0013-7227/99/$03.00/0
Endocrinology

Vol. 140, No. 5
Printed in U.S.A.

The Effects of Estrogen and Progesterone on Corticotropin-Releasing Hormone and Arginine Vasopressin Messenger Ribonucleic Acid Levels in the Paraventricular Nucleus and Supraoptic Nucleus of the Rhesus Monkey

BRENDA N. ROY, ROBERT L. REID, AND DEAN A. VAN VUGT

Departments of Physiology (B.N.R., D.A.V.V.) and Obstetrics and Gynecology (R.L.R., D.A.V.V.), Queen's University, Kingston, Ontario, Canada K7L 3N6

ABSTRACT

Ovarian steroids increase hypothalamic-pituitary-adrenal (HPA) axis activity and sensitize the hypothalamic-pituitary-ovarian (HPO) axis to stress-induced inhibition. The present study investigated the effect of ovarian steroids on CRH and arginine vasopressin (AVP) messenger RNA (mRNA) levels in the rhesus monkey hypothalamus, as both neuropeptides have been shown to stimulate the HPA axis and inhibit the HPO axis in this species. This was accomplished by measuring CRH and AVP mRNA in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) by *in situ* hybridization histochemistry. Menstrual cycles were simulated in ovariectomized (OVX) rhesus monkeys by sequential addition and removal of SILASTIC brand (Dow Corning Corp.) tubing containing either 17β-estradiol (E_2) or progesterone (P_4). On the morning of day 11 of the simulated follicular phase (E_2 alone) or day 21 of the luteal phase ($E_2 + P_4$), animals were anesthetized, and the brains were perfused with paraformaldehyde via the carotid artery. Coronal sections (30 μm) were cut, and mRNA for CRH and AVP in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) were semiquantified by *in situ* hybridization. CRH mRNA in the PVN of E_2-replaced OVX animals (n = 7) was 2-fold greater than that in untreated OVX controls (n = 4), whereas CRH mRNA after $E_2 + P_4$ (n = 4) was no different from that in controls (optical density ± SEM, 0.38 ± 0.06, 0.13 ± 0.08, and 0.14 ± 0.09 for OVX + E_2, OVX + E_2 + P_4, and OVX, respectively; $P = 0.02$). CRH in the SON was undetectable. In contrast to CRH, AVP mRNA in the PVN and the SON was similar in the three treatment groups. We conclude that E_2 and $E_2 + P_4$ replacement to OVX monkeys exert different effects on CRH and AVP gene expression, as estrogen stimulation of CRH mRNA in the PVN was abrogated by progesterone, whereas no effect of ovarian steroids on AVP mRNA in either the PVN or SON was observed. We postulate that ovarian steroid regulation of CRH synthesis and release may in part explain the central nervous system mechanisms by which ovarian steroids affect the HPA and HPO axes during basal and stress conditions. (*Endocrinology* **140:** 2191–2198, 1999)

VARIOUS components of the hypothalamic-pituitary-adrenal (HPA) axis have been shown to exert inhibitory effects at several levels of the hypothalamic-pituitary-ovarian (HPO) axis. These effects include inhibition of LH releasing hormone (LHRH) (1) and LH (LH) (2) by CRH, vasopressin (3), and glucocorticoids (4) and glucocorticoid inhibition of LHRH-induced LH secretion (5). Direct inhibition of gonadal steroid production by CRH and glucocorticoids (6, 7) and ACTH-induced decreased gonadal sensitivity to LH have been reported (8). The HPA axis is, in turn, influenced by the HPO axis. Stress-induced activation of the HPA axis and the sympathetic nervous system are more pronounced in females (9). Melancholic depression and anorexia nervosa, which are associated with hypersecretion of the HPA axis, occur more commonly in women (10). Ovariectomized (OVX) rats primed with 17β-estradiol (E_2), but not those given E_2 and progesterone (P_4), had significantly higher plasma ACTH levels immediately after a 20-min restraint stress compared with OVX controls (11).

Received August 14, 1998.
Address all correspondence and requests for reprints to: Dean Van Vugt, Ph.D., Department of Obstetrics and Gynecology, 3022 Etherington Hall, Queen's University, Kingston, Ontario, Canada K7L 3N6. E-mail: vanvugtd@post.queensu.ca.

It is suggested that ovarian steroids modulate the HPA axis, as some of the gender differences described above can be reduced by estrogen administration to men (12) or ovariectomy in rats (13). Estrogen may stimulate the HPA axis by an effect predominantly on CRH. Estradiol benzoate treatment increased CRH messenger RNA (mRNA) levels in the paraventricular nucleus (PVN) of OVX female rats (14). Additionally, both basal and endotoxin-induced CRH and CRH_1 receptor mRNA levels in the PVN were greatest on the morning of proestrus in association with elevated 17β-estradiol levels (15, 16). As the CRH gene contains estrogen response elements, these effects of estrogen may result from a direct effect on the CRH gene (17). However, the literature is not unanimous in its support of a stimulatory effect of estrogen on CRH. Although ovariectomy decreased the hypothalamic CRH content, this effect was not reversed by E_2 replacement (18). Paulmyer-Lacroix *et al.* (19). observed decreased CRH mRNA levels in the PVN after implantation of E_2 capsules to OVX rats, whereas administration of ovarian steroids did not significantly affect CRH mRNA levels in the PVN of OVX ewes (20). Estrogen also has been reported to decrease CRH synthesis in the hypothalamus of the rat (21).

Because arginine vasopressin (AVP) synergizes with CRH to release ACTH from the anterior pituitary (22), ovarian

steroids may influence HPA axis activity through an effect on AVP. The majority of studies suggest a positive effect of estrogen on AVP. Serum AVP levels paralleled estrogen levels during the rat estrous cycle and the human menstrual cycle (23, 24) and were decreased in OVX rats (25). At the level of the hypothalamus, a significant increase in AVP content was reported in the PVN at proestrus (26) and after an injection of estradiol-benzoate to OVX rats (14). *In vitro* studies demonstrated that E_2 increased hypothalamic AVP release (27).

There is a growing body of evidence that ovarian steroids sensitize the HPO axis to the inhibitory effect of stress. Hypoglycemia-induced LH inhibition is more pronounced in ovary-intact rhesus monkeys than in OVX monkeys (28, 29). Fasting-induced suppression of LH secretion is observed in OVX rats only after estrogen priming (30). In the absence of ovarian steroid priming, the LHRH pulse generator, as measured by multiunit electrical activity and pulsatile LH secretion, is not compromised in monkeys that are restrained in primate chairs, but is reduced after ovarian steroid replacement (31, 32).

We postulate that the mechanisms by which ovarian steroids modulate HPO axis responsiveness to stress may involve ovarian steroid modulation of CRH and AVP. This is supported by studies that report that both peptides increase during stress and inhibit LH secretion (33–35). In an attempt to clarify the effect of ovarian steroids on the HPA axis, as it may, in turn, influence the HPO axis of the primate, CRH and vasopressin mRNA levels were semiquantified by *in situ* hybridization in OVX rhesus monkeys after an ovarian steroid replacement regimen that mimicked the menstrual cycle.

Materials and Methods

Animal husbandry

This study was conducted in 15 adult female rhesus monkeys (*Macaca mulatta*) that were 7–15 yr old and weighed between 4.7–8.9 kg. All animals were OVX at least 1 month before this study. Animals were individually housed in a light (lights on, 0600–1800 h)- and temperature (21–23 C)-controlled room. Their diet consisted of water *ad libitum* and twice daily rations of monkey chow supplemented with fruits and vegetables. All husbandry practices and experimental procedures conformed to the guidelines of the Canadian Council on Animal Care and were approved by the Queen's University animal use committee.

Menstrual cycle simulation

The menstrual cycle was simulated by the addition and removal of E_2 (Sigma Chemical Co., St. Louis, MO) and P_4 (Sigma Chemical Co.)-filled SILASTIC brand capsules (Dow Corning Corp., Midland, MI). E_2 or P_4 was packed into 5-cm (E_2) or 6-cm (P_4) SILASTIC tubing (id, 3.3 mm; od, 4.6 mm), which was sealed at both ends with a 1-cm elastomer plug (Dow Corning Corp.). Before implantation, E_2 and P_4 capsules were incubated separately for 12 h in PBS to avoid an initial surge of steroid due to the accumulation within the SILASTIC wall.

Monkeys were immobilized on the day of implantation with an i.m. injection of ketamine HCl (5–10 mg/kg; Rogarsetic, Rogar/STB, Montréal, Québec, Canada) and were sedated with an iv infusion of a ketamine (100 mg/ml) and valium (5 mg/ml) solution (1:1 volume ratio; 0.15 ml/kg). A 2-cm incision was made between the scalpula, and one E_2 capsule was placed sc (day 1). On the 11th day, two additional E_2 capsules were added to produce an exponential rise in estrogen during the next 48 h that mimics the preovulatory estradiol surge. On day 16, two P_4 capsules were inserted, and one of the E_2 capsules was removed to simulate E_2 and P_4 levels typical of the luteal phase. All capsules, except one E_2 capsule, were removed on day 28, completing the first simulated menstrual cycle. Blood was collected each time a capsule was added or removed. All experiments were performed either on day 10/11 (midfollicular; n = 7) or day 21/22 (midluteal; n = 4) of the second or third simulated menstrual cycle. OVX animals without ovarian steroid replacement served as controls (n = 4). The untreated OVX monkeys were not subjected to sham procedures because there was a significant time period between steroid manipulations and perfusion (5 days in the case of luteal phase perfusions and 11 days in the case of follicular perfusions).

Perfusion

Animals were lightly sedated with ketamine and placed in primate restraint chairs between 1400–1600 h. An angiocatheter was inserted into a femoral vein, and the animals were left undisturbed in the chair overnight. All animals had been acclimated to the chair and were paired with another animal during the above procedure. Animals were rapidly anesthetized with a solution of Saffan (Pittman-Moore, Middlesex, UK; 1.2 mg/kg) and ketamine (10 mg/kg; 1:1, vol/vol) between 1000–1100 h on the next day. Both right and left carotid arteries were cannulated, and the brain was perfused with 100 ml PBS (4 C) followed by 4% paraformaldehyde with 0.1 M borax (pH 9.5) at a rate of 10 ml/kg·min for 20 min. Approximately 15 min elapsed between induction of anesthesia and initiating perfusion.

In situ hybridization histochemistry

The brain was removed from the skull and postfixed in 300 ml 4% paraformadehyde and 0.1 M borax buffer at 4 C. Brains were transferred to 4% paraformaldehyde-borax buffer containing 10% sucrose at 4 C for 48 h before sectioning. Thirty-micron coronal sections were cut from the olfactory bulb to the caudal medulla. The slices were placed in cryoprotectant [0.05 M sodium phosphate buffer (pH 7.3), 30% ethylene glycol, and 20% glycerol] and stored at −20 C until mounted.

Hybridization histochemical localization of each transcript was carried out in a one in six series (every sixth section) of slices from regions encompassing the supraoptic nucleus (SON) and the PVN. Slices were mounted onto gelatin- and poly-L-lysine-treated slides and dried overnight. All prehybridization solutions were treated with diethylpyrocarbonate (DEPC) and autoclaved to eliminate ribonuclease (RNase) activity. Tissue sections were then fixed for 20 min in 4% paraformaldehyde solution, incubated in potassium PBS for 10 min, and digested by proteinase K (10.0 μg/ml dissolved in 100 mM Tris-HCl, pH 8.0, and 50 mM EDTA, pH 8.0) at 37 C for 25 min. Slices were then rinsed with sterile DEPC-treated water followed by triethanolamine (100 mM, pH 8.0) and acetylated for 10 min in 0.25% acetic anhydride in 100 mM triethanolamine. Thereafter, tissue slices were washed for 5 min in standard saline citrate (2 × SSC) and dehydrated in 50%, 70%, 95%, and 100% ethanol.

Protocols for riboprobe synthesis and hybridization of mRNA were adapted from the report by Simmons *et al.* (36). After vacuum drying for at least 2 h, brain slices were spotted with 130 μl ^{35}S-labeled complementary RNA (cRNA) probes [10^7 cpm/ml], coverslipped, and incubated at 60 C on a slide warmer for 12–36 h depending on the probe. Coverslips were removed, and the tissues were rinsed in 4 × SSC, followed by digestion with RNase A (20 μg/ml, 37 C, 30 min) in RNase buffer (0.5 M NaCl, 10 mM Tris-HCl, and 0.5 mM EDTA). Tissues were then rinsed in descending concentrations of SSC and dithiothreitol (DTT; 2, 1, and 0.5 ×), followed by 0.1 × SSC for 30 min at 60 C, and dehydrated in ascending concentrations of ethanol (50%, 70%, 95%, and 100%). After being vacuum dried for at least 2 h, sections were exposed at 4 C on Biomax MR x-ray film (Eastman Kodak Co., Rochester, NY) for 12–84 h, depending on the probe. Some slides were then defatted in xylene, dipped in NTB2 nuclear emulsion (Eastman Kodak Co.; diluted 1:1 with distilled water), and exposed for 14 days at 4 C before being developed in D19 developer (Eastman Kodak Co.) for 3.5 min at 14–15 C and fixed in rapid fixer (Eastman Kodak Co.) for 5 min. Thereafter, tissues were rinsed in running distilled water for 1–2 h, counterstained with thionine (0.25%), dehydrated through graded concentrations of alcohol, cleared in xylene, and coverslipped with DPX.

All tissues were processed in two CRH and two AVP *in situ* hybrid-

ization assays. Groups were evenly represented in the two assays as a precaution against interassay variation. In addition, each x-ray film contained sections from all three treatment groups so as to protect against potential intraassay variation due to processing, exposure, and development.

cRNA probe synthesis and preparation

The CRH antisense riboprobe was generated from the *Eco*RI fragment of CRF complementary DNA (1.2 kb; Dr. K. Mayo, Northwestern University, IL), subcloned into pGEM4 plasmid, and linearized with *Hin*dIII. The AVP complementary DNA (230 bp) was generated from an *Sma*I-*Pst*I fragment, subcloned into a pSP65 plasmid, and linearized with *Hin*dIII (Dr. D. Richter, Universitat Hamburg, Hamburg, Germany).

Radioactive cRNA copies were synthesized by incubating 250 ng linearized plasmid in 6 mM $MgCl_2$, 40 mM Tris (pH 7.9), 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 0.2 mM ATP/GTP/CTP, 200 μCi [α-^{35}S]UTP (DuPont NEN, Boston, MA; NEG 039H), 40 U RNAsin (Promega Corp., Madison, WI), and 20 U SP6 RNA polymerase for 60 min at 37 C. After the transcription reaction, the DNA template was removed by adding 100 μl deoxyribonuclease solution [1 μl deoxyribonuclease, 2.5 μl transfer RNA (tRNA), 10 mg/ml 94 μl 10 mM Tris-10 mM $MgCl_2$, and 2.5 μl DEPC-treated water] and incubated at room temperature for 10 min. An extraction was then performed with 100 μl phenol-chloroform (1:1, vol/vol), and the probes were precipitated with 80 μl ammonium acetate (5 M; pH 5.5) and 500 μl 100% ethanol for 20 min on dry ice. After centrifugation, the pellet was dried and resuspended in 100 μl 10 mM Tris-1 mM EDTA (pH 8.0), counted, and then stored at −20 C. On the day of the hybridization, the probe was diluted to 10^7 cpm/ml hybridization solution [1 ml = 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 × Denhart's solution, 200 μl 50% dextran sulfate, 50 μl 10 mg/ml transfer RNA, 10 μl 1 M DTT, and 118 μl DEPC-treated water volume of probe used). The above solution was vortexed and incubated at 65 C for 5 min immediately before 130 μl were spotted onto each slide.

Quantitative analysis

The hybridization signal intensity of CRH and AVP mRNA was semiquantified for the PVN and SON (AVP mRNA only) for each animal from the x-ray films. Anatomically matched sections (one or two slices per animal for CRH and AVP mRNA in the PVN and two to four slices per animal for AVP mRNA in the SON) were bilaterally digitized for a total of 192 observations (PVN CRH mRNA, n = 60; PVN AVP mRNA, n = 44; SON AVP mRNA, n = 88). Transmittance values [referred to here as optical density (OD)] of the hybridization signals were measured under a Northern Light Desktop Illuminator (Imaging Research, Inc., St, Catharines, ON) using a Sony Camera Video System attached to a MicroNikkor 55 mm-Vivitar extension tube set for a Nikon lens (Nikon, Melville, NY) and coupled to a Macintosh computer (Power Macintosh 7100/66) and NIH software version 1.59/ppc (written by W. Rasband at the U.S. NIH and available from the Internet by anonymous ftp from zippy.nih.gov). OD values for each pixel were calculated using a known standard of intensity and distance measurements from a logarithmic specter adapted from Bioimage Visage 110s (Millipore Corp., Ann Arbor, MI). The wedge was calibrated before correcting for film saturation, which was determined by sampling the darkest PVNs and adjusting the light source and exposure time. Samples were evaluated within the linear OD curve to avoid pixel saturation and underestimation. The PVN and SON were digitized and subjected to densitometric analysis, yielding measurements of the mean density per area. The OD of the PVN and SON was then corrected for the average background signal by subtracting the OD of areas without positive signal located immediately outside the digitized nuclei.

Blood collection

Blood samples (2 ml) were collected whenever E_2- or P_4-filled SILASTIC capsules were added or removed and at the time of perfusion. Samples were left overnight to clot at 4 C before centrifugation for 15 min at 1500 × *g*. Serum was separated and stored at −20 C until assayed.

RIA

RIAs for E_2 and P_4 were performed to confirm that the steroid replacement regimen simulated physiological ovarian steroid concentrations found during the follicular and luteal phase of the menstrual cycle. Estrogen and P_4 levels were measured in duplicate using assay kits (Diagnostic Products Corp., Los Angeles, CA). The assay sensitivity for E_2 was 1.4 pg/ml, and that for P_4 was 0.02 ng/ml. The intraassay coefficients of variation for E_2 and P_4 were 5% and 3.8%, respectively, and the interassay coefficients of variation for E_2 and P_4 were 4.9% and 5.1%, respectively.

Data analysis

The data are expressed as the mean ± SEM and were analyzed by a one-way ANOVA followed by Tukey's protected *post-hoc* test using GB-Stat version 5.01 (Dynamic Microsystems, Inc., Silver Spring, MD).

Results

RIA

Serum E_2 and P_4 levels achieved by the steroid replacement regimen are shown in Table 1. The mean E_2 levels achieved during the simulated follicular, midcycle, and luteal phases were 37.2 ± 3.1, 145.6 ± 9.7, and 93.6 ± 10.3 pg/ml, respectively, whereas P_4 levels were 2.6 ± 0.4, 2.5 ± 0.7, and 13.1 ± 1.3 ng/ml. These values are similar to those reported for E_2 and P_4 during a natural menstrual cycle (37). Similar E_2 and P_4 levels were seen at the time of perfusion with the exception that E_2 levels in the luteal phase were approximately half the levels achieved in preceding simulated luteal phases. E_2 and P_4 levels in untreated OVX animals at the time of perfusion were undetectable (<1.4 pg/ml) and 2.15 ng/ml, respectively. We conclude from these results that the steroid replacement regimen achieved physiological concentrations of both steroids in OVX monkeys

CRH mRNA

CRH mRNA was detectable in the PVN, but not in the SON (Fig. 1A). Ovarian steroid priming had a significant effect on CRH mRNA signal intensity in the PVN of the nonhuman primate ($F = 6.1$; $P = 0.018$). CRH mRNA levels in the OVX + E_2 animals were 2-fold higher than those in OVX animals (0.38 ± 0.06 *vs.* 0.14 ± 0.09, respectively; $P < 0.05$). In contrast, CRH mRNA levels in OVX + E_2 + P_4 animals were not different from those in OVX controls (0.13 ± 0.08 *vs.* 0.14 ± 0.09).

AVP mRNA

AVP mRNA was detectable in both the PVN and the SON (Fig. 2A). No significant effect of ovarian steroid priming on

TABLE 1. Serum estrogen (E_2) and progesterone (P_4) levels achieved during the simulated menstrual cycle and at the time of perfusion

	OVX (4)	Days 5–12 (17–18)	Days 14–16 (8–10)	Days 19–29 (13)	Perfusion follicular (6)	Perfusion luteal (4)
E_2 (pg/ml)	<1.4	37.2 ± 3.1	145.6 ± 9.7	93.6 ± 10.3	46 ± 6.2	42 ± 4.3
P_4 (ng/ml)	2.15	2.6 ± 0.4	2.5 ± 0.7	13.1 ± 1.3	2.5 ± 0.9	14.3 ± 2.2

The number in *parentheses* represents number of blood samples. Values are the mean ± SEM.

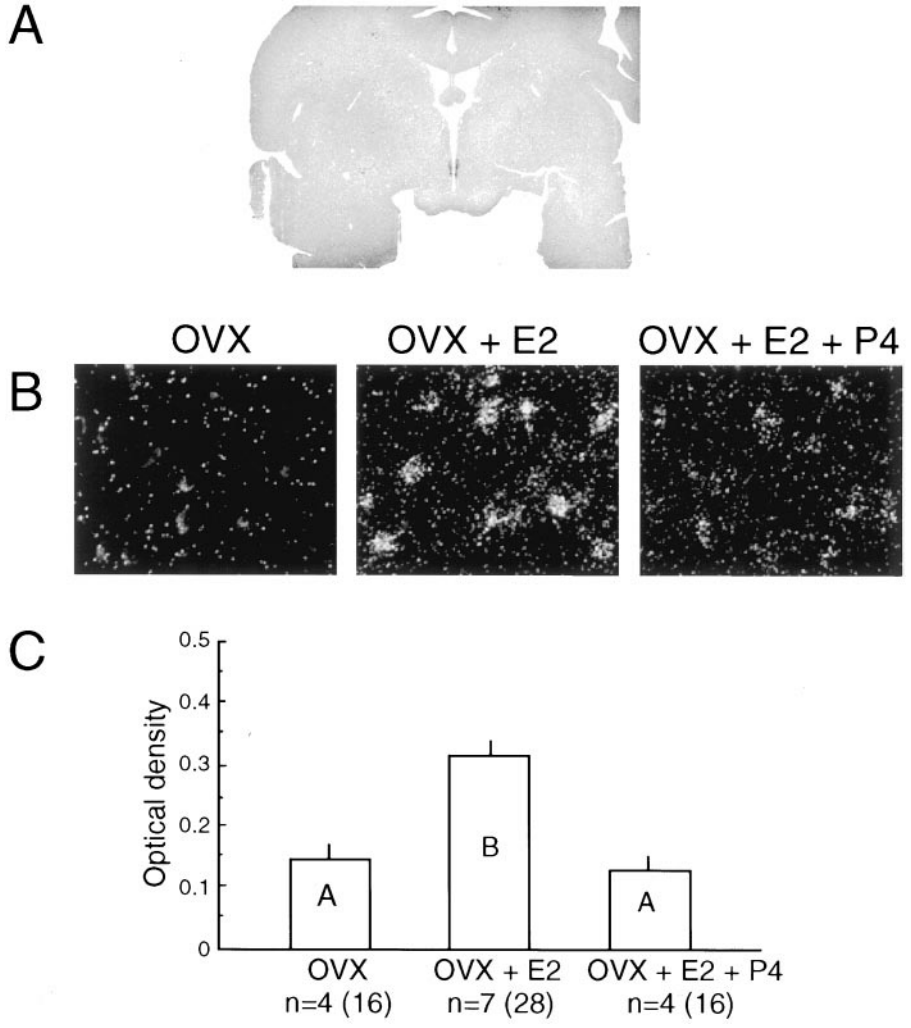

FIG. 1. The effect of ovarian steroids on CRH mRNA in the PVN of the nonhuman primate. A, An example of hybridization signal for CRH mRNA in the PVN of an OVX monkey primed with E_2 taken from x-ray film. B, Darkfield photomicrographs of emulsion-dipped 30-μm sections through the PVN. Note the higher density of silver grains in the OVX + E_2-primed animal (*middle frame*) compared with those in OVX (*left frame*) and OVX + E_2 + P_4 (*right frame*) animals. C, Mean OD measurements for CRH transcript in PVN of OVX, OVX + E_2m and OVX + E_2 + P_4 animals. Values are the mean ± SEM. N, The number of animals. The number in *parentheses* is the number of observations. *Different letters* denote significantly different values among groups ($P < 0.05$).

AVP mRNA levels in the PVN was observed. The relative ODs of AVP in the PVN of OVX, OVX + E_2, and OVX + E_2 + P_4 groups were 0.25 ± 0.03, 0.24 ± 0.03, and 0.175 ± 0.04, respectively (F = 1.26; $P = 0.33$). The signal for AVP mRNA transcript in the SON was more abundant than that in the PVN. No effect of ovarian steroid priming was found. The relative ODs in the OVX, OVX + E_2, and OVX + E_2 + P_4 groups were 1.2 ± 0.16, 1.2 ± 0.13, and 1.2 ± 0.14 (F = 0.19; $P = 0.83$).

Discussion

CRH mRNA levels in the PVN of OVX monkeys sequentially primed with E_2 and P_4 to simulate the menstrual cycle were significantly higher in the follicular phase compared with CRH mRNA levels in either the luteal phase or in the absence of any steroid priming. In contrast, AVP mRNA levels in the PVN and SON were unaffected by the ovarian steroid milieu. These effects of ovarian steroids on CRH mRNA in the monkey are in agreement with the findings of several studies conducted in rats. CRH mRNA in the PVN was significantly elevated on proestrous afternoon before the time of the LH surge (15). This increase was followed by a pronounced decline in CRH mRNA at the expected time of the LH surge. A similar effect of the estrous cycle on the number of immunoreactive CRH neurons in the PVN expressing the c-*fos* gene was reported (38). Furthermore, an increase in CRH heteronuclear RNA and c-*fos* mRNA in the PVN in response to an endotoxin challenge was more pronounced on proestrus compared with diestrus (38). CRH receptor $mRNA_1$, which appears to be positively regulated by CRH, was increased in the PVN of rats after immobilization stress to a greater extent on the morning of proestrus compared with diestrous day 2 (16). Increased CRH production in acetylcholine-stimulated hypothalamic slices in the presence of E_2 has been reported (39). Because our studies were conducted in monkeys seated in primate chairs, a condition that activates the HPA axis, the observed effects of ovarian steroids may reflect changes in CRH mRNA transcript in response to a challenge, and in that sense are in agreement with the above studies that used immobilization or an endotoxin challenge. To our knowledge, this is the first report that examines the potential effects of ovarian steroids on gene expression of CRH and AVP in the nonhuman primate.

However, not all studies support a positive effect of E_2 on CRH. Paulmyer-Lacroix *et al.* (19) observed decreased CRH

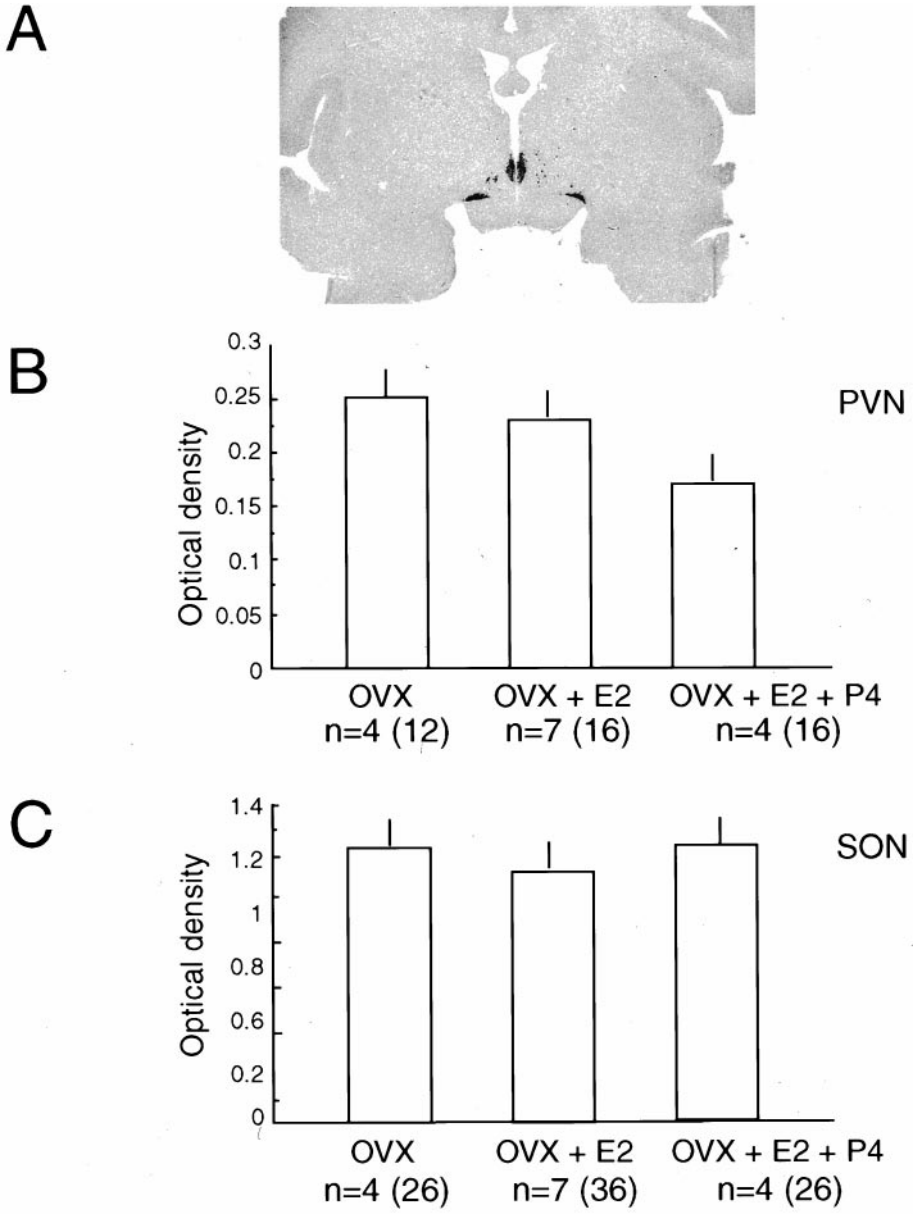

FIG. 2. The effect of ovarian steroids on AVP mRNA in the PVN or SON of the nonhuman primate. A, A representative 30-μm coronal section demonstrating AVP mRNA signal in the PVN and SON in an animal that was primed with E_2. B, Mean OD measurements for AVP transcript taken from x-ray films of the PVN of OVX, OVX + E_2, and OVX + E_2 + P_4 animals. Values are the mean ± SEM. N, The number of animals. The number in *parentheses* is the number of observations. There were no statistically significant differences among treatment groups. C, Mean OD measurements of AVP mRNA levels from the SON. Details are the same as in B.

mRNA levels in the PVN after implantation of E_2 capsules to OVX rats, whereas the administration of ovarian steroids to OVX ewes or OVX/adrenalectomized rats did not significantly affect CRH mRNA levels in the PVN (20, 40). Differences in the above results may be attributable to species, sex, and length of steroid treatment, as each of these potentially confounding variables may influence glucocorticoid secretion and glucocorticoid feedback on the central nervous system (40).

AVP mRNA in either the PVN or SON was unaffected by the different ovarian steroid priming conditions employed in the present study. This finding agrees with a study conducted by Van Tol *et al.* (41), who reported that AVP mRNA in the SON did not vary across the estrous cycle. Similarly, Nappi *et al.* reported that AVP heteronuclear RNA in the parvocellular PVN in response to an endotoxin challenge was not influenced by the estrous cycle (38).

The present results may provide insight into the mechanism of increased sensitivity of the LHRH-LH axis to the inhibitory effects of stress during the estrogen-dominated phases of the cycle. We postulate that estrogen-stimulated CRH gene transcription increases CRH synthesis and leads to increased CRH release with stress onset. Increased CRH release may mechanistically explain why stress-induced inhibition of LHRH/LH secretion occurs more readily in the presence of estrogen priming (30, 32). It is particularly interesting to note that restraint-induced inhibition of LH secretion occurred in the follicular phase, but not the luteal phase, of the nonhuman primate menstrual cycle (42), and that multiunit activity of the LHRH pulse generator in OVX monkeys was impeded by chair restraint only in the presence of estrogen priming (31). Thus, the LHRH/LH response to restraint correlates with the CRH mRNA response to ovarian steroids that we observed, as CRH mRNA levels in the PVN

of OVX monkeys were increased during the simulated follicular phase, but not in the simulated luteal phase. The observation that stress-induced inhibition of LH secretion in male rats was not blocked by lesions of the PVN suggests that CRH neurons in the PVN are not the sole mediators of stress-induced inhibition of the LHRH-LH axis (43). Ongoing studies in our laboratory are examining the effects of ovarian steroids on CRH, vasopressin, and POMC gene expression in hypothalamic and extrahypothalamic areas.

Ovarian steroid regulation of CRH gene expression may be an elementary component to ovarian modulation of HPA axis activity. Although the literature consistently indicates that ovarian steroids positively affect ACTH/corticosterone secretion in rodents, the literature is less consistent regarding the influence of the menstrual cycle on HPA axis activity. However, a definitive study by Smith and Norman indicates that, like the rodent, the amplitude of HPA axis activity is positively regulated by the ovaries and specifically by estrogen (44, 45). We have demonstrated that the ovarian steroid regimen used in the current study increases basal cortisol secretion. However, this difference in cortisol associated with steroid priming is lost when monkeys are restrained in primate chairs (46). Indeed, cortisol levels at the time of perfusion were the same in the three treatment groups (data not shown). Animals were chaired and catheterized in the current study to avoid the stress associated with protracted ketamine immobilization, which was shown to increase AVP levels in hypophyseal portal blood of sheep (47). Therefore, although CRH mRNA levels did not correlate with cortisol levels at the time of perfusion, the estrogen-induced increase in CRH mRNA does correspond with estrogen-induced cortisol secretion, which we and others have documented in the nonhuman primate under basal conditions. The decline in CRH mRNA seen with E_2 + P_4 priming does not correspond to cortisol secretion in the primate. However, it has been shown in the rat that the ACTH response to immobilization stress is enhanced by estrogen, whereas P_4 in combination with estrogen blocked estrogen-induced sensitization of the ACTH response to immobilization (11). Although an effect on the corticotroph cannot be ruled out, inhibition of CRH synthesis and release by P_4 is plausible and supported by the current results.

Steroid receptors function as ligand-induced transcription factors and bind to hormone response elements usually located in the 5′-flanking region of the respective gene. Localization of response elements and receptors within a neuron are suggestive of a direct effect of steroid hormones on gene expression in that particular cell. The 5′-flanking region of the CRH gene contains estrogen-responsive elements and would therefore allow for a direct effect of estrogen on CRH gene transcription (48). *In situ* hybridization measurement of estrogen receptor (ER) mRNA indicated that the PVN expressed high levels of ERβ in contrast to low expression of ERα (49). However, although ERβ colocalized with 60–80% of magnocellular CRH neurons, only 5% of neuroendocrine CRH neurons contained mRNA for ERβ.

The decrease in CRH mRNA observed in the simulated luteal phase may be another example of P_4 antagonism of E_2. An antagonistic effect of P_4 has been documented in the brain, uterus, and MC-7 cells and may result from inhibition of the expression of ER or molecular actions of ER (50, 51). P_4 receptors and P_4 receptor mRNA are localized in the PVN, ventral medial nucleus (VMN) and arcuate nucleus of the rhesus monkey (51, 52). P_4 significantly reduced the number of ER-positive cells in the PVN and VMN of estrogen-primed OVX rhesus monkeys as well as ER mRNA in the VMN, but not the PVN. We also have considered the possibility that the decreased CRH mRNA signal observed in the OVX + E_2 + P_4 animals may result from P_4 binding to the glucocorticoid receptor. P_4 binding to the glucocorticoid receptor could mimic cortisol-negative feedback on CRH synthesis (53).

We have considered the hypothesis that E_2 and P_4 exerts their effects on CRH via β-endorphin. β-Endorphin-containing neurons in the arcuate nucleus concentrate both E_2 and P_4 and contain a significant level of mRNA for both receptors (54, 55). Ovariectomy increased POMC mRNA in the arcuate nucleus of rats, whereas estrogen reversed this effect (56–58). As opiates may inhibit CRH (59), perhaps as a form of negative feedback in response to CRH stimulation of β-endorphin, it follows that estrogen-induced inhibition of POMC could increase CRH gene expression. P_4 antagonism of ERs (specifically on POMC neurons) in the arcuate would block estrogen stimulation of CRH gene expression. P_4 produced a biphasic effect on POMC levels in OVX estrogen-primed rats. P_4 caused an initial decrease in POMC mRNA, which correlated temporally with the onset of the LH surge, followed by a significant increase in POMC (60). A similar biphasic pattern of POMC mRNA was observed on proestrus (61). P_4 stimulation of POMC neurons in the primate is supported by portal blood measurements of β-endorphin, which are elevated in the luteal phase and in OVX monkeys primed with both estrogen and P_4 (62).

Ovarian steroids may affect CRH gene expression through extrahypothalamic afferent inputs to the PVN. The PVN is innervated by many regions, including the brain stem, the amygdala, and the bed nucleus of the stria terminalis (49, 63, 64). Each of these areas is rich in estrogen and P_4 receptors (65, 66) and is associated with catecholamine neurons, which are known to regulate both the HPA and HPO axes. A link between catecholamines, CRH, and estrogen is supported by the observations that fasting-induced suppression of LH secretion in estrogen-primed (but not unprimed) OVX rat was blocked by a CRH antagonist or by pretreatment with α_2-antagonists (67, 68).

Although our observations were limited to CRH gene expression in the PVN, extrahypothalamic CRH systems may be similarly affected by ovarian steroids. CRH appears to subserve a multitude of functions, ranging from its neuroendocrine regulation of adrenal, gonadal, and immune function to behavior, cognition, and memory. Therefore, modulation of CRH gene expression by ovarian steroids has the potential of producing wide ranging effects.

Acknowledgments

The authors greatly appreciate Dr. Serge Rivest for both the use of his laboratory facilities and for his insightful comments in the preparation of this manuscript. We also thank Nataly Laflamme, Sajani Swamy, and Dr. Alicja Krzemien for their technical assistance.

References

1. **Petraglia F, Sutton S, Vale W, Plotsky P** 1987 Corticotropin-releasing factor decreases plasma luteinizing hormone levels in female rats by inhibiting gonadotropin-releasing hormone release into hypophysial-portal circulation. Endocrinology 120:1083–1088
2. **Gindoff PR, Ferin M** 1987 Endogenous opioid peptides modulate the effect of corticotropin-releasing factor on gonadotropin release in the primate. Endocrinology 121:837–842
3. **Heisler LE, Tumber AJ, Reid RL, Van Vugt DA** 1994 Vasopressin mediates hypoglycemia-induced inhibition of luteinizing hormone secretion in the ovariectomized rhesus monkey. Neuroendocrinology 60:297–304
4. **Dubey AK, Plant TM** 1985 A suppression of gonadotropin secretion by cortisol in castrated male rhesus monkeys (*Macaca mulatta*) mediated by the interruption of hypothalamic gonadotropin-releasing hormone release. Biol Reprod 33:423–431
5. **Rosen H, Jameel ML, Barkan AL** 1988 Dexamethasone suppresses gonadotropin-releasing hormone (GNRH) secretion and has direct pituitary effects in male rats: differential regulation of GNRH receptor and gonadotropin responses to GNRH. Endocrinology 122:2873–2880
6. **Calogero AE, Burrello N, Negri-Cesi P, Papale L, Palumbo MA, Cianci A, Sanfilippo S, D'Agata R** 1996 Effects of corticotropin-releasing hormone on ovarian estrogen production in vitro. Endocrinology 137:4161–4166
7. **Welsh THJ, Bambino TH, Hsueh AJ** 1982 Mechanism of glucocorticoid-induced suppression of testicular androgen biosynthesis in vitro. Biol Reprod 27:1138–1146
8. **Mann DR, Free C, Nelson C, Scott C, Collins DC** 1987 Mutually independent effects of adrenocorticotropin on luteinizing hormone and testosterone secretion. Endocrinology 120:1542–1550
9. **Young EA** 1995 The role of gonadal steroids in hypothalamic-pituitary-adrenal axis regulation. Crit Rev Neurobiol 9:371–381
10. **Chrousos GP, Gold PW** 1992 The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis. JAMA 267:1244–1252
11. **Viau V, Meaney MJ** 1991 Variations in the hypothalamic-pituitary-adrenal response to stress during the estrous cycle in the rat. Endocrinology 129:2503–2511
12. **Kirschbaum C, Schommer N, Federenko I, Gaab J, Neumann O, Oellers M, Rohleder N, Untiedt A, Hanker J, Pirke KM, Helhammer DH** 1996 Short-term estradiol treatment enhanced pituitary-adrenal axis and sympathetic response to psychological stress in healthy young men. J Clin Endocrinol Metab 81:3639–3643
13. **Coyne M, Kitay JI** 1969 Effect of ovariectomy on pituitary secretion of ACTH. Endocrinology 85:1097–1102
14. **Patchev VK, Hayashi S, Orikasa C, Almeida OFX** 1995 Implications of estrogen-dependent brain organization for gender differences in hypothalamic-pituitary-adrenal regulation. FASEB J 9:419–423
15. **Bohler CL, Zoeller RT, King JC, Rubin BS, Weber R, Merriam GR** 1990 Corticotropin releasing hormone mRNA is elevated on the afternoon of proestrus in the parvocellular paraventricular nuclei of the female rat. Mol Brain Res 8:259–262
16. **Nappi RE, Rivest S** 1995 Ovulatory cycles influences the stimulatory effect of stress on the expression of corticotropin-releasing factor receptor messenger ribonucleic acid in the paraventricular nucleus of the female rat hypothalamus. Endocrinology 136:4073–4083
17. **Vamvakopoulos NC, Chrousos GP** 1993 Evidence of direct estrogenic regulation of human corticotropin-releasing hormone gene expression: potential implications for the sexual dimorphism of the stress response and immune/inflammatory reaction. J Clin Invest 92:1896–1902
18. **Haas DA, George SR** 1987 Gonadal regulation of corticotropin-releasing factor immunoreactivity in hypothalamus. Brain Res Bull 20:361–367
19. **Paulmyer-Lacroix O, Hery M, Pugeat M, Grino M** 1996 The modulatory role of estrogens on corticotropin-releasing factor gene expression in the hypothalamic paraventricular nucleus of ovariectomized rats: role of the adrenal gland. J Neuroendocrinol 8:515–519
20. **Broad KD, Keverne EB, Kendrick KM** 1995 Corticotropin-releasing factor mRNA in the sheep brain during pregnancy, parturition and lactation and following exogenous progesterone and estrogen treatment. Mol Brain Res 29:310–316
21. **Haas DA, George SR** 1989 Estradiol or ovariectomy decreases CRH synthesis in hypothalamus. Brain Res Bull 23:215–218
22. **Gillies GE, Linton EA, Lowry PJ** 1982 Corticotropin releasing activity of the new crf is potentiated several times by vasopressin. Nature 299:355–357
23. **Skowsky WR, Swan L, Smith P** 1979 Effects of sex steroid hormones on arginine vasopressin in intact and castrated male and female rats. Endocrinology 104:105–108
24. **Punnonen R, Viinamaki O, Multamaki S** 1983 Plasma vasopressin during normal menstrual cycle. Horm Res 17:90–92
25. **Forsling ML, Kelestimur H, Windle R** 1991 The influence of reproductive status on vasopressin release in the rat. J Endocrinol 130:387–393
26. **Greer ER, Caldwell JD, Johnson MF, Prange AJ, Pedersen CA** 1986 Variations in concentration of oxytocin and vasopressin in the paraventricular nucleus of the hypothalamus during the estrous cycle in rats. Life Sci 38:2311–2318
27. **Forsling ML** 1993 The influence of gonadal steroids on the release and actions of neurohypophysial hormones. Regul Pept 45:193–196
28. **Heisler LE, Pallotta CM, Reid RL, Van Vugt DA** 1993 Hypoglycemia-induced inhibition of luteinizing hormone secretion in the rhesus monkey is not mediated by endogenous opioid peptides. J Clin Endocrinol Metab 76:1280–1285
29. **Chen MD, O'Byrne KT, Chiappini SE, Hotchkiss J, Knobil E** 1992 Hypoglycemic 'stress' and gonadotropin-releasing hormone pulse generator activity in the rhesus monkey: role of the ovary. Neuroendocrinology 56:666–673
30. **Cagampang FR, Maeda KI, Tsukamura H, Ohkura S, Ota K** 1991 Involvement of ovarian steroids and endogenous opioids in the fasting-induced suppression of pulsatile LH release in ovariectomized rats. J Endocrinol 129:321–328
31. **Kesner JS, Wilson R, Kaufman J, Hotchkiss J, Chen Y, Yamamoto H, Pardo R, Knobil E** 1987 Unexpected response of the hypothalamic gonadotropin-releasing hormone "pulse-generator" to physiological estradiol inputs in the absence of the ovary. Proc Natl Acad Sci USA 84:8745–8749
32. **O'Byrne K, Thalabard JC, Grosser PM, Wilson R, Williams CL, Chen MD, Ladendorf D, Hotchkiss J, Knobil E** 1991 Radiotelemetric monitoring of hypothalamic gonadotropin-releasing hormone pulse generator activity throughout the menstrual cycle of the rhesus monkey. Endocrinology 129:1207–1214
33. **Shalts E, Xia L, Xiao E, Ferin M** 1994 Inhibitory effects of arginine-vasopressin on LH secretion in the ovariectomized rhesus monkey. Neuroendocrinology 59:336–342
34. **Shalts E, Feng YJ, Ferin M** 1992 Vasopressin mediates the interleukin-1α-induced decrease in luteinizing hormone secretion in the ovariectomized rhesus monkey. Endocrinology 13:153–158
35. **Van Vugt DA, Heisler LE** 1995 Stress and reproduction. In: Barnes CD, Sarker DK (eds) The Reproductive Neuroendocrinology of Aging and Drug Abuse. CRC Press, Boca Raton, pp 189–214
36. **Simmons DM, Arriza JL, Swanson LW** 1989 A complete protocol for in situ hybridization of messenger RNAs in brain and other tissues with radiolabeled single-stranded RNA probes. J Histotechnol 12:169–181
37. **Speroff L, Glass RH, Kase NG** 1994 Regulation of the menstrual cycle In: Mitchell C (ed) Clinical Gynecologic Endocrinology and Infertility, ed 5. Williams and Wilkins, Baltimore, pp 183–230
38. **Nappi RE, Bonneau MJ, Rivest S** 1997 Influence of the estrous cycle on c-*fos* and CRF gene transcription in the brain of endotoxin-challenged female rats. Neuroendocrinology 65:29–46
39. **Buckingham JC** 1982 Effects of adrenocortical and gonadal steroids on the secretion *in vitro* of corticotropin and its hypothalamic releasing factor. J Endocrinol 93:123–132
40. **Patchev VK, Almeida OFX** 1996 Gonadal steroids exert facilitating and "buffering" effects on glucocorticoid-mediated transcriptional regulation of corticotropin-releasing hormone and corticosteroid receptor genes in rat brain. J Neurosci 16:7077–7084
41. **Van Tol HH, Bolwerk EL, Liu B, Burbach JP** 1988 Oxytocin and vasopressin gene expression in the hypothalamo-neurohypophyseal system of the rat during the estrous cycle, pregnancy, and lactation. Endocrinology 122:945–951
42. **Norman RL, McGlone J, Smith CJ** 1994 Restraint inhibits luteinizing hormone secretion in the follicular phase of the menstrual cycle in rhesus macaques. Biol Reprod 50:16–26
43. **Rivest S, Rivier C** 1991 Influence of the paraventricular nucleus of the hypothalamus in the alteration of neuroendocrine functions induced by intermittent footshock or interleukin. Endocrinology 129:2049–2057
44. **Smith CJ, Norman RL** 1987 Influences of the gonads on cortisol secretion in female rhesus macaques. Endocrinology 121:2192–2198
45. **Norman RL, Smith CJ, Pappas JD, Hall J** 1992 Exposure to ovarian steroids elicits a female pattern of plasma cortisol levels in castrated male macaques. Steroids 57:37–43
46. **Heisler LE, Reid RL, Van Vugt DA,** Ovarian steroid modulation of CRH stimulation of cortisol in rheus monkeys. 20th Annual Meeting of the Society of Neuroscience, St. Louis MO, 1990 (Abstract)
47. **Engler D, Pham T, Fullerton MJ, Ooi G, Funder JW, Clarke IJ** 1989 Studies of the secretion of corticotropin-releasing factor and arginine vasopressin into the hypophysial-portal circulation of the conscious sheep. I. Effect of an audiovisual stimulus and insulin-induced hypoglycemia. Neuroendocrinology 49:367–381
48. **Vamvakopoulos NC, Chrousos GP** 1993 Structural organization of the 5′ flanking region of the human corticotropin releasing hormone gene. DNA Sequence 4:197–206
49. **LaFlamme N, Nappi RE, Drolet G, Labrie C, Rivest S** 1998 Expression and neuropeptidergic characterization of estrogen receptors (ERα and ERβ) throughout the rat brain: anatomical evidence of distinct roles of each subtype. J Neurobiol 36:357–378
50. **Graham JD, Clarke CL** 1997 Physiological actions of progesterone in target tissues. Endocr Rev 18:502–519
51. **Bethea CL, Brown NA, Kohama SG** 1996 Steroid regulation of estrogen and progestin receptor messenger ribonucleic acid in monkey hypothalamus and pituitary. Endocrinology 137:4372–4383
52. **Goldsmith PC, Boggan JE, Thind KK** 1997 Estrogen and progesterone receptor expression in neuroendocrine and related neurons of the pubertal female monkey hypothalamus. Neuroendocrinology 65:325–334
53. **Svec F** 1991 Comparison of glucocorticoid receptors liganded with dexamethasone or progesterone. Proc Soc Exp Biol Med 198:811–817

54. **Fox SR, Harlan RE, Shivers BD, Pfaff DW** 1990 Chemical characterization of neuroendocrine targets for progesterone in the female rat brain and pituitary. Neuroendocrinology 51:276–283
55. **Morrell JI, McGinty JF, Pfaff DW** 1985 A subset of β-endorphin- or dynorphin-containing neurons in the medial basal hypothalamus accumulates estradiol. Neuroendocrinology 41:417–426
56. **Treiser SL, Wardlaw SL** 1992 Estradiol regulation of proopiomelanocortin gene expression and peptide content in the hypothalamus. Neuroendocrinology 55:167–173
57. **Wilcox JN, Roberts JL** 1985 Estrogen decreases rat hypothalamic proopiomelanocortin messenger ribonucleic acid levels. Endocrinology 117:2392–2396
58. **Tong Y, Zhao HF, Labrie F, Pelletier G** 1990 Regulation of proopiomelanocortin messenger ribonucleic acid content by sex steroids in the arcuate nucleus of the female rat brain. Neurosci Lett 112:104–108
59. **Plotsky PM** 1986 Opioid inhibition of immunoreactive corticotropin-releasing factor secretion into the hypophysial-portal circulation of rats. Regul Pept 16:235–242
60. **Petersen SL, Keller ML, Carder SA, McCrone S** 1993 Differential effects of estrogen and progesterone on levels of POMC mRNA levels in the arcuate nucleus: relationship to the timing of LH surge release. J Neurobiol 36:357–378
61. **Bohler HC, Tracer H, Merriam GR, Petersen SL** 1991 Changes in proopiomelanocortin messenger ribonucleic acid levels in the rostral periarcuate region of the female rat during the estrous cycle. Endocrinology 128:1265–1269
62. **Ferin M, Van Vugt DA, Wardlaw SL** 1984 The hypothalamic control of the menstrual cycle and the role of endogenous opioids peptides. Recent Prog Horm Res 40:441–485
63. **Simerly RB, Chang C, Muramatsu M, Swanson LW** 1990 Distribution of androgen and estrogen receptor mrna-containing cells in the rat brain: an *in situ* hybridization study. J Comp Neurol 294:76–95
64. **Shughrue PJ, Lane MV, Merchenthaler I** 1997 Comparative distribution of estrogen receptor-α and -β mRNA in the rat central nervous system. J Comp Neurol 388:507–525
65. **Li HY, Blaustein JD, De Vries GJ, Wade GN** 1993 Estrogen-receptor immunoreactivity in hamster brain: preoptic area, hypothalamus and amygdala. Brain Res 631:304–312
66. **Blaustein JD, Turcotte JC** 1989 Estradiol-induced progestin receptor immunoreactivity is found only in estrogen receptor-immunoreactive cells in guinea pig brain. Neuroendocrinology 49:454–461
67. **Tsukamura H, Nagatani S, Cagampang FR, Kawakami S, Maeda K** 1994 Corticotropin-releasing hormone mediates suppression of pulsatile luteinizing hormone secretion induced by activation of α-adrenergic receptors in the paraventricular nucleus in female rats. Endocrinology 134:1460–1466
68. **Cagampang FR, Ohkura S, Tsukamura H, Coen CW, Ota K, Maeda K** 1992 α_2-Adrenergic receptors are involved in the suppression of luteinizing hormone release during acute fasting in the ovariectomized estradiol-primed rats. Neuroendocrinology 56:724–728