Stress-Induced Genetic Expression of a Selective Corticotropin-Releasing Factor-Receptor Subtype Within the Rat Ovaries: An Effect Dependent on the Ovulatory Cycle

Rossella E. Nappi and Serge Rivest

Laboratory of Molecular Endocrinology, CHUL Research Center and Laval University
Québec, Canada G1V 4G2

ABSTRACT

The identification of several sources of corticotropin-releasing factor (CRF) outside the brain, including the gonads, has suggested the intriguing possibility that CRF of systemic origin can influence gonadal function under both normal and stressful conditions. However, the exact sites of action and the type of cells targeted by this stress-related neuropeptide in the ovaries remain unknown. The present study therefore investigated the effect of acute immobilization stress on the distribution of the recently cloned CRF receptor (CRF1a, CRF1b, and CRF2a) genes in the ovaries of adult cycling female rats (200–250 g; 14 h light, lights-on at 0600 h). Reproductive stages were verified by daily vaginal smears taken each morning for a minimum of 3–4 cycles prior to the experiment. Three hours after the start of a 90-min immobilization session, rats were deeply anesthetized and transcardially perfused with a solution of 4% paraformaldehyde on the morning (1100 h) and on the afternoon (1700 h) of proestrus and diestrus-2. Frozen ovaries were mounted on a microtome, cut into 30-μm slices, and then processed for the detection of mRNAs encoding CRF1a, CRF1b, or CRF2a receptors by in situ hybridization histochemistry using 35S-labeled riboprobes. Whereas the ovaries displayed barely detectable levels of CRF1 receptor mRNA in control and in stressed animals on the morning of proestrus and the day of diestrus-2, a positive signal for this transcript was detected in stroma cells and in the theca surrounding the ovulatory follicles during the afternoon of proestrus. Excluding the cumulus oophorus, which showed a light expression of the mRNA encoding the type 1 CRF receptor, granulosa cells were completely devoid of transcript in Graafian follicles as well as in CL, regardless of the stage of maturation. Interestingly, immobilization stress induced a marked expression of CRF2a receptor mRNA in the stroma cells in the afternoon of proestrus, suggesting that the ovaries may be sensitive to acute neurogenic challenge during the preovulatory stage. On the other hand, CRF1b and CRF2a receptor mRNAs were undetectable both in control and stressed animals throughout the estrous cycle. These results provide clear evidence that the gene encoding the CRF2a receptor, but not the type 2 receptors can be finely induced in selective ovarian compartments in both control and stressful conditions during the gonadal life cycle. The temporal and anatomic selectivity of the ovarian periovulatory CRF2a receptor gene expression may suggest a critical biological action of CRF during the ovulatory process and suggests that the intraovarian environment may influence the stress-induced transcription of a selective CRF receptor subtype within the ovary.

INTRODUCTION

The neuroanatomical distribution and regulation of the gene encoding corticotropin-releasing factor (CRF) in the rat brain are consistent with the pivotal role of this neuropeptide in the stress response [1–4]. The antireproductive effect of stress-related hormones has been extensively studied at all three levels of the hypothalamic-pituitary-gonadal axis, and CRF is recognized as a potent mediator of the stress-related reproductive failure influencing LH-RH-secreting neuronal activity [5–7]. Moreover, the concept of a strict bidirectional linkage between activity of the hypothalamic-pituitary-adrenal (HPA) axis and ovarian steroidogenesis is supported by variations in the HPA axis response to stress during the estrous cycle [8] and our recent report [9] indicating that the estrous cycle can interfere with the capacity of immobilization stress to stimulate the biosynthesis of CRF type 1 receptor in the hypothalamic paraventricular nucleus (PVN). The identification of several systemic sources of CRF [10–14] also suggests that activation of the CRF system outside the brain can influence the ovulatory cycle and interfere locally with gonadal function under stressful conditions. Indeed, immunoreactive CRF (CRF-ir) and CRF mRNA were identified in rat testes, and the fact that CRF secreted by Leydig cells negatively affects testicular steroidogenesis provided in vitro evidence that this peptide can act as an antireproductive hormone in the gonads [13–17].

A large variety of neuropeptides, most of which seem to act as putative intraovarian regulators, were localized in the follicular unit by immunohistochemistry, in situ hybridization, and receptor studies in several species [18]. Recently, Mastorakos et al. [19] detected CRF-ir and CRF binding sites in rat ovaries; positive immunostaining for CRF and autoradiographic localization of CRF receptor were present primarily in the ovarian theca and stroma [19], the functional counterpart of the Leydig cells in the testis. No apparent differences were observed in CRF-ir staining and CRF binding throughout the estrous cycle [19]. Likewise, CRF-ir was found in normal and polycystic human ovaries and in the follicular fluid [20]. It is possible that CRF is involved as a proinflammatory agent within the ovary during the ovulatory process, luteal development, luteolysis, and follicular
ataresia because CRF is expressed in inflammatory sites [11, 21, 22], and the ovary is now considered a crucial site of interaction between the immune and the endocrine systems [23, 24]. White blood cells, mainly macrophages, constitute a major cellular component of the interstitial ovarian compartment [25], whereas migrating leukocytes are a potential source of cytokines involved in the inflammatory-like ovulation-associated phenomena [23, 24]. In particular, apart from the largely recognized effects on gonadal steroidogenesis in several species [26–28], cytokines promote ovulation in the perfused rat ovary and show cyclic variations at various stages of the estrous cycle [29]. A similar physiological involvement of CRF during the proestrus stage may thus be proposed. The possible importance of ovarian CRF in the local regulation of steroid biosynthesis is supported by the presence of CRF-ir in the human polycystic ovary, which may reflect a role in the occurrence of hyperandrogenism [20]. Moreover, CRF inhibits in a dose-dependent fashion FSH-stimulated estradiol production from rat granulosa cells in vitro [30].

The study of the ovarian distribution of the recently cloned CRF receptor subtypes (types 1, 2α, and 2β) [31–34] is a powerful approach to investigating the potential role of CRF and the exact sites of action of the peptide in the ovaries during both normal and emergency situations for the organism. Thus, in the present study we evaluated the effect of acute neurogenic stress immobilization on expression of CRF₁, CRF₂α, and CRF₂β receptor transcripts in the ovaries of adult female cycling rats.

MATERIALS AND METHODS

Animals

Adult female Sprague-Dawley rats (~200–250 g) were acclimated to standard laboratory conditions (14L:10D cycle; lights-on at 0600 h) and given free access to rat chow and water. Reproductive stages were verified by daily vaginal smears taken every morning (700–730 h) for a minimum of 3–4 cycles prior to the experiment. Three to six rats were used for each group, and all protocols were approved by the Laval University Animal Welfare Committee. This experiment was conducted twice.

Acute Immobilization Session

Rats were placed in individual immobilizers for 90 min and, at 1100 h and 1700 h on the morning and afternoon of proestrus and diestrus-2, were killed 3 h after the beginning of the session. The time for killing the rats was chosen on the basis of our previous results in male and female rats in which a strong hybridization signal for the CRF₁ receptor mRNA was observed in the hypothalamic PVN 90 min after a 90-min stress session. Immobilization was accomplished by use of adjustable restraining cages (Centrap Cages, 01–282–10; Fisher Scientific Co., Pittsburgh, PA). Cages were adjusted tightly so that the animals were unable to move when in the immobilizer. The rats were anesthetized with an i.p. injection of 0.3 ml of a mixture of ketamine hydrochloride (80 mg/kg) and xylazine (10 mg/kg) and were rapidly perfused transcardially with saline, followed by 4% paraformaldehyde (PF) in 0.1 M borax buffer (pH 9.5 at 4°C). Ovaries were removed, cleaned of fat, postfixed, and then placed in a solution of 4% PF-10% sucrose overnight at 4°C. Frozen ovaries were mounted on a microtome (Reichert-Jung, Cambridge Instruments Company, Deerfield, IL) and cut into 30-µm sections. The slices were collected in a cold cryoprotectant solution (0.05 M sodium phosphate buffer, 30% ethylene glycol, 20% glycerol) and stored at −20°C. The mRNAs encoding each specific CRF receptor subtype were assayed by in situ hybridization histochemistry.

In Situ Hybridization Histochemistry

Localization by histochemical hybridization of each transcript (CRF₁, CRF₂α, and CRF₂β receptor mRNAs) was carried out in a 1-in-4 series (every fourth section) of ovary slices with use of 35S-labeled cRNA probes. Protocols for riboprobe synthesis, hybridization, and autoradiographic localization of mRNA signal were adapted from Simmons et al. [35]. All solutions were treated with diethyl pyrocarbonate (DEPC) and sterilized to prevent RNA degradation. Tissue sections mounted onto poly-L-lysine-coated slides were desiccated under a vacuum overnight, fixed in 4% PF for 30 min, and digested by proteinase K (10 µg/ml in 100 mM Tris HCl, pH 8.0, and 50 mM EDTA) at 37°C for 25 min. Thereafter, the ovary sections were rinsed in sterile DEPC water followed by a solution of 0.1 M triethanolamine (TEA, pH 8.0), acetylated in 0.25% acetic anhydride in 0.1 M TEA, and dehydrated through graded concentrations of alcohol (50, 70, 95, and 100%). After vacuum drying for a minimum of 2 h, 90 µl of a hybridization mixture (10⁷ cpm/ml) was spotted onto each slide, sealed under a coverslip, and incubated at 60°C overnight (~15–20 h) in a slide warmer. Coverslips were then removed, and the slides were rinsed in 4-strength SSC at room temperature. The sections were digested by Ribonuclease A (20 µg/ml, 37°C, 30 min), rinsed in descending concentrations of SSC (double-strength, single-strength, 0.5-strength; single-strength SSC = 0.15 M NaCl, 15 mM trisodium citrate buffer, pH 7.0), washed in 0.1-strength SSC for 30 min at 65°C, and dehydrated through graded concentrations of alcohol. After being dried for 2 h under a vacuum, sections were exposed at 4°C to x-ray film (XAR5; Eastman Kodak, Rochester, NY) for 24–48 h, defatted in xylene, and dipped in NTB2 nuclear emulsion (Kodak; diluted 1:1 with distilled water). Slides were exposed for 10–14 days, developed in D19 developer (Kodak) for 3.5 min at 15°C, and fixed in rapid fixer (Kodak) for 5 min. Thereafter the tissues were rinsed in running distilled water for 1–2 h, counterstained with thionin (0.25%), dehydrated through...
graded concentrations of alcohol, cleared in xylene, and coverslipped with DPX (Electron Microscopy Sciences, Fort Washington, PA).

**Complementary RNA Probe Synthesis and Preparation**

Specific rat CRF<sub>2</sub> receptor probe (1.3 kb) was generated from the PstI-PstI fragment of rat prCRF PPL3-BS cDNA (Dr. W. Vale, Peptide Biology Laboratory, The Salk Institute) subcloned into pBluescript II SK (Stratagene, La Jolla, CA) and linearized with BamHI and HindIII (Pharmacia) for antisense and sense probes, respectively. These two probes (CRF<sub>2a</sub> receptor cDNA or a 200-bp insert of a rat CRF<sub>2a</sub> receptor cDNA (Dr. T. Lovenberg, Neurocrine Biosciences Inc., San Diego, CA [34]) were linearized with HindIII and BamHI to generate antisense and sense probes, respectively. These two probes (CRF<sub>2a</sub> and CRF<sub>2b</sub> probes) have no overlap with one another and have no similarity to the CRF<sub>2</sub> receptor probe (T. Lovenberg, personal communication). Radioactive cRNA copies were synthesized by incubation of 250 ng linearized plasmid in 6 mM MgCl<sub>2</sub>, 40 mM Tris (pH 7.9), 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 0.2 mM ATP/GTP/CTP (Fisher; Promega), [α<sup>35</sup>S]UTP, 40 U Rnasin (Promega), and 20 U T7 (CRF<sub>1</sub> receptor antisense; CRF<sub>2a</sub> and CRF<sub>2b</sub> receptor sense probes) and T3 (CRF<sub>2</sub> receptor sense; CRF<sub>2a</sub> and CRF<sub>2b</sub> receptor antisense probes) RNA polymerase (Fisher; Promega) for 60 min at 37°C. Unincorporated nucleotides were removed by the ammonium-acetate method; 100 μl of DNase solution (1 μl DNase, 5 μl of 5 mg/ml RNA, 94 μl of 10 mM Tris/10 mM MgCl<sub>2</sub>) was added, and 10 min later an extraction was accomplished by use of a phenol-chloroform solution. The cRNA was precipitated with 80 μl of 5 M ammonium acetate and 500 μl of 100% ethanol for 20 min on dry ice. The pellet was washed with 500 μl ethanol, dried in a vacuum centrifuge for 10 min, and resuspended in 100 μl of 10 mM Tris/1 mM EDTA. A concentration of 10<sup>6</sup> cpm probe was mixed into 1 ml of hybridization solution (500 μl formamide, 60 μl 5 M NaCl, 10 μl 1 M Tris [pH 8.0], 2 μl 0.5 M EDTA [pH 8.0], 50 μl 20-strength Denhardt’s solution, 200 μl 50% dextran sulfate, 50 μl 10 mg/ml tRNA, 10 μl 1 M dithiothreitol [DTT], 118 μl DEPC water—volume of probe used). This solution was mixed and heated for 5 min at 65°C before being spotted on slides.

**RESULTS**

**Distribution of Type 1 and Type 2 (α and β) CRF-Receptor mRNAs in the Rat Ovary**

Figure 1 illustrates representative examples of CRF<sub>1</sub> receptor gene expression in the ovaries of stressed (left) and control (right) female rats on the morning and the afternoon of proestrus and diestrus-2. Almost no positive hybridization signal for the mRNA encoding the type 1 CRF receptor was observed on x-ray film in the ovaries of control or stressed animals on the morning of proestrus or on the day of diestrus-2. In the afternoon of proestrus, however, a low to moderate endogenous expression of CRF<sub>1</sub> receptor transcript was detected in the stroma cells and theca surrounding the ovulatory follicles of control rats (Pro-17, Control). Interestingly, immobilization stress caused a marked activation of CRF<sub>1</sub> receptor gene transcription in the stroma cells during this ovulatory period without affecting the genetic expression of this receptor in other periods of the estrous cycle. Tissues hybridized with sense probe did not exhibit detectable signal in any of the sites that showed positive signal with antisense probe (Fig. 2, top). In contrast to CRF<sub>1</sub>, no positive signal was hybridized with antisense probes to detect CRF<sub>2a</sub> and CRF<sub>2b</sub> receptor mRNAs in the ovaries of control (data not shown) and immobilized rats (Fig. 2, bottom panels). These findings indicate that CRF of type 2 receptors are not present in the rat ovary throughout the estrous cycle under both control and stressful conditions.

**Site-Specific Induction of CRF<sub>1</sub> Receptor Transcription in the Ovaries of Stressed Female Rats during the Afternoon of Proestrus**

Figure 3 shows an example of Graafian follicles of control (right) and stressed (left) female rats in the afternoon of proestrus. The mRNA encoding the type 1 receptor was spontaneously expressed in the theca surrounding the ovulatory follicles of control rats at this specific time of the cycle. No differences were found in such endogenous expression of the CRF<sub>1</sub> receptor transcript in animals killed 3 h after the beginning of the 90-min immobilization session. The selectivity of CRF<sub>1</sub> receptor gene expression within the theca surrounding ovulatory follicles in the afternoon of proestrus is presented by Figure 4. Excluding the cumulus oophorus, which demonstrates a light expression of the gene encoding the CRF<sub>1</sub> receptor, granulosa cells were completely devoid of this transcript in the ovaries of both control and stressed animals.

Immobilization stress caused activation of CRF<sub>1</sub> receptor transcription in a defined ovarian compartment during the afternoon of proestrus (Fig. 5, left). Regardless of the stage of maturation, the CL did not express CRF<sub>1</sub> receptor mRNA, whereas the interstitial compartment exhibited a positive hybridization signal 3 h after the acute immobilization session (Fig. 5, bottom left). In fact, strong levels of silver grains were observed in the ovarian stroma (Fig. 6, right) and interstitial cells in anatomic proximity to blood vessels (Fig. 6, left) 90 min after the end of the acute stress session accomplished in the afternoon of proestrus.

**DISCUSSION**

The present study provides evidence that the gene encoding the type 1 but not the type 2 (α and β) CRF receptors can
be finely induced in selective ovarian compartments in both control and stressful conditions during the gonadal life cycle. Indeed, whereas the ovary displayed barely detectable levels of CRF_1 receptor mRNA in control and stressed animals on the morning of proestrus and the day of diestrus-2, a clear signal for this transcript was detected in stroma cells and the theca surrounding the ovulatory follicles during the afternoon of proestrus. Excluding the cumulus oophorus, which showed a light expression of CRF_1 receptor mRNA, granulosa cells were completely devoid of this particular transcript in Graafian follicles, as well as in CL, regardless of the stage of maturation. Interestingly, immobilization stress induced a marked expression of the gene encoding the type 1 CRF receptor in the stroma cells in the afternoon of proestrus, suggesting that the ovary may be sensitive to acute neurogenic challenge during the preovulatory stage.

The ovary has been demonstrated to be a peripheral source of CRF [19, 20]. The chromatographic mobility of rat and human extracts can be superimposed on that of synthetic rat/human CRF ([19, 20] and personal observation), indicating that ovarian CRF is similar to that produced by the rat and human hypothalamus, human placenta, rat Leydig cells, and peripheral inflammatory sites [2, 12, 14, 21, 36]. Also reported were CRF immunostaining in stroma and theca cells surrounding follicles at different stages of maturation, in a subpopulation of cells within the CL, and in the oocytes of the antral follicles, as well as a sporadic positive staining in granulosa cells of ovulatory follicles [19]. Although the presence of CRF-ir in the ovaries of cycling rats is clearly established, the potential roles of such neuropeptides remain to be elucidated. This picture is further complicated by the fact that we can only hypothesize about the possible site(s) of CRF biosynthesis and release within the ovary. The pattern of distribution of its own receptor and the present data showing a strong selective induction of the CRF_1 receptor transcription in the thecal layer surrounding the ovulatory follicles and, more lightly, in the cumulus oophorus in the afternoon of proestrus may indicate that CRF constitutes an additional neuropeptidergic signal involved in the gonadal reproductive cycle. Indeed, the striking temporal and anatomic selectivity of the ovarian periovulatory CRF_1 receptor gene expression emerging from our study indicates that a critical biological action for this neuropeptide may be identified mainly during the ovulatory process.

One of the possible interpretations of the physiological relevance of an up-regulation of the CRF, receptor mRNA in the afternoon of proestrus is that CRF, through this particular receptor subtype, may actively participate in the final maturation and rupture of the follicular unit and in the initial luteinization phenomena as well as exert a potential effect on steroid biosynthesis during ovulation. Hence, it is likely that CRF may behave as a proinflammatory factor and be part of the whole cascade of concomitant and/or coordinated periovulatory events [37]. This phenomenon includes a complex cohort of immune-related substances, namely cytokines, whose participation in the ovulatory process and in ovarian steroid hormone regulation has been strongly investigated. Indeed, macrophage-derived cytokines can promote ovulation in the perfused rat ovary [38] and stimulate the production of progesterone and androgens from granulosa and thecal layers, respectively, in preovulatory follicles [27, 39]. On the other hand, a gonadotropin-dependent preovulatory induction of interleukin-1 (IL-1) transcript was reported in the rat as well as human ovary [28, 40], and LH seems to be the key factor leading to the production of cytokines and chemotactic stimuli in the ovary. Expression of IL-1β mRNA in human peripheral leukocytes is sensitive to gonadal hormones [41], confirming that steroid-secreting cells may exert a modulatory influence on immune-related peptides. Complex interplay between the hormonal intraovarian environment and expression of intragonadal regulators is a constant feature of ovarian physiology and the pivotal role of gonadotropins in the promotion of follicular development. The demonstration of a preovulatory-induced transcription of CRF_1 receptor could suggest that the acquisition of CRF responsiveness is central to the final maturative processes of the Graafian follicle. An example of the reciprocal interactions existing between the hormonal intraovarian environment and the expression and regulation of another neuropeptide operating in the rat ovary is given by the GnRH system, which includes GnRH gene transcript, GnRH binding sites, and GnRH receptor mRNAs [42, 43]. The importance of ovarian GnRH during the periovulatory period is further supported by a recent report indicating a marked decline in ovarian GnRH receptor gene expression 12 h after HCG injection in eCG-primed immature rats [44].

However, whether CRF participates in the biochemical ovulation-associated events more as a proinflammatory factor or as a neuropeptidergic signal involved in the modulation of steroidogenic activity is still an open question. Also remaining unknown is the nature of the modulatory factors influencing the transcription and stability of ovarian CRF_1 receptor mRNA and the biological activity of CRF. On the other hand, because CRF-ir is present throughout the entire estrous cycle, we cannot rule out the possibility that the peptide may be potentially involved in gonadal biological functions other than ovulation directly or via other me-
FIG. 2. Stress-induced genetic expression of selective CRF receptor subtype in ovaries of stressed cycling female rats on afternoon of proestrus. These sections (30 μm) displayed positive signal on x-ray film for CRF₁ receptor mRNA selectively on afternoon of proestrus (A). Hybridized tissues with sense probe (B) did not exhibit detectable signal in any site that showed positive signal with antisense probe (A). In contrast, no positive signal was hybridized with antisense probes to detect CRF₂α (C) and CRF₂β (D) receptor mRNAs in ovary of immobilized rats on afternoon of proestrus.

A  CRF₁ receptor (antisense)  B  CRF₁ receptor (sense)

C  CRF₂α receptor  D  CRF₂β receptor
FIG. 3. Representative example of Graafian follicle expressing gene encoding CRF, receptor in control and stressed female rats on afternoon of proestrus. Brightfield (top) and darkfield (bottom) photomicrographs of dipped autoradiographs of hybridized 30-μm sections with CRF, receptor cRNA probe of similar ovulatory follicles. Note that mRNA encoding CRF, receptor was spontaneously expressed in theca surrounding follicle of control rats (bottom right). No significant differences were found in such endogenous expression of CRF, receptor transcripts in animals killed 3 h after 90 min immobilization session began (bottom left). ×24.
FIG. 4. Selectivity of CRF1 receptor gene expression within theca surrounding ovulatory follicle on afternoon of proestrus. Brightfield (A) and darkfield (B) photomicrographs of hybridized 35-μm sections with CRF1 receptor cDNA probe of Graafian follicles (×24). High-power brightfield photomicrographs of dipped autoradiographs show details of same follicle (×64). C, D. Although granulosa cells were completely devoid of CRF1 receptor transcripts, strong hybridization signals were detected in theca cell nuclei surrounding the ovulatory follicle (×640). Follicle gc, granulosa cells; t, theca.
FIG. 5. Representative example of site-specific induction of CRF₁ receptor transcription in ovary of stressed female rat on afternoon of proestrus. Brightfield (top) and darkfield (bottom) photomicrographs of hybridized 30-μm sections with CRF₁ receptor cRNA probe showing stromal and luteal compartments. Stromal compartment exhibited positive hybridization signal after stress session (bottom left), whereas CL did not express CRF₁ receptor mRNA. cl, CL, s, stroma. × 24.
FIG. 6. Intensity of expression of gene encoding CRF1 receptor in stromal and interstitial compartments of ovaries of stressed female rats on afternoon of proestrus. High-power brightfield photomicrographs of dipped autoradiographs of hybridized 30-μm sections with CRF1 receptor cRNA probe. Note strong levels of silver grains in ovarian stroma (right) and in interstitial cells in anatomical proximity to blood vessels (left). *Blood vessels; gc, granulosa cells; s, stroma; t, theca. Top, x94; bottom, x235.
diators, such as proopiomelanocortin (POMC)-related peptides. The ovarian content of immunoreactive \( \beta \)-endorphin (\( \beta \)-END-ir) and POMC mRNA increases after eCG administration [45], and granulosa cells located near the oocytes exhibit positive signals for \( \beta \)-END-ir [46]. The presence of CRF\(_{1}\) receptor transcript and binding sites in the cumulus oophorus, together with evidence of CRF-ir in mature oocytes, provides anatomic evidence of a possible interaction between POMC-derived peptides and CRF within selective compartments of the ovaries during normal and emergency circumstances in the female organism.

CRF mRNA and peptide were detected in peripheral leukocytes [11, 21, 22]. A large population of immune-related cell types is normally resident in the interstitial ovarian compartment in anatomic vicinity to stroma blood vessels and perifollicular capillaries [23]. Whether selective cell types in the ovaries contain the biosynthetic machinery to produce CRF is still under debate. Indeed, we (unpublished results) and others (C. Rivier, personal communication) were unable to detect a positive hybridization signal for CRF mRNA in somatic ovarian cells. However, in situ hybridization histochemistry might not be sensitive enough to detect very low levels of transcript that could be visible only by polymerase chain reaction or other highly sensitive techniques. On the other hand, the massive ovarian infiltration by several types of white blood cells [47, 48] and the concomitant inflammatory-like phenomena occurring at the time of ovulation [37] could be responsible for delivering CRF locally, where it could exert, throughout interaction with its receptor, paracrine effects at the level of the different ovarian compartments. Our data showing a relative abundance of CRF\(_{1}\) receptor transcripts in the loose connective tissue areas, in the medullary regions surrounding the blood vessels, and in the thecal layer near perifollicular capillaries support such a hypothesis. Moreover, the absence of a clear co-localization between the cells expressing the CRF\(_{1}\) receptor mRNA and the CRF-ir cells during the afternoon of proestrus (data not shown) seems to rule out the presence of autocrine events in the ovarian CRF system.

The sympathetic nervous system could represent another potential source of CRF delivery within the ovary [49]. Immunocytochemical localization of CRF in the rat spinal cord has been identified [50], and CRF may be released locally by the fibers innervating blood vessels, interstitial tissues, and developing follicles [51]. A potential modulatory role of adrenergic agents in follicular development, steroid secretion, and ovulation remains possible. Using isolated rat ovaries, Ferruz et al. [52] observed a profile of norepinephrine release during the estrous cycle, and this phenomenon is influenced by gonadotropins, particularly during the day of proestrus. However, little is known about the functional interactions between the activity of the sympathetic nervous system and the ovarian CRFergic system.

The fact that immobilization can stimulate transcription of the gene encoding the type 1 receptor in the stroma cells in the afternoon of proestrus, apparently without affecting the spontaneous CRF\(_{1}\) receptor mRNA expression in the thecal layer around the ovulatory follicles, supports the concept of stress-induced CRFergic activity within selective ovarian subdivisions. These results also suggest that the ovary is more susceptible to the local antireproductive influence of CRF during a specific period of the ovulatory cycle in severely stressed animals. Indeed, the intraovarian environment may somehow influence the gonadal responsiveness to the immobilization session. The exact physiological significance of a selective reactivity of the ovaries limited to the afternoon of proestrus and the pathways involved in such stress-induced up-regulation of the CRF\(_{1}\) receptor mRNA in the theca/interstitial compartment must be clarified. A possible involvement of local dynamic vascular and/or neural changes occurring during stressful conditions may be proposed. We recently reported that immobilization stress can selectively induce the gene encoding the type 1 CRF receptor in the parvicellular division of the PVN and that such stress-induced CRF-R\(_{1}\) transcription was significantly higher on the morning of proestrus [9]. Consequently, the gonadal steroid milieu may modulate the neuroendocrine CRFergic system in the female rat brain during stress. The observation that estradiol can stimulate human CRF gene transcription [53] also supports a direct influence of steroid hormones on the local activity of the ovarian CRFergic system. A better understanding of the relationship between the ovulatory cycle and the local influence of stressful stimuli will help to clarify the biological significance of the presence of CRF-ir cells in the gonadal life cycle and the possible sites of biosynthesis and release of the peptide within the ovary.

In conclusion, the mRNA encoding the type 1 CRF receptor can be finely induced in selective ovarian compartments under both control and stressful conditions during the gonadal life cycle. The temporal and anatomic selectivity of the ovarian periovulatory CRF\(_{1}\) receptor gene expression indicates that a critical biological action for CRF may be identified during the ovulatory process and that the intraovarian environment may influence the stress-induced transcription of that particular CRF receptor subtype in rat ovaries.

**ACKNOWLEDGMENTS**

We thank Dr. Wylie W. Vale (PBL, The Salk Institute, La Jolla, CA) for the generous gift of rat CRF receptor cDNA, Dr. Timothy W. Lovenberg (Department of Molecular Biology, Neurocine Biosciences, Inc., San Diego, CA) for the gift of plasmids containing rat CRF\(_{1}\) and CRF\(_{2}\) receptor cDNAs, and Miss Nathalie Laflamme for invaluable technical assistance.

**REFERENCES**


