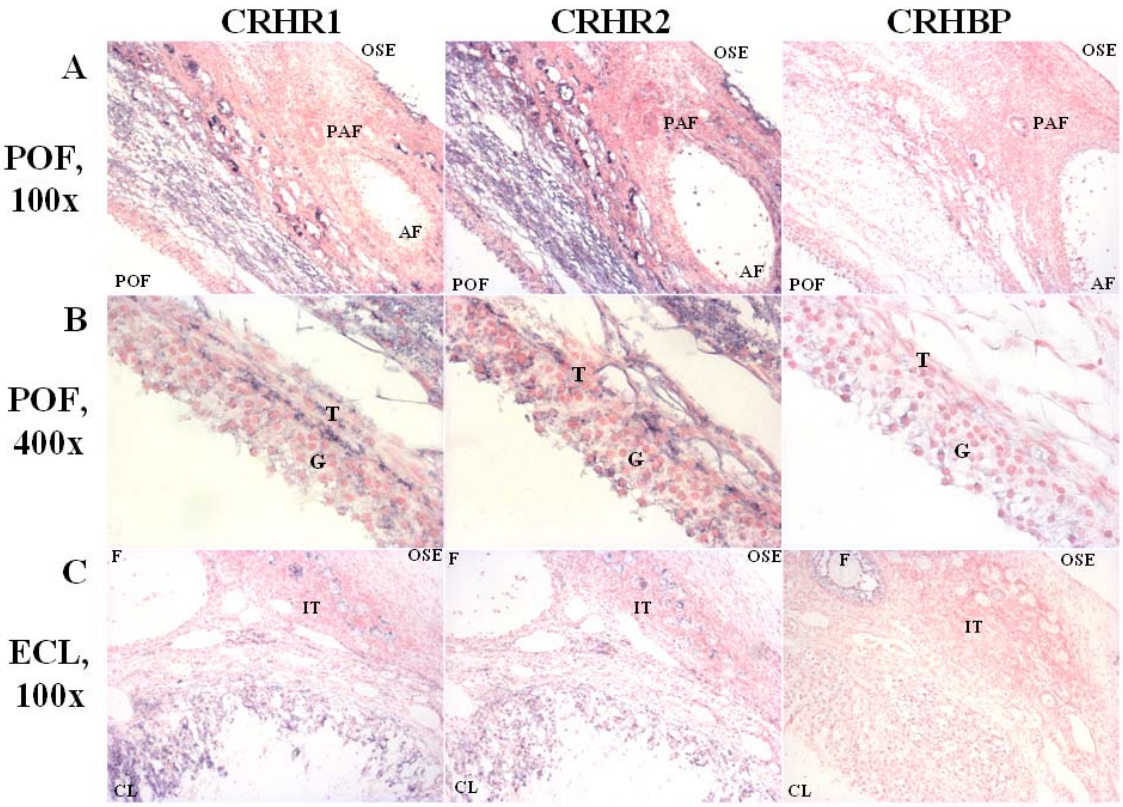


Fig. 5.3 In situ localization of CRH/UCN receptor and binding protein mRNAs in the rhesus macaque ovary containing the preovulatory follicle (POF) at original magnification ×100 (A) or ×400 (B) and the early CL (C; ECL, original magnification ×100). POF, preovulatory follicle; PAF, preantral follicle; F, antral follicle; AF, atretic follicle; OSE, ovarian surface epithelium; G, granulosa cells; T, theca cells; CL, corpus luteum; IT, interstitial tissue.

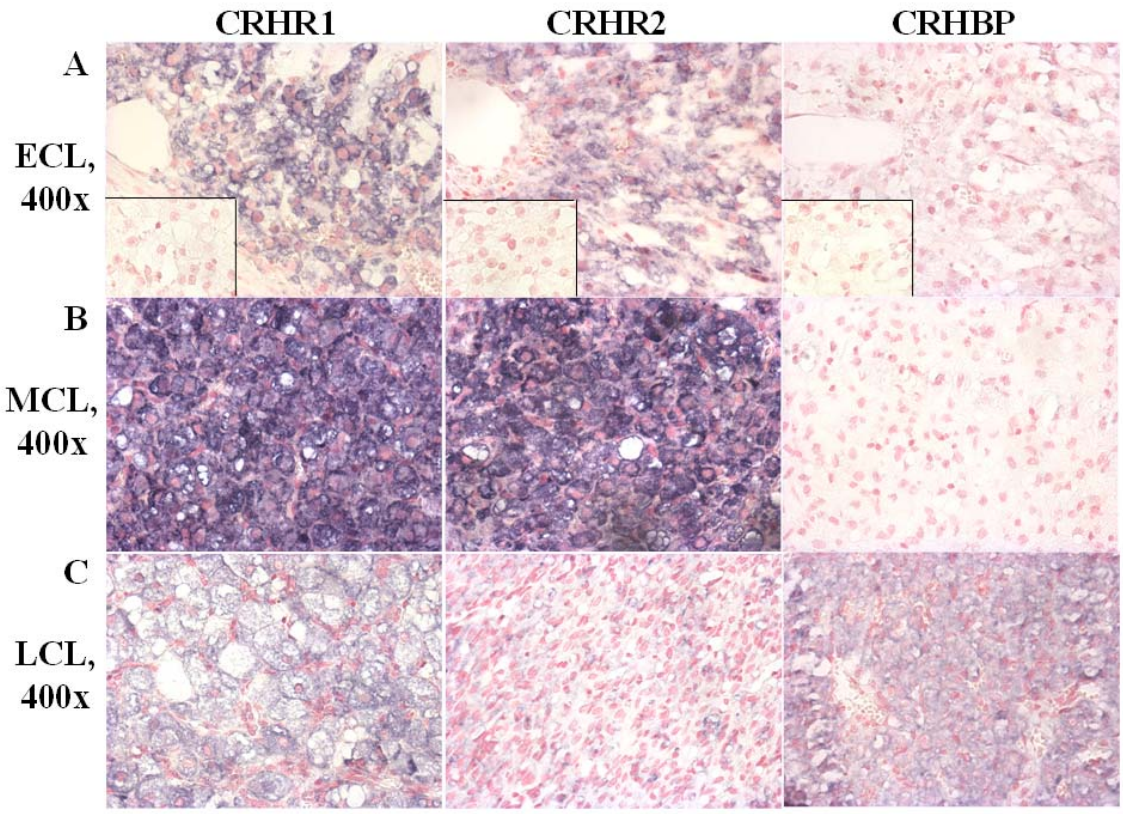


Fig. 5.4 In situ localization of CRH/UCN receptor and binding protein mRNAs in the rhesus macaque CL during the early (A; ECL, original magnification ×400), mid (B; MCL, ×400), and late (C; LCL, ×400) stage of the luteal phase. The non-specific staining associated with the pre-absorbed primary antibody is illustrated in the inset of panel A.

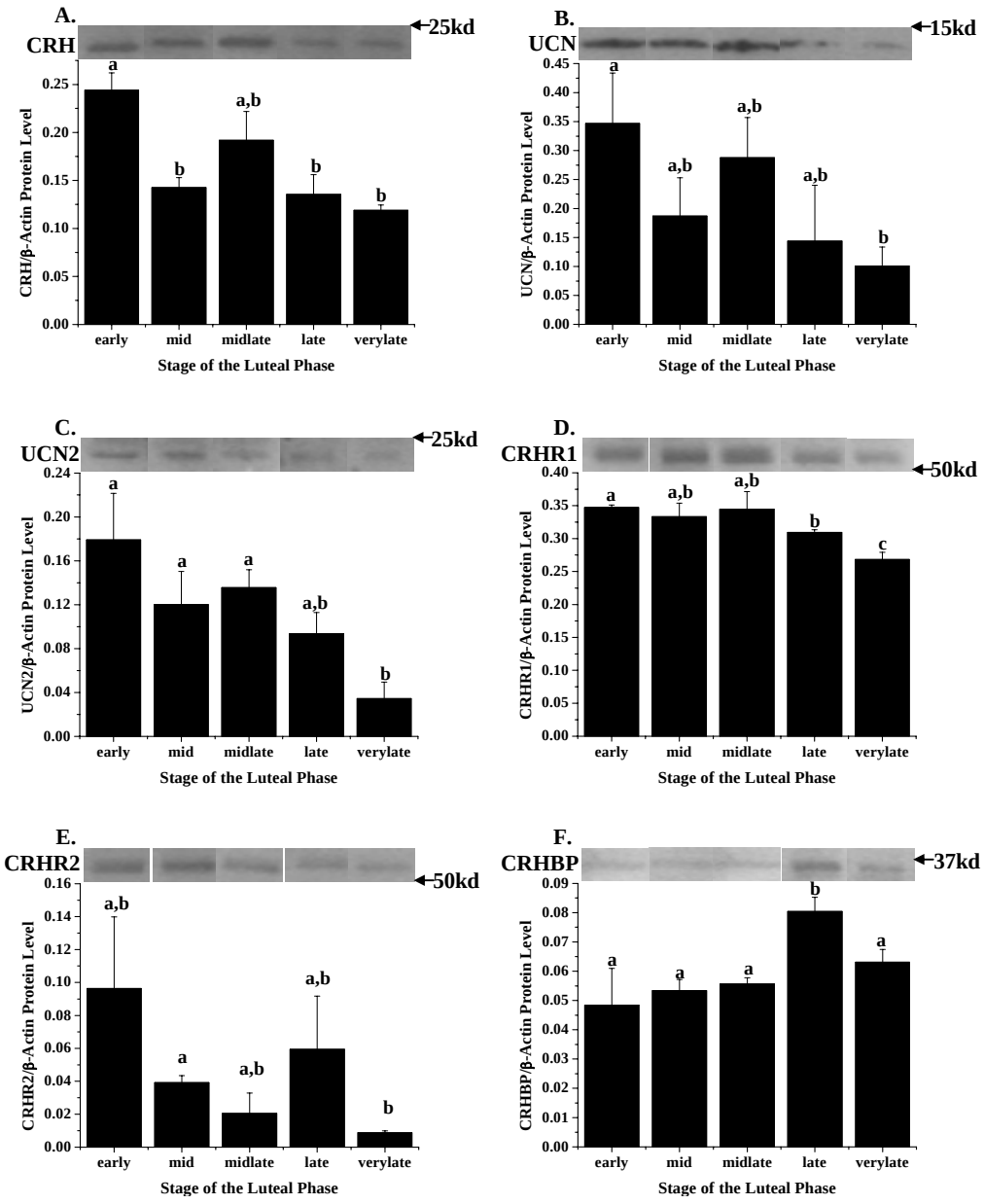


Fig. 5.5 Mean (± SEM) protein levels for CRH/UCN-R-BP components relative to β-actin in the rhesus macaque CL throughout the luteal phase of the natural menstrual cycle. The total protein for western blotting was generated from CL (n = 4/stage) collected during the early (day 3-5 post LH surge), mid (day 6-8), midlate (day 10-12), late (day 14-16), and very late (day 18-19; menses) luteal phase. Values were standardized to respective β-actin protein control values. Columns with different letters are significantly ($P < 0.05$) different.

were expressed in the macaque CL throughout its lifespan in the menstrual cycle. Likewise, proteins for the CRHR1, CRHR2, as well as CRHBP, were identified in macaque CL.

When normalized to β-actin (which were invariant during the CL lifespan) after densitometric analysis, protein levels of CRH were high in the ECL and then decreased in the MCL ($P < 0.05$) (Fig. 5.5A). UCN protein levels were detectable throughout, but decreased ($P < 0.05$) in the VLCL compared to ECL (Fig. 5.5B). Compared to the early-midlate stage, protein levels for UCN2 was decreased significantly ($P < 0.05$) in the VLCL (Fig. 5.5C). CRHR1 protein levels were highest from the ECL to MLCL before gradually declined in the LCL to VLCL ($P < 0.05$) (Fig. 5.5D). CRHR2 protein levels decreased ($P < 0.05$) in the VLCL relative to MCL (Fig. 5.5E). In contrast, CRHBP levels were higher ($P < 0.05$) at the LCL than the other stages (Fig. 5.5F).

5.4.3 Effects of Injection into the Preovulatory Follicle

All the control and Astressin-treated monkeys (n = 5/group) displayed a typical stigmata/rupture site on the ovary bearing the antecedent preovulatory follicle by 3 days post-injection (Fig. 5.6A, B). After the preovulatory surge, E levels declined by early luteal phase (day 2) in both groups. Thereafter, serum E concentrations increased gradually through the luteal phase in control monkeys, whereas the E levels of the Astressin-treated monkeys remained at baseline and were diminished ($P < 0.05$) in midlate luteal phase (20-30 pg/ml) (Fig. 5.7A). Serum P concentrations increased in both control and Astressin-treated monkeys during the early luteal lifespan (day 1-4). However, Astressin administration significantly ($P < 0.05$) suppressed P levels during the mid (day 5) to midlate (day 13) stage of the luteal phase, compared to those in control monkeys (Fig. 5.7B). All the control monkeys began menses on day 17 of the luteal phase, whereas menstruation in Astressin-treated monkeys began on day 13 ± 1.8 ($P < 0.06$) with two of them mensing at day 8 and 9 respectively.

On the day of ovariectomy (day 9), P levels were significantly lower in Astressin-treated versus control animals (2.2 ± 0.5 vs. 4.6 ± 0.0 ng/ml; n = 3; $P < 0.05$). There was an empty cavity lined by a single layer of cells in the CL following Astressin treatment

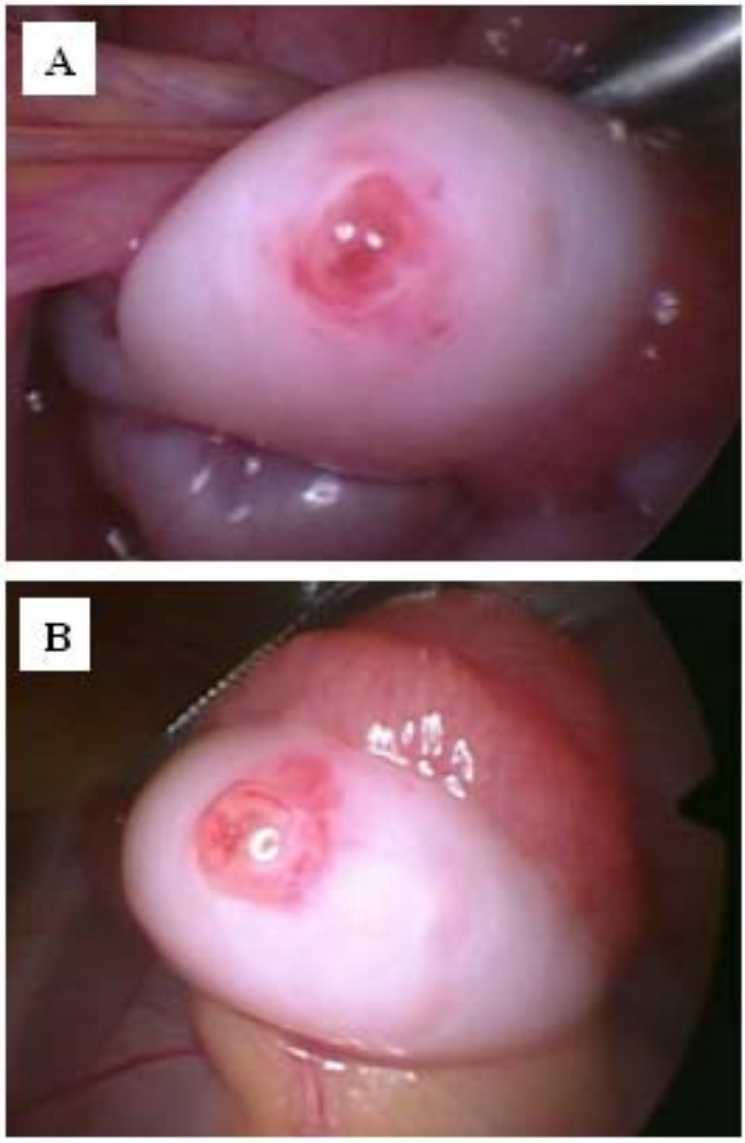


Fig. 5.6 Ovarian indices of follicular rupture (ovulation) by laparoscopic evaluation on day 3 after intrafollicular treatment with vehicle (0.1% BSA/PBS, A) and Astressin (5 μg/follicle, B).

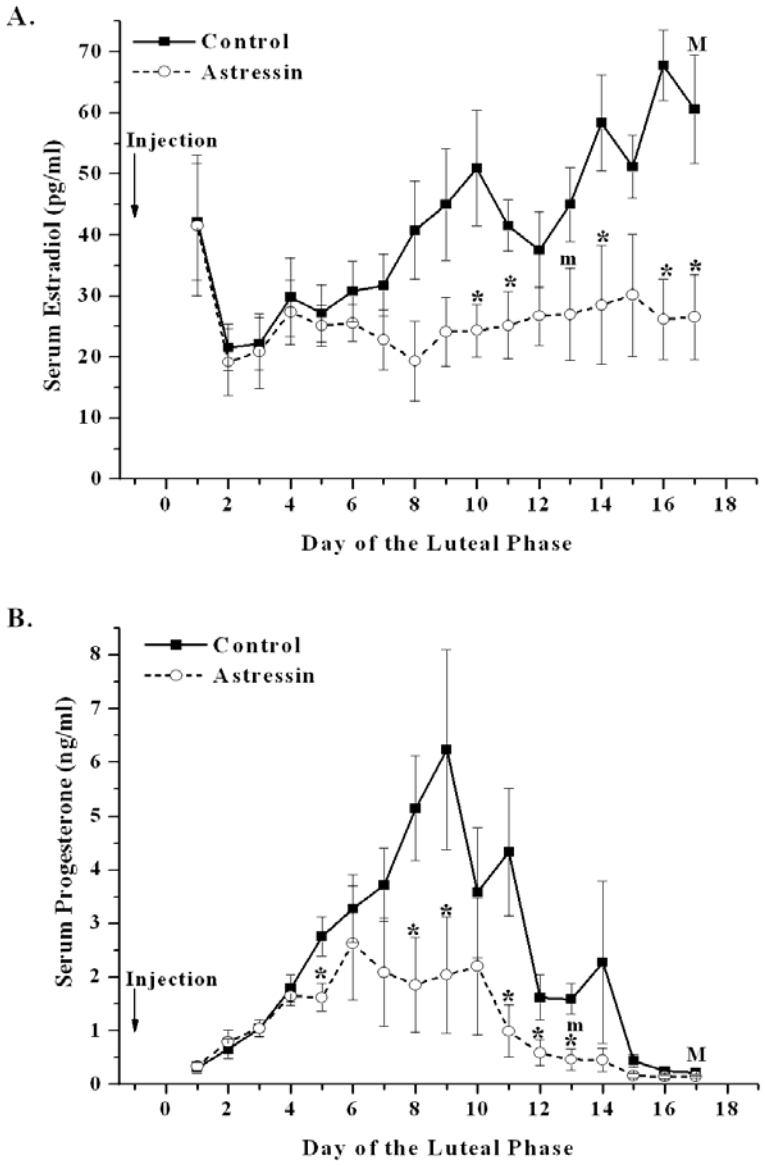


Fig. 5.7 Serum estradiol (A) and progesterone (B) concentrations (mean ± SEM) during the luteal phase in rhesus macaque (n = 5/group) receiving an intrafollicular injection (arrow) of 5 µg Astressin compared with that of vehicle-controls (0.1% BSA/PBS). Small (m) depicts the average length of luteal phase for Astressin-treated animals, while the large (M) denotes the average length of luteal phase for the controls. Asterisks represent significant differences ($P < 0.05$) between groups in estradiol or progesterone concentrations on specific days.

(Fig. 5.8A, B), whereas control CL was filled with luteal cells (not shown). There was no obvious histologic difference between luteal tissue of both groups, as judged by H & E staining (not shown). However, TUNEL staining showed that there were large cells with brown nuclei (Fig. 5.8D, open arrows) and small cells with condensed fragmented nuclei (Fig. 5.8D, black arrows) in the CL following Astressin treatment. TUNEL staining was not found in the CL following PBS/BSA treatment (Fig. 5.8C), negative control without the TdT enzyme (Fig. 5.8D), nor in the surrounding stroma (not shown) where the healthy nuclei were stained blue.

5.5 Discussion

This is the first report detailing the localization of CRH/UCN-R-BP mRNAs in the primate ovary during the preovulatory stage and luteal phase of the ovarian cycle. There was no obvious mRNA staining for ligands in the macaque ovary during the preovulatory phase. However, *CRHR1* and *CRHR2* mRNAs were detected in the theca and, to a lesser extent, the granulosa cells of the preovulatory follicle, as well as the interstitial cells in the ovarian stroma. Thus, if the CRH/UCN-R system is active in the preovulatory follicle, it may require circulating ligands originating from outside the ovary. Notably, both ligand (*CRH*) and *CRHR* mRNAs were detected in small preantral follicles in the macaque ovary during the early luteal phase, but not the preceding preovulatory stage. *CRH* and *CRHR*s expression after the midcycle LH/FSH surge suggests that ligand-receptor action modulates early follicle growth. Previous studies focused on selected components of the CRH/UCN-R-BP system in developing antral follicles. For example, *CRHR1* was localized to theca cells of human antral (7-8 mm in diameter) follicles (Asakura *et al.*, 1997) and the stroma and theca cells of the rat ovulatory follicles (Nappi and Rivest, 1995), which is consistent with our observation.

In contrast to the preovulatory follicle, ligand (*CRH*, *UCN*, and *UCN2*) and receptor (*CRHR1* and *CRHR2*) mRNAs were localized to the granulosa-lutein cells, but not theca-lutein and endothelial cells, in the CL by the early luteal phase. This suggests that differentiating steroidogenic cells in the CL are the sites of *CRH*/*UCN*s synthesis and

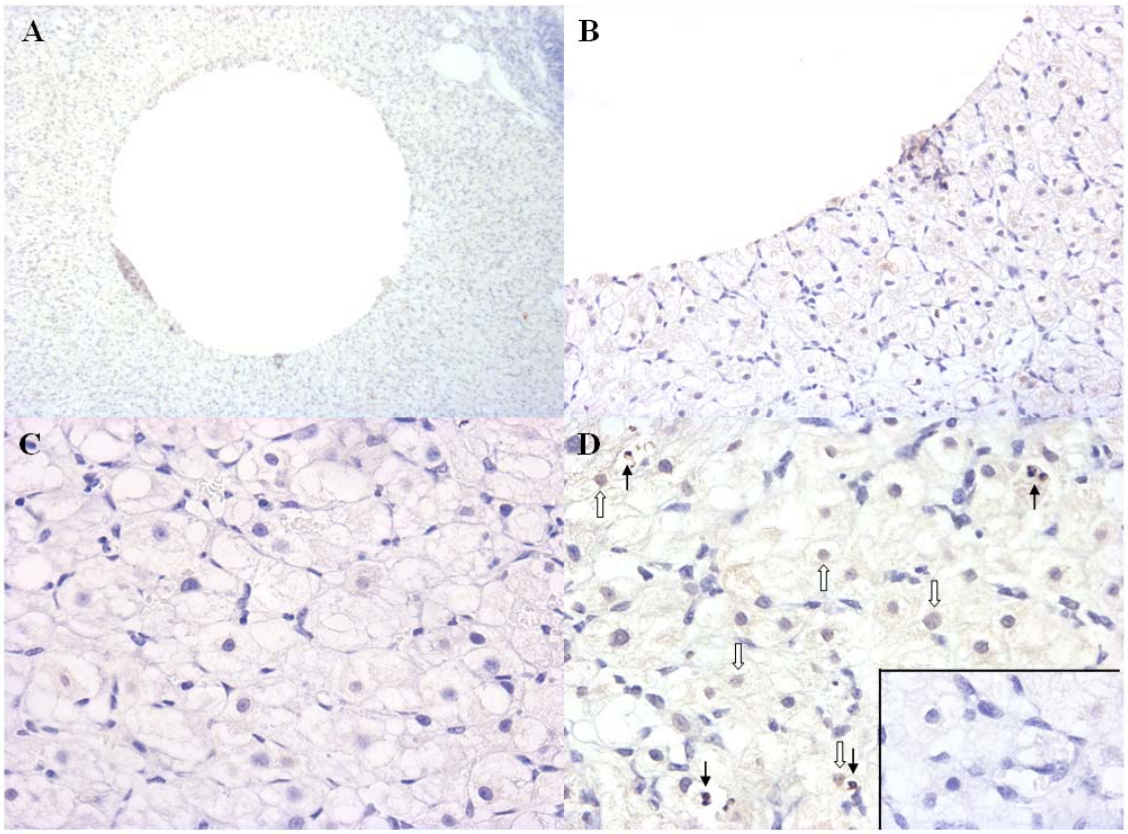


Fig. 5.8 Terminal deoxynucleotidyl transferase nick end labeling (TUNEL) of CL obtained from control (PBS/BSA, C) and Astressin-treated (A, B, and D) rhesus macaques. The negative control without the TdT enzyme is illustrated in the inset of panel D. A, original magnification×50; B, ×200; C and D, ×400; open arrows, large cells with brown nuclei; black arrows, small cells with condensed nuclei.

CRHR-mediated action. Except *CRH*, a high level of staining intensity for other ligands and receptors was observed in luteal cells in the early-to-mid stage of the luteal phase, suggesting that processes causing (e.g., the mid cycle LH surge) or associated with (e.g., LH-induced local factors) follicle luteinization and/or luteal development influence *UCN*s-*R* expression. Subsequently, *UCN*s and receptor staining declined in the CL during the later stages of the luteal phase. In general, the ISH localization of ligand/receptor mRNAs to luteal cells, particularly in the early-to-mid luteal phase, is consistent with immunohistochemical localization of the corresponding proteins in the macaque CL (Xu *et al.*, 2006).

In contrast, *CRHBP* mRNA was detected in both the theca and granulosa layer of the preovulatory follicle and the theca-lutein and granulosa-lutein cells of the CL, whereas CRHBP immunostaining was limited to only theca and theca-lutein cells (Xu *et al.*, 2006). Since CRHBP is a secreted protein, it may be sequestered in the theca layer of the follicle or paraluteal region of the CL through unknown mechanisms. Unlike ligand-receptor mRNAs, *CRHBP* mRNA staining in the CL increased in the late luteal phase compared to other stages. Asakara et al. (Asakura et al., 1997) could not detect *CRHBP* mRNA expression in the human follicle, and proposed that CRHBP protein in the ovary originated from the circulation. However, based on our current findings and published studies (Xu *et al.*, 2005b; 2006), *CRHBP* is expressed in the macaque antral follicle and CL.

The western blotting data were consistent with our previous RT- and real-time PCR results (Xu *et al.*, 2006) indicating that all ligands, except UCN3 (data not shown), were expressed in the rhesus macaque CL. The expression of both receptors and the binding protein was also detected. The CRH/UCN-R-BP mRNAs (Xu *et al.*, 2006) and proteins (current study) were dynamically expressed during the luteal lifespan in the menstrual cycle. CRH protein levels were high at the early stage of the luteal phase compared with the later stages, much like our previous immunohistochemistry (IHC) results (Xu *et al.*, 2006). However, *CRH* mRNA levels didn't change throughout the luteal lifespan according to our real-time PCR (Xu *et al.*, 2006) and current ISH analysis. Thus, the dynamic expression of CRH may due to post-transcriptional regulation. Both mRNA and

protein levels of the UCNs and CRHRs were high at early stage of the luteal phase before declining at later stages, which are supported by the IHC and ISH results. However, the decrease in protein levels lagged to some extent behind the mRNA. For example, *UCN* and *CRHR1* mRNA levels declined abruptly at midlate luteal phase (Xu *et al.*, 2006), whereas protein levels did not decline significantly until the late-to-very late luteal phase. In contrast, the protein level changing pattern of CRHBP corresponded well with the mRNA data, which was supported by IHC and ISH analysis. Collectively, the data indicate that *CRH*/*UCN* and *CRHR1*/*CRHR2* mRNA and protein expression peaked during either CL development or optimal progesterone production (i.e. through the early-to-midlate stages). When CL regression was underway or complete (i.e. late-to-very late luteal phase), however, the expression of these proteins declined. In contrast, *CRHBP* mRNA and protein expression increased significantly in the CL during luteal regression. Previous studies demonstrated that significant CRH immunostaining was observed in developing CL and less prominent or totally absent in regressing CL of rodents and women (Mastorakos *et al.*, 1993; 1994), which are consistent with our observations. However, Muramatsu et al. (Muramatsu *et al.*, 2001) reported higher levels of immunoreactive CRH and UCN in the regressing CL relative to the developing CL in women. This discrepancy may due to the differences between species, methods for classifying stages of the luteal phase, and/or techniques employed.

The increased expression of CRH/UCN-CRHR1/CRHR2 in CL during early-to-mid luteal phase and its potential neutralization by increased CRHBP expression during late luteal phase suggests that this ligand-receptor system may promote CL structure and function. Evidence that the primary luteotropin LH increases UCN2 expression and suppresses CRHBP expression supports this hypothesis (Xu *et al.*, 2006). Therefore, an in vivo study was designed to block local CRH/UCN-R action in the preovulatory follicle at midcycle, to determine if periovulatory events or subsequent CL development were altered. Astressin is a CRH-modified analog, a general antagonist with high affinity for both CRHR1 and CRHR2 (Gulyas *et al.*, 1995). It has proven particularly potent at inhibiting CRH function in the hypothalamo-pituitary-adrenal axis in rhesus monkeys (Broadbear *et al.*, 2004). Notably, acute exposure of Astressin, by injection into the

preovulatory follicle 2-3 days prior to expected ovulation had no effect on follicle rupture. This negative result contrasts with intrafollicular injection of antagonists to other ovarian local factors, e.g. prostaglandins (PGs, Duffy and Stouffer, 2002) and angiopoietin (ANGPT2, Xu and Stouffer, 2005a), which blocked ovulation. Therefore, CRH/UCN ligands do not appear to regulate ovulation in monkeys.

However, exposure of the ovulatory, luteinizing follicle to CRHR antagonist reduced circulating E and P levels by the mid-to-midlate luteal phase of the menstrual cycle. Thus, CRH/UCN-R-BP system ligand-receptor action may promote luteal steroidogenic function. Several reports indicate CRH can stimulate steroidogenesis in mammals. CRH increases estrogen biosynthesis in cultured human placental cells (You *et al.*, 2006) and also stimulates, while CRH-antagonist inhibits, progesterone release in cultured rat granulosa cells (Roguski *et al.*, 2000). CRH treatment of mouse Leydig cell cultures stimulates steroidogenesis by increasing the synthesis of the steroidogenic acute regulatory (StAR) protein that plays a critical role in facilitating the transfer of cholesterol across the mitochondrial membrane (Huang *et al.*, 1995; 1997). In human fetal adrenal cells, CRH induced cholesterol side chain cleavage cytochrome P450 (CYP11A), which initiates steroid hormone biosynthesis and 3β-hydroxysteroid dehydrogenase (HSD3B), which is also involved in progesterone biosynthesis in the CL (Sirianni *et al.*, 2005). However, there are also reports that CRH may suppress ovarian steroidogenesis. CRH exerts an inhibitory effect on estrogen and progesterone production in cultured human granulosa-lutein cells (Calogero *et al.*, 1996; Ghizzoni *et al.*, 1997), and inhibited androgen production in isolated theca cells (Erden *et al.*, 1998).

The cavity observed in the CL following Astressin treatment at midcycle also suggests that the CRH/UCN-R action promotes luteal development. One of the hallmarks of luteinization is the further differentiation of the follicle wall into CL through tissue remodeling (Chaffin and Stouffer, 2000). A previous study indicated that CRH is a potent inducer of cell differentiation in fetal lung explants of baboons (Emanuel *et al.*, 2000). In addition, the expression and activity of matrix metalloproteinases (MMPs) have been implicated in the remodeling of extracellular matrix that occurs in the CL throughout the cycle (Smith *et al.*, 1999; Young *et al.*, 2002). CRH and UCN were found to induce

secretion of MMPs by cultured cells from human placenta and fetal membranes (Li and Challis, 2005). Based on these and the results of the current study, CRH/UCN ligands may promote luteal formation and development perhaps via proteases.

Astressin treatment also shortened the luteal lifespan in some animals and appeared to promote luteal cell DNA degradation as observed by TUNEL analysis. This suggests that blocking CRH/UCN-R action promoted early luteolysis, probably via apopotosis, as early as mid luteal phase. Increasing evidence supports the theory that apoptosis is an important mechanism controlling involution of the CL. Apoptotic indices have been reported during structural involution of the CL in rodents and primates (Bowen *et al.*, 1999; Peluffo *et al.*, 2005; Shikone *et al.*, 1996). In addition, CRH/UCN peptides reportedly protect diverse cell types from environmental insults, a so-called cytoprotective effect, which could maintain cell health and prevent apoptosis. CRH/UCN was proposed to provide protection to rat hippocampal neurons (Fox *et al.*, 1993; Pedersen *et al.*, 2002) and cardiac myocytes from necrotic and apoptotic death (Brar *et al.*, 1999; 2000). CRH also acts as a cytoprotective agent in the X. *laevis* tadpole tail, with CRHBP blocking CRH action and hastening tail muscle cell death (Boorse *et al.*, 2006). Thus, CRH/UCN ligands may provide protection to luteal cells during luteal development and function at the early stage as a luteotropic factor. Also, CRHBP neutralization of CRH/UCN bioactivity may promote luteal cell death through apoptosis during luteal regression.

In summary, the current study provides detailed information regarding the specific ovarian cell types synthesizing the CRH/UCN-R-BP components, and the dynamic expression of the CRH/UCN-R-BP protein in the primate CL during the menstrual cycle. The pattern of the CRH/UCN-R-BP protein expression during the CL lifespan suggests that CRH/UCN regulate luteal processes primarily during the early-to-mid luteal phase when there is greater expression of ligands/receptors and less expression of binding protein. By the late luteal phase, however, their luteal activities may be restricted as the expression of the ligands/receptors decreases and CRHBP increases. This is consistent with the hypothesis that ligand-receptor action promotes luteal development and/or structure-function, and its loss is associated with luteal regression. Evidence from direct

injection of a CRHR antagonist into the preovulatory follicle supports the concept that the CRH/UCN-R system promotes luteal development and function, but not the ovulatory process in primates. However, whether CRH/UCN-R-BP system acts directly or indirectly and which cellular processes are regulated by this local system is unknown. Many issues remain to be resolved at the systemic, cellular, and molecular levels to understand the activities of the primate CL and its regulation by endocrine and local factors. CHR/UCN-R-BP system may represent a novel regulatory system that functions in luteinization and luteolysis.

CHAPTER 6
SUMMARY

The events that promote luteal development after the LH surge or maintain luteal structure-function in a luteotropic milieu remain poorly understood. The luteinizing follicle and CL in primates contain specific high-affinity receptors for LH (Cameron *et al.*, 1982). But how LH receptor-stimulated signal transduction, e.g. cAMP-mediated pathways (Eyster *et al.*, 1985), controls luteal structure-function is unclear. Researchers are discovering that local factors combine with, or mediate actions of, luteotropic hormones, e.g. LH, to control the functional lifespan of the CL. The current results indicate that the local CRH/UCN-R-BP system in the CL warrants further scrutiny. Nevertheless, a physiologic role for this system in regulating the primate CL during the menstrual cycle remains unsubstantiated.

Evidence for diverse biological functions of the CRH/UCN-R-BP system in peripheral tissues may be relevant to its proposed local luteotropic action in the CL.

6.1 Steroidogenesis

This dissertation research suggests that the LH-regulated CRH/UCN-R-BP system functions during the luteal phase to promote progesterone production by the primate CL. A number of reports indicate that CRH can stimulate steroidogenesis in mammals. CRH elicited steroidogenesis in cultured human adrenal cells in a dose-dependent manner and the inclusion of an antagonist blocked CRH-induced stimulation (Parker *et al.*, 1999). CRH increased estrogen biosynthesis in cultured human placental cells (You *et al.*, 2006) and also stimulated, while CRH-antagonist inhibited, progesterone release in cultured rat granulosa cells (Roguski *et al.*, 2000).

To achieve steroidogenic activity, it appears that at least two major pathways are

enhanced in luteal cells: (1) cell-specific amplification of steps in the steroidogenic pathway converting cholesterol to progesterone; and (2) increased ability to sequester blood-borne sources of cholesterol and to store cholesterol-rich lipid deposits. CRH treatment of mouse testis Leydig cell cultures results in a dose-dependent stimulation of steroidogenesis by acting through the cAMP/PKA second messenger pathway (Huang *et al.*, 1995). It occurs as a result of an increase in the synthesis of the steroidogenic acute regulatory (StAR) protein that plays a critical role in trophic hormone-stimulated steroid biosynthesis by facilitating the transfer of cholesterol across the mitochondrial membrane (Huang *et al.*, 1997). In human fetal adrenal cells, CRH induced cholesterol side chain cleavage cytochrome P450 (CYP11A) and 3 beta-hydroxysteroid dehydrogenase (HSD3B) which initiate steroid hormone biosynthesis by converting cholesterol to progesterone (Sirianni *et al.*, 2005). However, whether CRH acts via cAMP-mediated pathways to stimulate steroidogenesis at similar steps in the primate CL is unknown.

There are also reports that CRH may suppress ovarian steroidogenesis. CRH exerts an inhibitory effect on estrogen and progesterone production in cultured human granulosa-lutein cells (Calogero *et al.*, 1996; Ghzzoni *et al.*, 1997), and inhibited androgen production in cultured theca cells from human follicles (11-13 mm in diameter) (Erden *et al.*, 1998). This discrepancy may due to the differences between species (e.g. in CRHR expression), methods for classifying ovarian/luteal stages, and/or techniques employed.

6.2 Cell Differentiation and Tissue Remodeling

One of the hallmarks of luteinization is the further differentiation of the follicle wall into a progesterone-secreting gland, the CL. The luteal cavity discovered in this dissertation research after intrafollicular injection of Astressin suggests that CL development is incomplete or disrupted by blocking CRH/UCN ligand-receptor action. A previous study indicated that CRH is a potent inducer of cell differentiation in baboon fetal lung explants (Emanuel *et al.*, 2000). In addition, proteinases are involved in tissue remodeling processes, e.g. matrix metalloproteinases (MMPs), which participate in

extracellular matrix remodelling and degradation (Hijova *et al.*, 2005). The expression and activity of MMPs have been implicated in the remodeling of extracellular matrix that occurs in the ovarian follicle and CL throughout the cycle (Chaffin and Stouffer, 1999; Young *et al.*, 2002). CRH and UCN were found to induce secretion of MMPs by cultured cells from human placenta and fetal membranes (Li and Challis, 2005). Based on these and the results of this dissertation research, CRH-like peptides may promote luteal formation by regulating tissue remodeling, including proteases.

6.3 Cytoprotection and Cell Death

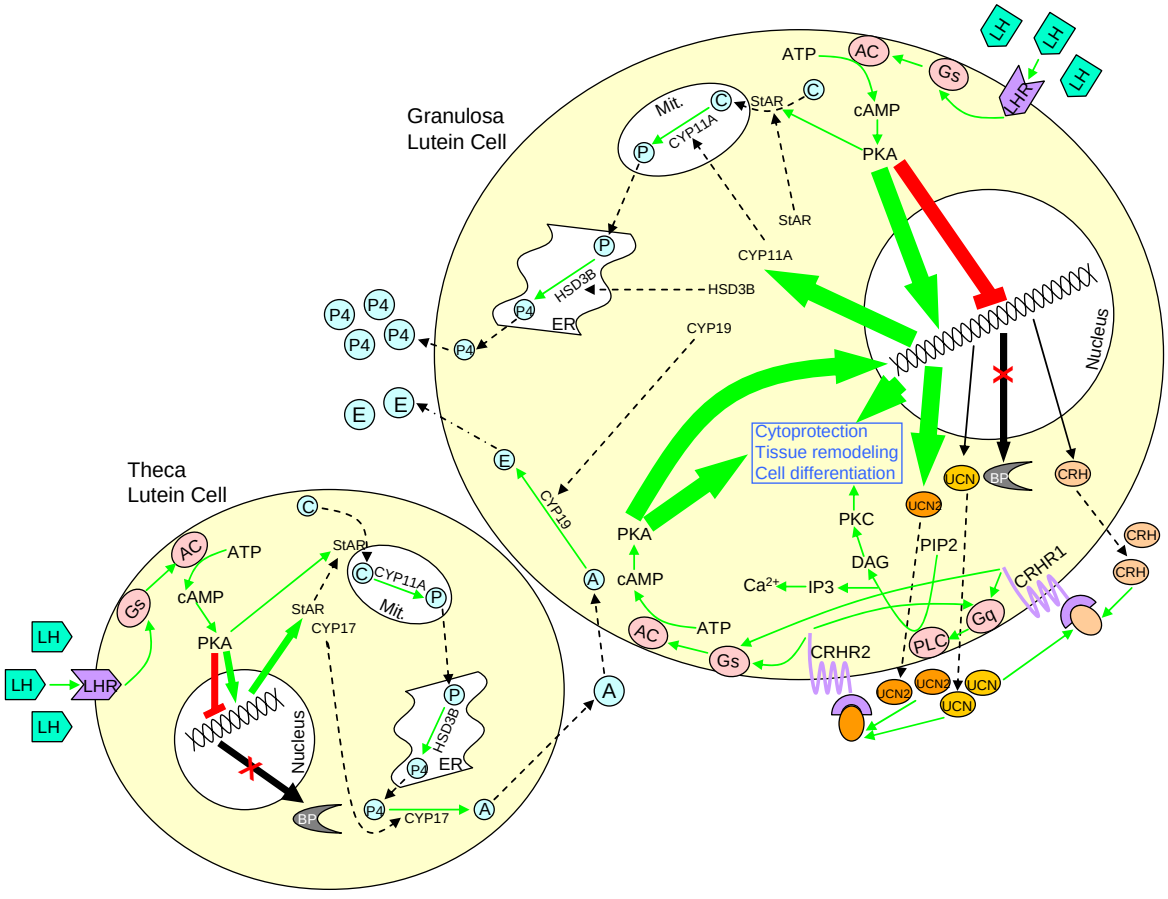
According to the *in vivo* data, CRH/UCN ligand-receptor antagonism caused luteal cell DNA damage, and the CL degenerated early before the midlate luteal phase. CRH-like peptides are known to protect diverse cell types from environmental insults, a so-called cytoprotective action. CRH treatment provided protection to hypoxic hippocampal neurons in the rat brain (Fox *et al.*, 1993) and UCN exerted a potent protective action in cultured rat hippocampal neurons (Pedersen *et al.*, 2002). These authors hypothesized that CRH-like peptides act as an endogenous neuroprotective hormone. UCN was also potent in protecting rat cardiac myocytes from necrotic and apoptotic death in primary cell cultures via a MAPK-dependent pathway, and the cardioprotective effects are abrogated by CRH-like peptide antagonists (Brar *et al.*, 1999; 2000). Besides mammals, it was reported recently that CRH acted as a cytoprotective agent in the tail of the X. *laevis* tadpole and CRHBP could block CRH action and hasten tail muscle cell death. The expression of CRHBP is strongly upregulated in the tadpole tail at metamorphic climax where it may neutralize CRH bioactivity, thus promoting tail resorption (Boorse *et al.*, 2006). In primates, there is evidence suggesting that prostaglandin F2α (PGF2α) may promote luteolysis without use of the uterus as an intermediate (Fuchs, 1987). CRH was reported to exert a tonic stimulatory effect on the activity of prostaglandin dehydrogenase (PGDH), which is the main inactivating enzyme for prostaglandins, in human chorion trophoblast cells (McKeown and Challis, 2003). Thus, CRH may stimulate PGDH expression during early luteal development to reduce the amount of PGF2α. However,

during late luteal phase with less CRH-R activity, PGF2α increases due to less PGDH presence and promotes lyteolysis.

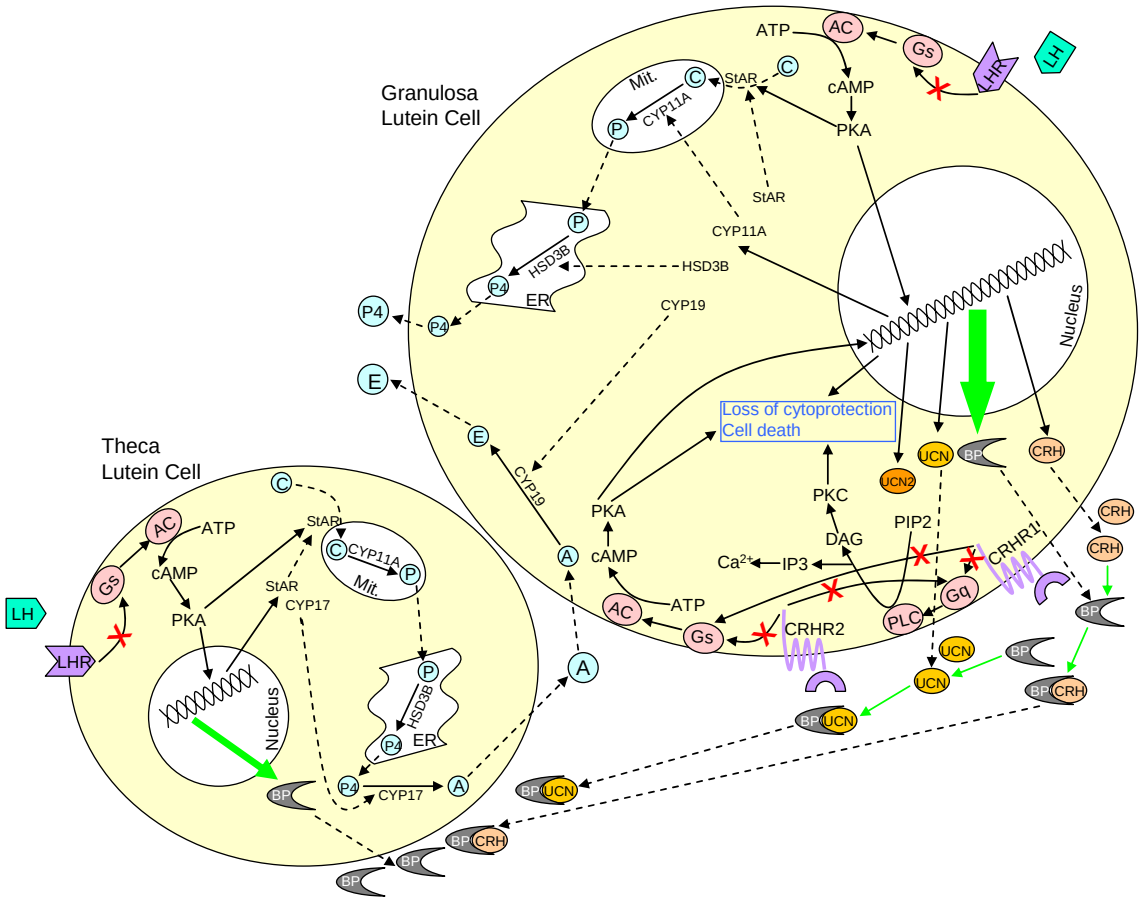
In primates, such as Old World Monkeys and women, it is clear that the demise of the CL near the end of the nonfecund menstrual cycle is not due to a uterine luteolytic factor. Nevertheless, the factors responsible for luteolysis in primates remain an enigma. The lack of a uterine or pituitary role in signaling luteolysis led investigators to propose "self-destruct" mechanisms whereby local factors synthesized in the ovary or CL lead to loss of luteal structure-function (Knobil, 1973; Olofsson and Leung, 1996). There is mounting evidence, both circumstantial and direct, suggesting that luteal cells transfer molecules that are important in cell signaling or as hormone precursors (Grazul-Bilska *et al.*, 1997). Increasing evidence supports the theory that apoptosis is an important mechanism controlling involution of the CL. Apoptotic indices have been reported during structural involution of the CL in rodents and primates (Bowen *et al.*, 1999; Shikone *et al.*, 1996) and progesterone inhibits apoptosis of human luteinizing granulosa cells during *in vitro* cluture (Makrigiannakis *et al.*, 2000). According to this dissertation study, CRH-like peptides may provide protection to luteal cells during luteal development and function at the early stage as a luteotropic factor. Subsequently, increased levels of CRHBP may neutralize ligand bioactivity and promote luteal cell death through apoptosis during luteal regression. However, whether CRH/UCN-R-BP system acts directly or indirectly and how cellular processes are activated are not clear. Although some progress has occurred in evaluating local death ligands and apoptotic regulatory proteins in the CL (Davis and Rueda, 2002), there is little information on the molecular pathways that execute apoptosis and degrade cellular targets. Further research is needed to evaluate the regulation and processes of apoptosis, as well as other forms of cell death, in the CL and to discern their role(s) in functional as well as structural luteolysis.

Thus, the CRH/UCN-R-BP system may represent a novel regulatory system in the CL that functions in luteinization and luteolysis. Since all its various components are expressed in the primate CL, one might expect that the signal transduction pathways mediated by ligand binding to CRHR1 and CRHR2 may function in the same or different cell types during the luteal lifespan. Fig. 6.1 represents a hypothesized schematic of the

A. Early-to-mid luteal phase



B. Late luteal phase



C. Astressin treatment

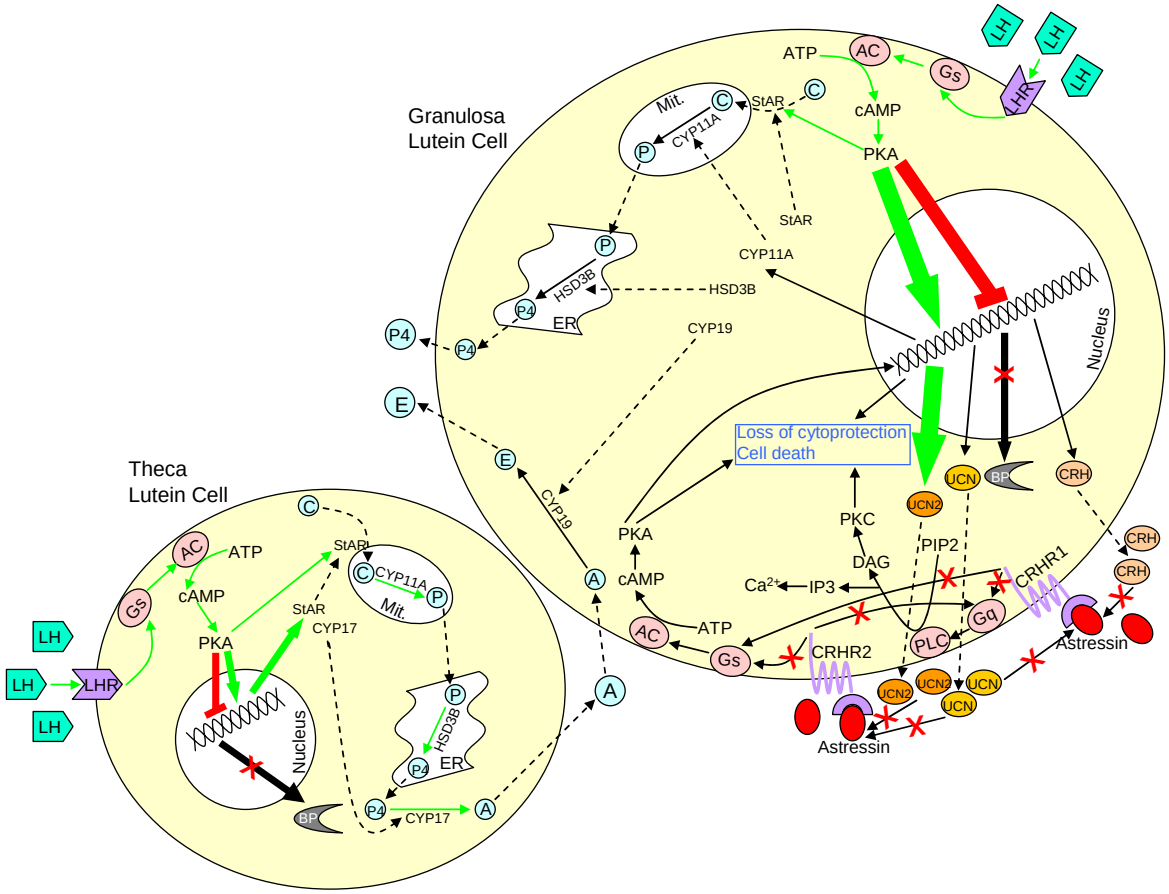


Fig. 6.1 Proposed interaction between the luteotropic hormone LH and the CRH/UCN-R-BP system in controlling the structure-function of the primate CL during the early-to-mid luteal phase (A), late luteal phase (B), and after the Astressin treatment (C). Green arrows indicate processes promoted by LH and/or CRH/UCN. Red line and "X"s denote processes suppressed by LH (panel A, C) or loss of LH (panel B) or CRH/UCN (panel B, C) action. A, androgen; AC, adenylate cyclase; ATP, adenosine triphosphate; BP, CRH binding protein; C, cholesterol; Ca^{2+}, calcium; cAMP, cyclic adenosine monophosphate; CRH, corticotropin releasing hormone; CRHR1 and 2, CRH receptor 1 and 2; CYP11A, cholesterol side chain cleavage cytochrome P450; CYP17, cytochrome P450 17α-hydroxylase/17,20-lyase; CYP19, aromatase; DAG, diacylglycerol; E, estradiol; Gq and Gs, G protein q and s; HSD3B, 3 beta-hydroxysteroid dehydrogenase; IP3, inositol triphosphate; LH, luteinzing hormone; LHR, LH receptor; P, pregnenolone; P4, progesterone; PIP2, phosphatidylinositol bisphosphate; PKA and PKC, protein kinase A and C; PLC, phospholipase C; StAR, steroidogenic acute regulatory protein; UCN, urocortin; UCN2, urocortin 2.

interaction between LH and the CRH/UCN-R-BP system to control the primate CL during the menstrual cycle. In the early-to-mid luteal phase (Fig. 6.1A), circulating LH acts on its receptors on luteal cells and may promote CRH/UCN-receptor action to assist in stimulating steroidogenesis and/or luteal development. At the same time, LH-receptor action suppresses *CRHBP* expression to allow CRH/UCN-receptor actions. By the late luteal phase (Fig. 6.1B), fewer LH pulses and/or reduced luteal cell sensitivity to LH may inhibit CRH/UCN-receptor action due to the loss in promotion of *UCN2* and disinhibition of *CRHBP* expression. CRHBP neutralizes CRH/UCN ligands to suppress steroidogenesis and luteal structure. CRHBP appears to be sequestered near the theca-lutein cells by an unknown mechanism. Administration of the CRHR antagonist Astressin (Fig. 6.1C) blocks CRH/UCN-receptor action in the CL during early luteal stage regardless of the presence of LH, and thereby suppresses steroidogenesis and luteal development. However, whether CRH/UCN ligand signal transduction via CRHR1 and CRHR2 are complementary or counteractive in regulating cell activity during luteal development or regression is unknown. Also, whether the luteotropic or luteolytic actions of the CRH/UCN-R-BP system are directed or mediated by other factors, e.g. interleukin (Ghzzoni *et al.*, 1997), is unclear. Further studies are needed to examine the direct effects of CRH, UCN, and UCN2 on individual cell types, e.g., nonluteinized or luteinized granulosa cells, dispersed luteal cells, or luteal microvascular cells. The use of highly selective CRHR1 and CRHR2 antagonists should help to elucidate the roles of the CRH/UCN-R-BP system in regulating luteal structure-function and lifespan *in vivo*. Likewise, investigations regarding the role of CRHBP in the primate CL, particularly during luteolysis, are also warranted.

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BIOGRAPHICAL SKETCH

Jing Xu was born in China on May 14th, 1973. She did the undergraduate program in the Department of Biochemistry and Molecular Biology, College of Life Science, Nankai University, China. In 1996, she received the B.S. Degree in Biochemistry and Molecular Biology. As a Research Assistant II, she worked in the Neuroendocrinology and Stem Cell Application Laboratory, Division of Reproductive Sciences, Oregon National Primate Research Center, Oregon Health & Science University for two years. Then, she joined the Department of Biochemistry and Molecular Biology, now known as the Department of Environmental & Biomolecular Systems, OGI School of Science and Engineering, Oregon Health & Science University for graduate study in 2002. The next year she received the M.S. Degree in Biochemistry and Molecular Biology and continued her dissertation research in the Ovarian Biology Laboratory of Dr. Richard L. Stouffer, Division of Reproductive Science, Oregon National Primate Research Center, Oregon Health & Science University. She is interested in ovarian biology and endocrinology.

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