ACTA NEUROBIOLOGIAE EXPERIMENTALIS

Acta Neurobiol. Exp. 1997, 57: 91-99

The effect of corticotropin-releasing factor (CRF) on the gonadotropin hormone releasing hormone (GnRH) hypothalamic neuronal system during preovulatory period in the ewe

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Abstract. Effects of infusions of corticotropin releasing factor (CRF) into the 3rd ventricle of the brain of ewes during the proestrus on the immunoreactive (ir) gonadotropin hormone releasing hormone (GnRH) neuronal system, pituitary luteinizing hormone(LH) producing cells and LH concentrations in the blood plasma were studied. None of the CRF-treated sheep displayed the estrous activity nor ovulated on the day of estrus (17th day of the cycle), and two days later when they were slaughtered. The GnRH center of CRF treated ewes situated in the preoptico-septal area was well organized , but irGnRH stores in the median eminence were low in comparison to the controls (sheep from the late follicular phase of the estrous cycle). The feature and the number of LH-cells in CRF-treated ewes were typical for the preovulatory phase of the cycle but the plasma concentrations of LH did not exceed basal levels. These results suggest that CRF induced decrease of irGnRH stores in the nerve terminals of the ME can be responsible for the blockade of the preovulatory surge of GnRH/LH in the sheep.

Key words: CRF, GnRH, ovulation, ewe

INTRODUCTION

Recent evidence has shown that the hypothalamic corticotropin releasing factor (CRF) primarily involved in the control of pituitary ACTH, and thus connected with stress-induced responses, also has an effect upon secretion of reproductive hormones in mammals. It affects the reproductive axis by decreasing the concentration of LH in the blood plasma of rats (Rivier and Vale 1984), humans (Barbarino et al. 1989) and primates (Olster and Ferin 1987, Xiao et al. 1989). The observation that CRF inhibits the release of the gonadotropin hormone releasing hormone (GnRH) into the pituitary portal circulation *in vivo* (Pertaglia et al. 1987) and *in vitro* (Nikolarakis et al. 1986) suggests that the peptide acts at the central nervous system level. The role of CRF in GnRH/LH secretion in the sheep is controversial. Our previous experiment made on anestrous ewes suggests that stress-induced suppression of GnRH neuronal activity can be initiated by CRF discharge elicited by the acute stressful stimulation (Polkowska and Przekop 1992). On the other hand, infusions of CRF into the 3rd ventricle of the brain (ICV) of ovariectomized sheep were shown to stimulate basal LH release (Naylor et al. 1990) while Horton et al.(1988) did not find this effect. The mechanism through which CRF can influence GnRH secretion is not fully elucidated. The presence of synaptic connections between GnRH and CRF neurons suggests the possibility of direct action (MacLusky et al.1988). However, there is some evidence for indirect action, namely that CRF modulates the activity of catecholaminergic (Tomaszewska et al.1992, Levicki and Dunn 1993) and opioid (Shutt et al.1989) systems involved in GnRH regulation.

The present work was designed to examine the effect of ICV CRF infusions in ewes at the late follicular phase of the estrous cycle on estrous behavior and on the preovulatory surge of LH and to correlate them with the activity of hormones of the hypothalamo-pituitary gonadotropic (H-P-G) axis. The immunoreactive (ir) GnRH neuronal system in the hypothalamus, irLH-producing cells in the pituitary gland and plasma LH concentrations were examined.

METHODS

Animals

Studies were performed on three- year- old Polish Merino ewes in the follicular phase of the estrous cycle. The animals were exposed to a natural day/night cycle, were kept indoors in individual pens and had visual contact with their neighbors throughout the study. Animals were fed a standard diet of hay and concentrates. Food and water were available *ad libitum*. The estrous cycles were checked with a vasectomized ram. Only animals with regular 17-day estrous cycles were included in the experiment.

Sugical procedures

Implantations of cannulae were made at least three weeks prior to the infusion. Animals were pretreated with atropine (Polfa) given at a dose of 0.05 mg/kg of body weight s.c. and after 15 min anesthesia was induced with pentobarbital ("Vetbutal" Biowet, Puławy) given i.v. at a dose of 10-20 mg/kg of body weight until surgical levels of anesthesia were reached. Animals under deep anesthesia were fixed in a stereotaxic apparatus. Using the procedure of Traczyk and Przekop (1963), permanent stainless-steel guide cannulae (0.6 mm) were lowered towards the 3rd ventricle using the stereotaxic coordinate system of Welento et al. (1969) (position anterio-posterior 31 mm, lateral 0.5 mm along saggital plane 0.10). The cannulae were secured to the skull with dental cement and screws. Each guide tube was fitted with an indwelling stylette to prevent a backflow of the cerebrospinal fluid. After the surgery, the antibiotic Pen--Strep (Norbrook) 0.04 ml/kg of body weight was given s.c. during 4 consecutive days. One day before infusions a jugular venous catheter was inserted into each ewe and kept with heparinized saline (50 units heparin/ml in 0.9% w/v NaCl.

Injection procedure and blood sampling

GROUP 1

Control (Ringer solution) and experimental (CRF infusions) were applied to 5 regularly cycling ewes on the 16th and 17th days of the estrous cycle for 6 h starting at 0930 a.m. Immediately before each injection, the stylette was removed from the guide cannula and an injector needle connected to a lenght of tubing (Hythe, Kent, England) containing the solution to be injected was lowered to a depth approximately 2.5 mm above the base of the brain, at the level of the medial basal hypothalamus. A 3 h control period of continuous infusion of Ringer solution preceded CRF infusion. During the next

3 h each sheep received 25 μg CRF (Sigma) dissolved in Ringer solution. Both control and experimental infusions were performed at the flow rate of 5 μl/min using CMA 100 microinjection pump (CMA/Microdialysis AB, Stockholm, Sweden). Blood samples were drawn at 15 min intervals over the first 2 h of infusion, and at 30 min intervals over the next 4 h of infusion. Samples were centrifuged and collected plasma was stored at -20°C until analysis. Two days after the last infusion, i.e. on the 19th day after the last estrus, ewes were decapitated under pentobarbital anesthesia in the local licenced abattoir.

GROUP 2

Three regularly cycling ewes were continuously infused on the 16th, and 17th days of the cycle with Ringer solution for 6 hours between 0930 a.m and 0330 p.m. according the procedure described above. Peripheral blood samples were drawn as in group 1 and used for estimation of LH concentrations. This experiment was performed to show the effect of ICV vehicle infusions on plasma LH levels during late follicular phase of the estrous cycle and ovulation.

GROUP 3

Four regularly cycling ewes were infused with Ringer solution on the 16th day of the cycle as described above. They were slaughtered in the morning of the 17th day of the cycle when the first signs of estrous behavior were noticed, but before the ovulation. These ewes constituted the control for immunocytochemical purposes.

All these procedures were done with the consent of the Institutional Animal Care Committee.

Immunocytochemistry

Immediately after decapitation, the brains were perfused *via* internal carotid arteries with 0.1 M phosphate buffered saline (PBS) and subsequently with 0.1 M PBS containing 4% (w/v) paraformaldehyde and 15% (w/v) saturated picric acid solution, pH 7.4. The hypothalami and pituitaries were dissected out 30 min after the beginning of perfusion and fixed for a further 72 h by immersion in the same fixative. Pituitaries and 2 hypothalami from each group were washed with 0.01 M PBS (pH 7.4), dehydrated in graded alcohols, embedded in paraplast and then cut in coronal plane to 3 μm (pituitaries) or 7 μm (hypothalami) sections and processed for immunocytochemistry. Remaining hypothalami were cryoprotected in a 20% sucrose solution in 0.1 M PBS at 4°C for at least one week, cut on a cryostat (Leica, Jung CM 1500) in coronal planes at 30 μm thickness and processed for immunocytochemistry. Pituitaries were cut through the entire gland, hypothalami between the septum and the mamillary bodies. All sections were washed in 0.01 M PBS,and then incubated for 30 min in 0.1% hydrogen peroxide in 0.01 M PBS and 30 min in 2% preimmune lamb serum in 0.01 M PBS. Cryostat sections were incubated additionally in 0.5% Triton X-100 in 0.01 M PBS for 30 min. The collected brain sections were incubated in primary antiserum for 72 h at 4°C, pituitary sections for 24 h at 4°C. The following antisera were used: anti-(2-10) GnRH no. 1900; anti-(1-10) GnRH no. 8534, incubated at dilution 1:2000, anti-LHβ no. 1926 at dilution 1:200-1:500. All antibodies were kindly donated by dr M.P. Dubois from I.N.R.A, Nouzilly, France. Methodological details and the specificity of these antibodies were as described by Dubois and Barry (1974) and Dubois and Dubois (1974). After incubation with primary antibody the sections were rinsed in 0.01 PBS and incubated for 2 h at room temperature in secondary antibody (sheep anti-rabbit IgG [H+L] labeled with peroxidase, Institute Pasteur, Paris), dilution 1:40 with 0.1% normal lamb serum. The peroxidase was visualized by a 0.05% 3'3-diaminobenzidine tetrachloride chromogen (Sigma) with 0.001% hydrogen peroxide in 0.05 M TRIS buffer. Selected material was additionally stained by the silver intensification method of Liposits et al. (1984). As a control reaction, the inhibition of anti-hormone serum with its homologous antigen was used. Preincubation of GnRH antisera with 10 ug/ml synthetic GnRH (UCB Belgium) and the LH antisera with 4 μg/ml porcine LHβ (Courte and Willemont) blocked the immunostaining. Antigens and antisera were mixed and pre-incubated for 24 h at 4°C before use. A projection microscope (Nikon type 104) was used to observe hypothalamic and pituitary sections and count nuclei in the pituitary cells. The percentage of immunopositive LH-cells in the pituitary gland was determined in the population of six thousand cells counted in every fiftieth section of each pituitary gland. Average cell surface area of normal and hypertrophied LH cells was determined by capturing images of cells and measuring surface area of 40 cells in each pituitary using Lucia version 3.5 (Laboratory Imaging Ltd).

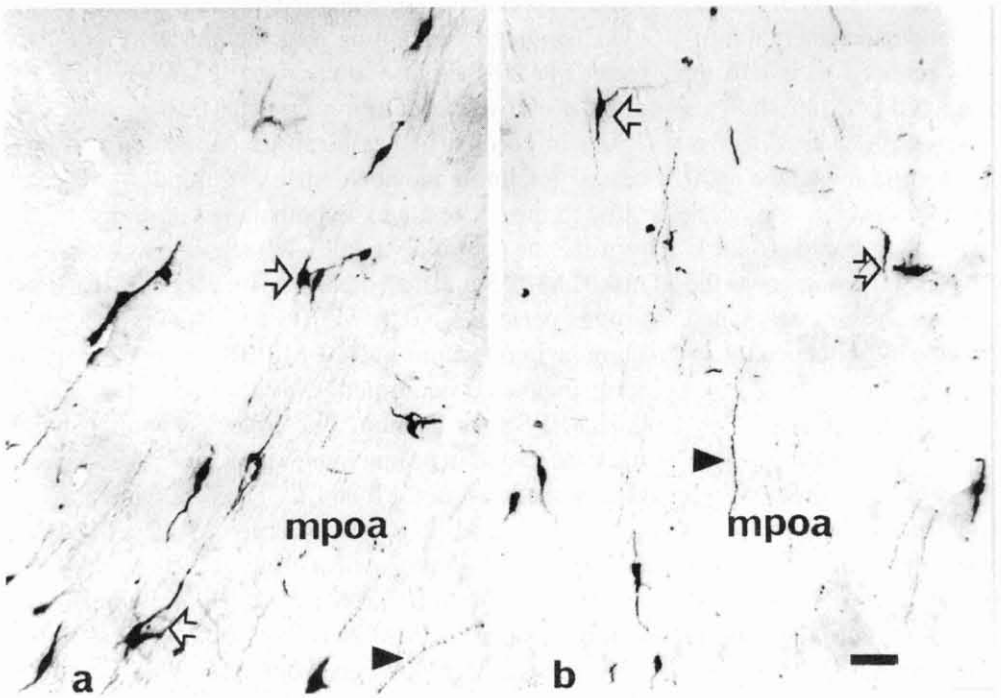

Fig. 1. IrGnRH neurons in the medial preoptic area of representative sheep from control (a) and CRF-infused (b) groups. Scale bar = 200 μm, mpoa-medial preoptic area. Note numerous irGnRH perikarya (open arrows) and dense network of immunoreactive axons (black arrow heads) in both groups.

Determination of LH in peripheral blood plasma

The concentration of LH was determined by routine double antibody radioimmunoassay (Stupnicki and Madej 1976). The assay detection limit was 0.06 ng/ml sample. The coefficient of variation calculated for control samples at concentration 1 ng/ml and 5 ng/ml of LH was 10 and 4% respectively.

Statistical analysis

Data are presented as the mean ± standard deviation. Differences between the groups (immunocytochemical data) or between different treatments in group 1 (LH concentrations) were determined with the Student's *t*-test. Statistical significance was taken at $P<0.01$.

RESULTS

Immunocytochemistry of the hypothalamus

In all ewes infused with CRF (group 1) and with Ringer solution (group 3), a characteristic distribution pattern of GnRH neurons was seen confirming our previous results (Polkowska et al. 1980) and those of others (Lehman et al. 1986, Caldani et al. 1988). The majority of GnRH neurons was found within the medial preoptic area at the level of the organum vasculosum of the lamina terminalis (OVLT). Scattered cells extended rostrally by the nucleus tractus diagonalis and caudally were spreading out in the direction of the lateral preoptic nucleus and the area dorsal to the nucleus supraopticus. Single cell bodies were seen in the medial basal hypothalamus, usually in the vicinity of the median eminence (ME). In the ME the highest concentrations of GnRH-containing terminals were located in the medial-basal and lateral portions of the external layer of this organ. The same distribution of GnRH neurons, high number of GnRH cell bodies (approximately 10 to 40 cells per slice) and excessive dense network of GnRH fibers within the area preoptica was observed in the control as well as in the CRF-infused groups (Fig. 1 a and b). Cell bodies displayed various morphologies. They were irregular, fusiform in shape, generally bi- or multipolar with extensive dendrite-like processes irregular in outline and branched. (Fig. 2 a-d). An intensive immunoreaction was observed within GnRH cell bodies and GnRH fibers. Striking differences in the density of GnRH material between both groups were observed in the region of the ME. In the control ewes there was a high accumulation of immunoreactive GnRH stores in all parts of the ME (Fig. 3 a and c) as compared to a very small density of irGnRH material in the ME of CRF-treated ewes. (Fig. 3 b and d).

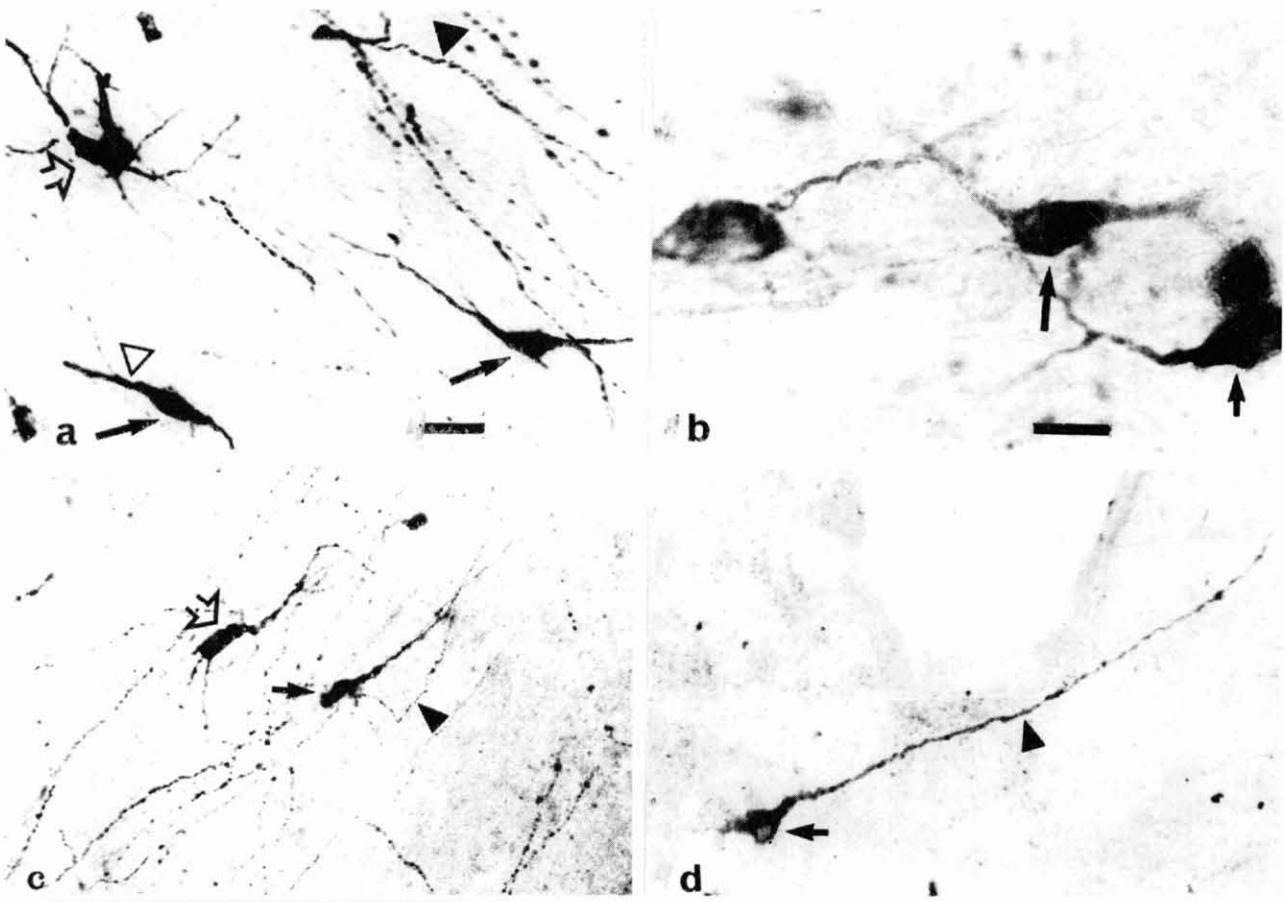

Fig. 2. Variety of morphological types of GnRH perikarya from uni- (short arrows) to bi- (long arrows) and multipolar (open arrows) cells, with extensive dendrites (white arrow heads) and axonal processes (black arrow heads). a-b, control sheep; c-d, CRF-treated sheep. Scale bars: a, c and d =100 μm; b = 50 μm.

Immunocytochemistry of LH-cells

The feature and the number of irLH-cells in pituitaries of the control (group 3) and CRF-treated (group 1) ewes were in both groups typical for the phase of the estrous cycle which they represented (late follicular phase of the cycle)(Polkowska et al. 1980). They showed an intense immunocytochemical reaction and some of them were hypertrophied (Fig. 4 a and b). There were no significant differences between the control ($n = 4$) and CRF-treated ($n = 5$) groups in: 1. the number of LH cells (expressed as percentage of counted adenohypophysial cells) (14.1 ± 1.39 *versus* 14.8 ± 1.82, mean ± SD); 2. cell surface area (μm^2) of normal LH cells (50.4 ± 15.1 *versus* 51.7 ± 12.1, mean ± SD); 3. cell surface area of hypertrophied cells (118.9 ± 21.2 *versus* 115 ± 20.5, mean ± SD).

LH concentrations

All ewes infused ICV with CRF (group 1) did not show estrous behavior on the 17th day of the cycle, nor during the next two days. Plasma levels of LH were unaffected by central injections of CRF. There were no significant differences between the mean LH concentrations following Ringer and CRF infusions ($P<0.01$) (Fig. 5). None of the sheep treated with CRF exhibited the preovulatory surge of LH. The mean concentration of LH following CRF infusions on the 16th day of the estrous cycle was not significantly different from that on the 17th day ($P<0.01$) (Fig. 5). The ewes infused only with Ringer solution (group 2) during the 16th and 17th days of the cycle showed normal estrous behavior in the morning of the 17th day of the cycle. On the 16th day, the mean basal levels of LH oscillated at about 6 ng/ml

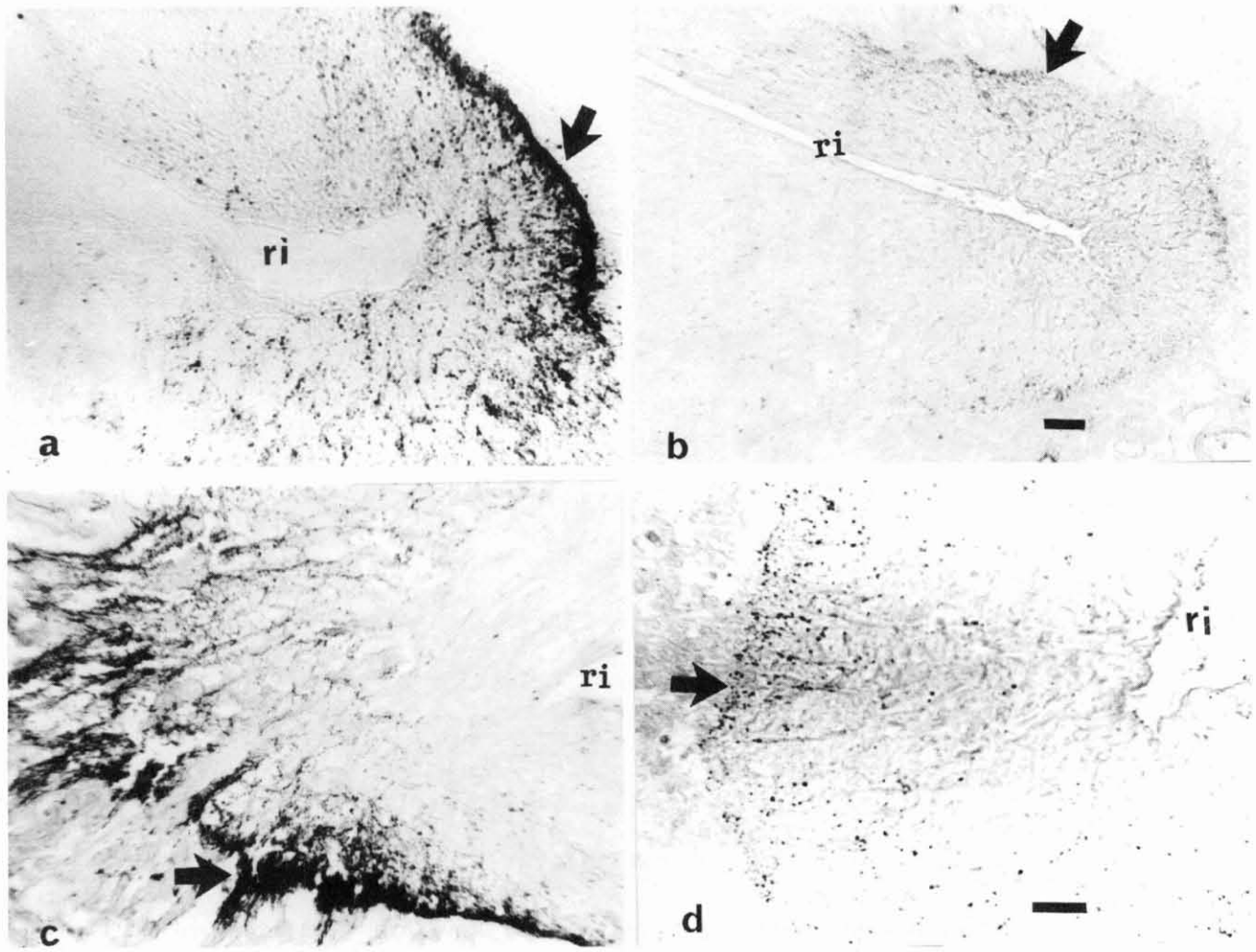

Fig. 3. IrGnRH material in the median eminence of four representative sheep from control (a, c) and CRF- treated (b, d) groups. Scale bars = 200 μm; r, recessus infundibularis. Note markedly diminished accumulation of irGnRH material in the median eminence of CRF-infused sheep in comparison to controls (arrows).

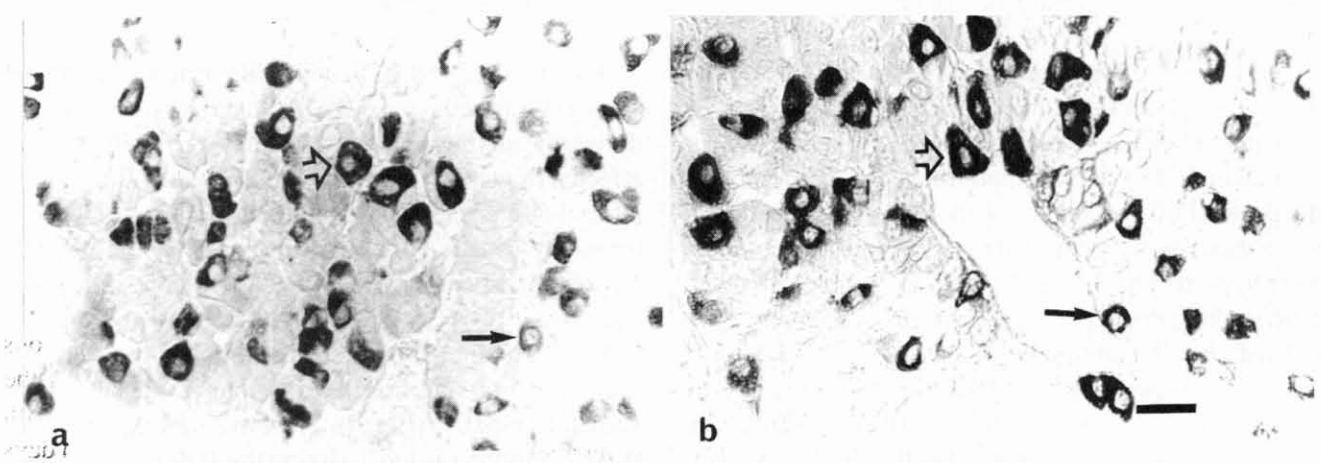

Fig. 4. IrLHβ cells in the adenohypophyses of representative sheep from control (a) and CRF-infused (b) groups, Scale bar = 20 μm. Note the accumulation of irLHβ material and hypertrophy of cells from both groups (normalcells, black arrows; hypertrophied cells, open arrows).

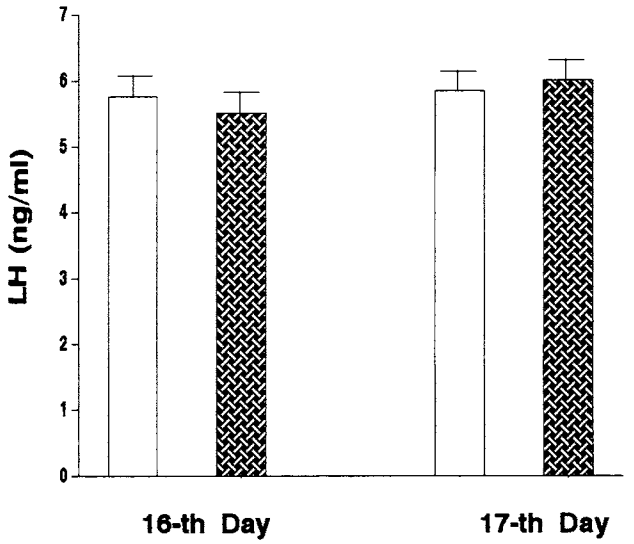

Fig. 5. Mean concentrations of LH (mean ± SD, $n = 5$) in the blood plasma of ewes infused ICV 3 h with saline (open bar) and next 3 h with CRF (hatching) on days 16 and 17 of the estrous cycle. Blood samples were collected from animals at 15 min intervals over the first 2 h and 30 min intervals over the next 4 h of the infusion.

(Fig. 6). The physiological pattern of the preovulatory LH surge with highest concentrations of about 200 ng/ml was observed on the 17th day of the cycle in three monitored sheep (Fig. 6).

DISCUSSION

It is well established that stress disrupts reproductive functions in mammals by modifying the secretion of gonadotropic hormones (reviewed by Collu et al. 1984). Many effects of CRF resembled those observed in stress which suggests that CRF may be an endogenous mediator of such responses (Koob and Bloom 1985). In this study, we have demonstrated that ICV infusions of CRF during the late follicular phase of ovine estrous cycle delayed the LH surge, and in consequence, estrous and ovulation did not occur. The analysis of GnRH neurons in brains of the CRF-treated sheep shows that the GnRH center in the area preoptica is typical for the preovulatory phase: rich in perikarya densely filled with the hormone. In contrast to preovulatory phase characteristics, (Polkowska et al. 1980) GnRH terminals in the ME revealed a shortage of hormonal stores. This suggest that CRF does not block biosynthesis but rather can affect

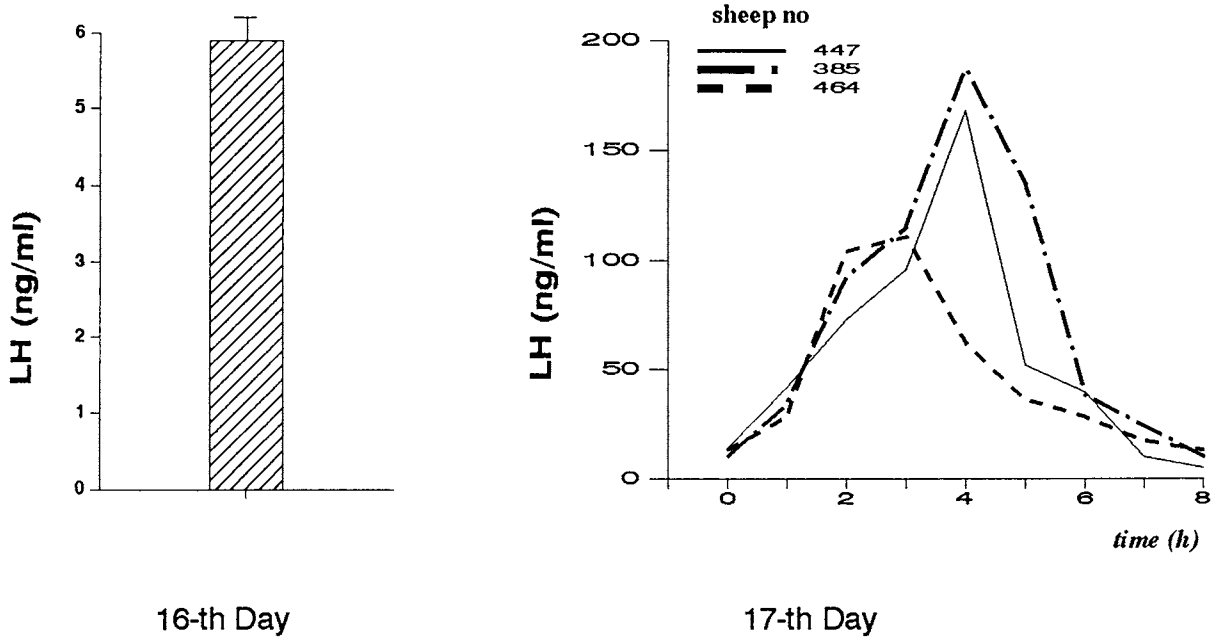

Fig. 6 . Mean basal levels of LH in the plasma of three ewes infused 6 h ICV with saline, during the 16th day of the estrous cycle (mean ± SD) and individual preovulatory LH profiles in the same three ewes treated 6 h with saline on the 17th day of the cycle. Sampling period 6 h, with 15 min intervals over first 2 h and 30 min intervals over next 4 h of the infusion.

GnRH release or its transport from perikarya to the terminals. In the present study we have not defined the pattern of GnRH release from the ME to the portal vessels. However, no changes in morphology, function or number of pituitary LH cells and basal LH concentrations in the CRF-treated ewes indicate that CRF does not affect basal GnRH release. In addition, it has been demonstrated that in ovariectomized estradiol-treated ewes ICV infusions of CRF did not change LH profiles (Horton et al. 1988). In contrast, Naylor et al. (1990) showed that ICV infusions of CRF resulted in an increase of dose-related LH secretion in the same experimental model. These discrepancies may be due to different experimental conditions. It seems that in the sheep evaluations of the functional relationship between CRF and GnRH in the basal release of LH require additional studies. In other species, like rat and monkey, the inhibitory effect of ICV infused CRF on basal and preovulatory LH or GnRH release has been well proved (Rivier and Vale 1984, Gindoff and Ferin 1987, Olster and Ferin 1987, Petraglia et al. 1987). It seems that lack of the preovulatory surge of LH in CRF-treated ewes can be due to a marked diminishing of GnRH stores in the nerve terminals of the ME. The pathways and mechanisms involved in the interactions between CRF and GnRH neurons are not known.Many results obtained on rats and primates indicate that the role of endogenous CRF in mediating the GnRH neuronal activity can be species-dependent (reviewed by Rivest and Rivier 1995).

The effect of infused CRF on the GnRH neuronal system resembles the effect of stressful stimulation on these neurons. We have recently observed that suppression in GnRH release did not occur after acute stressful stimulation when the release of CRF is the highest, but it did after prolonged stimulation when activities of the hypothalamo-pituitary -adrenal axis returned to control levels. This suggest that the increase of CRF activity can initiate changes in GnRH secretion but can not be considered as a major factor affecting its secretion (Polkowska and Przekop 1992). The delay of estrus and the preovulatory LH surge in the CRF-treated ewes suggests that CRF can disturb the neuronal components known as the preovulatory surge GnRH generator. Its action is associated with the complex interplays of various excitatory and inhibitory neurotransmitters and neuropeptides (Tanaka et al. 1995). Therefore we suggest that in the sheep during the preovulatory phase of the estrous cycle CRF affects the mechanism govering GnRH secretion rather than acting directly on GnRH neurons. In consequence, this action delays the preovulatory surge of GnRH/LH. Similarly to the stressful conditions, administered CRF can only interfere with other pathways such as biogenic amines or endogenous opiates which are involved in the regulation of GnRH secretion. This is supported by the observation that ICV infusions of CRF change the activity of catecholamines and serotonin, especially during the preovulatory phase of the estrous cycle in sheep (Tomaszewska et al. 1992), and that endogenous opiates are involved in the inhibition of LH by CRF in rats (Almeida et al. 1988).

In infusions of CRF into the 3rd ventricle of the brain in the follicular phase of the estrous cycle delay estrous behavior and ovulation in sheep. CRF blocks the preovulatory surge of LH but does not affect basal LH levels. It seems that during these experimental conditions the synthesis of GnRH and LH is not impaired. CRF induced reduction of irGnRH stores in the nerve terminals of the ME can be responsible for the blockade of the preovulatory surge of GnRH/LH. These results support the hypothesis that CRF can act on the central nervous system and by inhibiting GnRH secretion supress the activity of the H-P-G axis.

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Received 5 July 1996, accepted 14 February 1997