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EFFECTS OF CORTICOTROPIN RELEASING HORMONE AND CORTICOTROPIN RELEASING HORMONE ANTAGONIST ON BIOSYNTHESIS OF GONADOTROPIN RELEASING HORMONE AND GONADOTROPIN RELEASING HORMONE RECEPTOR IN THE HYPOTHALAMIC-PITUITARY UNIT OF FOLLICULAR-PHASE EWES AND CONTRIBUTION OF KISSPEPTIN

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This study aimed to determine the mechanisms governing gonadotropin releasing hormone (GnRH) biosynthesis and luteinising hormone (LH) secretion in follicular-phase sheep after infusion of corticotropin releasing hormone (CRH) and/or corticotropin releasing hormone antagonist (CRH-A) into the third cerebral ventricle. The study included two experimental approaches: first, we investigated the effect of CRH or CRH-A (α-helical CRH 9-41) on GnRH and GnRH receptor (GnRHR) biosynthesis in the preoptic area (POA), anterior (AH) and ventromedial hypothalamus (VMH), stalk/median eminence (SME), and on GnRHR in the anterior pituitary (AP) using an enzyme-linked immunosorbent assay (ELISA); second, we used real-time PCR to analyse the influence of CRH and CRH-A on the levels of kisspeptin (Kiss1) mRNA in POA and VMH including arcuate nucleus (VMH/ARC), and on Kiss1 receptor (Kiss1r) mRNA abundance in POA-hypothalamic structures. These analyses were supplemented by radioimmunoassay (RIA) and ELISA methods for measurement of LH and cortisol levels in the blood, respectively. Our results show that administration of CRH significantly decreased GnRH biosynthesis in the POA/hypothalamus. CRH also decreased GnRHR abundance in the hypothalamus and in the AP, but increased it in the POA. Furthermore, administration of CRH decreased plasma LH concentration and levels of Kiss1 mRNA in the POA and VMH/ARC as well as Kiss1r mRNA in these structures and in the SME. Significant increase in plasma cortisol concentration in the group treated with CRH was also observed. For CRH-A, all analysed effects were opposite to those induced by CRH. The study demonstrates that intracerebroventricular (i.c.v.) infusion of both CRH and CRH-A affects the GnRH/GnRHR biosynthesis and LH secretion in follicular-phase sheep conceivably via either central and peripheral mechanisms including Kiss1 neurons activity and cortisol signals. It has also been suggested that CRH and CRH-A infusion probably had effects directly at the AP.

Key words: *hypothalamus, corticotropin releasing hormone, gonadotropin releasing hormone/gonadotropin releasing hormone receptor, kisspeptin, luteinising hormone, follicular-phase*

INTRODUCTION

Corticotropin releasing hormone (CRH) is the central mediator of stress-induced changes in the pituitary-adrenal axis because it induces the release of adrenocorticotropic hormone (ACTH) and finally increases the levels of cortisol in the blood. CRH also affects the release of gonadotropin releasing hormone (GnRH) and it has been proposed as a mediator of the anti-reproductive effects of stress (1, 2). Most studies on reproduction in rats have shown that CRH suppresses GnRH release by acting directly on GnRH perikarya *via* synaptic contacts in the preoptic area (POA), as well as on GnRH nerve terminals in the stalk/median eminence (SME) (3, 4). The assumption that CRH interferes with GnRH neuronal activity was also confirmed in experiments in which the CRH antagonist (CRH-A) reversed or attenuated the inhibitory effect of CRH on GnRH and/or luteinizing hormone (LH) secretion after certain challenges in rats (5-7).

The relationship between CRH and GnRH neuronal activities is more complex in sheep and the precise mechanism by which CRH system affects GnRH secretory activity is not entirely clear. Smith and co-workers (8) have revealed that in sheep, CRH neurons in the paraventricular nucleus that project to the SME probably do not directly inhibit GnRH, but either afferent or parallel central pathways are involved. Subsequently, Ghuman *et al*. (9) established that the SME may provide a major site for direct modulation of GnRH release by CRH terminals, whereas CRH paraventricular neurons may involve interneurons to influence GnRH cell bodies in POA.

It is generally accepted that stress has usually a profound inhibitory influence on reproductive functions (10-13) through the suppression of GnRH/LH secretion. Since an important sign of stress is activation of the hypothalamic-pituitary-adrenal (HPA) axis, the components of this axis, mainly glucocorticoids, have been extensively studied to explain their interferences with reproductive function. It has been proven in many *in vivo* studies that various stress conditions activate CRH secretion in sheep (14-16), and that prolonged footshock stimulation evokes the persistent augmentation of CRH input to the GnRH/LH axis (17). However, central administration of CRH in ewes increased LH pulse frequency in some studies, but not in others in which CRH decreased or has no effect on pulsatile secretion of LH (1, 18-22).

There is also substantial evidence to suggest that intracerebroventricularly (i.c.v.) infused CRH could reach the pituitary and also enhanced cortisol release (1, 23).

Inhibitory effects of various stressors on LH release are usually associated with increases in the circulation levels of cortisol (24, 25). Studies in ovariectomised sheep have demonstrated that a stress-like elevation of plasma cortisol decreases pulsatile LH secretion in the absence of stress *via* suppression of pituitary responsiveness to GnRH and this effect is mediated *via* Type II glucocorticoid receptors in gonadotrophs (26, 27). Furthermore, cortisol may also act at the hypothalamic level to suppress GnRH secretion, but the presence of gonadal steroids is necessary for this cortisol effect (28). Such activity of cortisol may involve interneurons that provide afferents to GnRH system because GnRH neurons in sheep do not express the Type II glucocorticoid receptors (29).

Despite a number of studies on the influence of CRH on GnRH/LH release, there is no coherent evidence as to how this neurohormone affects the biosynthesis of GnRH and GnRH receptor (GnRHR). Actually, we (22) and others (30) demonstrated that CRH action may be involved in the regulation of GnRH transcription, but the relationships between transcription and translation processes are so far entirely unknown. However, identification of kisspeptin (Kiss1) and its G protein-coupled receptor 54 (Kiss1r) as an essential component of the hypothalamic-pituitary-gonadal axis controlling GnRH/LH release (31, 32) raises the possibility that Kiss1-Kiss1r signaling may play a critical role in the transduction of CRH-induced changes in GnRH and GnRHR genes expression.

To clarify this point, in the present study we investigated whether the central administration of CRH and/or CRH-A interferes (i) with levels of post-translational products of genes encoding GnRH and GnRHR, and with LH secretion, (ii) with expression of mRNAs encoding Kiss1 and Kiss1r. The level of cortisol in the blood was also determined as an indicator of adrenal gland activation. All these interferences were studied in the hypothalamic-pituitary unit of follicular-phase sheep.

MATERIAL AND METHODS

Animals

The experimental procedures were conducted in accordance with the Polish Guide for the Care and Use of Animals and were approved by a Local Ethics Committee of the Warsaw University of Life Sciences (No 11/2014).

The studies were performed on 3 – 4-year-old Polish Merino ewes in the middle of the breeding season (October-November). The sheep were kept indoors in individual pens and exposed to natural light. Food and water were available *ad libitum*. The sheep were well adapted to the experimental conditions; they always had visual contact with their neighbours, even during the process of blood collection, to prevent stress associated with social isolation.

The oestrous cycle in the ewes was tested by running them with a vasectomised ram twice daily; only ewes that showed two consecutive normal oestrous cycles were chosen for subsequent experiments. Six ewes were used in each group. The day of the onset of the oestrous cycle is referred to as day 0.

Surgical procedures

Guide cannulas were implanted into the third cerebral ventricle of ewes under general anaesthesia (xylazine: 40 µg/kg of body mass, intravenously; xylapan and ketamine: 10 to 20 mg/kg of body mass, intravenously; Bioketan; Vetoquinol Biowet, Pulawy, Poland). Using a stereotaxic procedure (33, 34), stainless steel guide cannulas were directed towards the third ventricle and secured to the skull with screws and dental cement. Each cannula was fitted with an indwelling stylette to prevent back flow of cerebrospinal fluid. After surgery, the ewes were given antibiotics subcutaneously for 4 consecutive days.

Infusion procedures and collection of material

One hour before infusion, cannulas were introduced through the guide cannula and secured in position with tips placed approximately 2.0 – 2.5 mm above the base of brain; when the tips of the cannulas were in the third ventricle, cerebrospinal fluid was seen to flow into the infusion cannula. For precise analysis of the effects of CRH or CRH-A on LH and cortisol secretion in ewes, each animal received two infusions: first, a control infusion with Ringer's solution on days 13 – 16 of the oestrous cycle; and second infusion of either CRH (20 µg CRH/ml Ringer's solution) or CRH-A (20 µg CRH-A/ml Ringer's solution) over the same period in the next oestrous cycle. The doses of drugs were selected based on our previous study and many years of experience (22). Thus, for LH and cortisol measurements each ewe served as its own control. The infusion of both Ringer's solution and drugs was performed at the rate of 2 µl/min for 20 min of every hour for 5 h daily (from 08.00 to 13.00). This infusion protocol resulted in daily doses of 4 µg CRH and 4 µg CRH-A per animal. Infusions were applied using calibrated glass-tight syringes and a CMA/100 microdialysis microinjection pump (CMA Microdialysis AB, Stockholm, Sweden). To determine the LH and cortisol concentrations, a series of blood samples were collected *via* an indwelling jugular catheter at 10-min intervals on the last day of infusion (Ringer's solution or CRH or CRH-A). Blood samples were taken into tubes containing 100 µl of heparine (100 U/ml) and centrifuged within 1 h from collection. Plasma was stored at –20°C until assay. Immediately after the last infusion of CRH and/or CRH-A, ewes were euthanised and brain tissue were taken for analysis of GnRH and GnRHR levels as well as for Kiss1 and Kiss1 receptor (Kiss1r) mRNAs. For this analysis, a separate group of ewes which received an infusion of Ringer's solution served as a control for CRH and CRH-A-infused animals. Control for Ringer's solution group was non-treated (intact) animals.

Following the euthanasia, brain was removed rapidly from the skull and the stalk/median eminences (SME) were isolated. Using surgical instruments (scalpel, scissors, narrow forceps and micrometer) fresh hypothalamic blocks were sectioned sagitally and dissected from both sides into three, 3 mm thick parts *i.e.* preoptic area (POA), anterior hypothalamus (AH), ventromedial hypothalamus including arcuate nucleus (VMH/ARC) according to the stereotaxic atlas of the ovine brain (34) for measuring the levels of GnRH/GnRHR and Kiss1/Kiss1r mRNAs. The pars distalis (PD) of the anterior pituitary gland (AP) was also taken for GnRHR analysis. The measurement of Kiss1 mRNA was limited to the POA and VMH/ARC. After fresh dissection samples were frozen in liquid nitrogen and stored at –80°C until assay.

Determining the GnRH, GnRHR and cortisol levels

The quantitative measurement of GnRH and GnRHR in the hypothalamic-anterior pituitary tissue and the analysis of cortisol level in the blood plasma was performed using an immunoassay ELISA. All steps of the assay were performed according to the manufacturer's instructions for GnRH (Cusabio Biotech Co., Wuhan, China, Cat. no. CSB-E15857B), GnRHR (Cusabio Biotech Co., Wuhan, China, Cat. no. CSB-EL009637SH) and cortisol (SunRed Biotech Co., Shanghai, China, Cat. no. 201-07-0067).

According to the manual guides, the detection ranges for GnRH and GnRHR were 100 – 4000 pg/ml and 40 – 1400 pg/ml, respectively. The range of determination for cortisol was 0.2 – 60 ng/ml. The minimum detectable dose for GnRH and GnRHR were less than 100 pg/ml and 20 pg/ml, respectively. Predicting detection unit for cortisol was less than 0.183 ng/ml. The absorbance was measured at 450 nm using a microplate reader (VersaMax Elisa Microplate Reader, Sunnyvale, CA 94089 USA).

For the normalisation of GnRH and GnRHR levels in the hypothalamus-pituitary tissue material, the Bradford assay (Merck KGaA, Darmstadt, Germany) was used to estimate total decapeptide and protein concentrations. The ratio of hormone and receptor were calculated and express as pg of GnRH and GnRHR/mg of total protein.

Three samples of known concentration were tested by producer twenty times on one plate to assess the precision of the assay. Independently, we have done 5 technical replicates per sample. The coefficient of variation for GnRH (in range of: 140 – 700 pg/mg of total protein) was 11%, for GnRHR (in a range of: 10 – 30 pg/mg of total protein) was 13% and for cortisol (in a range of: 15 – 55) was 10%. Additionally, we have done 5 biological replicates per sample. The coefficients of variation for GnRH, GnRHR, and cortisol were 19%, 16% and 17% respectively.

Quantitative gene expression assay

1. Isolation of RNA and cDNA synthesis

Total RNA from POA-hypothalamus structures and from AP was extracted using Miniprep Kit (RTN70-1KT, Sigma Aldrich, St. Louis, MO, USA) according to manufacturer's protocol. To remove any potential genomic DNA contamination, the extracted RNA was treated with RNAase free DNase I (AMPD1, Sigma Aldrich, St. Louis, MO, USA). The RNA integrity was checked in a 1.5% agarose gel. All pure and protein free samples were reverse transcribed using Enhanced Avian RT-PCR Kit (HSRT100-1KT, Sigma Aldrich, St. Louis, MO, USA) in a 20 µl reaction volume according to the user manual. A negative control (no reverse transcriptase added) was included for all samples. The cDNA was subsequently stored at –20°C until further analysis.

2. Primer design

Specific primer pairs for target amplification were designed using Primer3 software from sheep sequences available in GenBank. The oligonucleotides were synthesised by Oligo IBB PAN, Poland. Based on the work of Peletto and co-workers (35), a combination of two reference genes SDHA/YWHAZ (geometric mean) was selected for normalization of Kiss1 and Kiss1r mRNAs expression (*Table 1*).

3. Quantitative real-time PCR

Real-time PCR was carried out using Ct®SYBR®Green RT-PCR Kit (L6544-100RXN, Sigma-Aldrich, St. Louis, MO, USA) in reaction volume 20 µl. PCR cycling conditions were 95°C for 10 minutes and 40 cycles of 95°C for 10 seconds, 56 or 60°C for 10 seconds, and 75°C for 10 seconds. The reactions were performed in a Rotor-Gene Q apparatus (QIAGEN Company, Switzerland). The positive (1 µl cDNA each sample) and negative (no template and no RT) controls were run for each reaction.

4. Data analysis

All data were initially analysed using Rotor Gene Q software. The $2^{\Delta\Delta Ct}$ method (36) was used in calculating the relative ratio. We used a noise-resistant iterative nonlinear regression algorithm (real-time PCR miner; www.miner.ewindup.info) to determine the efficiency of the PCR reaction (37). The products were separated in agarose gel (1.5%) and visualised by ethidium bromide.

5. Determining the luteinising hormone concentration

Plasma LH concentrations were analysed by double-antibody radioimmunoassay using anti-ovine LH and anti-rabbit gamma

Table 1. Primers for experimental and housekeeping genes amplification.

Gene	Sequence	Temperature of annealing (ºC)
Kiss1 Kisspeptin	Forward ATCCTAGAACCACAGGCTCG Rewerse AAGGAGTTCCAGTTGTAGGCG	56
Kiss1r Kisspeptin receptor	Forward TACATCCAGCAGGTCTCGGTG Fewerse ACGTACCAGCGGTCCACACT	56
YWHAZF Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	Forward: AGACGGAAGGTGCTGAGAAA Rewerse: CGTTGGGGATCAAGAACTTT	60
SDHAF Succinate dehydrogenase complex, subunit A	Forward: CATCCACTACATGACGGAGCA Rewerse: ATCTTGCCATCTTCAGTTCTGCTA	56

globulin antisera and ovine LH standard (NIH-LH-SO18, Sigma-Aldrich), as described by Stupnicki and Madej (38). The sensitivity of the assay was 0.06 ng/ml and the intra and inter assay coefficients of variation were 9% and 11%, respectively.

Statistical analysis

All data were expressed as the mean ± standard error of the mean (SEM). The t-test and one-way ANOVA analyses of variance were used for statistical analysis of ELISA and RT-PCR data. The significance of differences in LH secretion between control and drugs-treated ewes was assessed by one-way ANOVA followed by the least significant differences (LSD) test (STATISTICA; Stat-Soft, Inc., Tulsa, OK, USA). Differences in LH pulse frequency and amplitude between groups were analysed with unilateral Wilcoxon test. The frequency of LH pulses was defined as the number of pulses identified per collection period (39). The amplitude of LH pulses was defined as the differences between peak and nadir values. P-values < 0.05 were considered significant.

RESULTS

Effects of CRH and CRH-A on the GnRH concentration in the preoptic area-hypothalamus region of follicular-phase ewes

In control ewes, GnRH was found in structures throughout the POA, AH, VMH and SME (*Fig. 1*). Following CRH treatment, GnRH levels decreased significantly in all POA-hypothalamic areas (POA: $P < 0.05$, AH: $P < 0.05$, VMH: $P < 0.01$, SME: $P < 0.01$), whereas administration of CRH-A resulted in a significant increase in GnRH quantities in all of the analysed tissue material (POA: $P < 0.01$, AH: $P < 0.01$, VMH:$P < 0.05$, SME: $P < 0.01$) compared with control values.

Effects of CRH and CRH-A on levels of GnRHR in the preoptic area-hypothalamus and in the anterior pituitary of follicular-phase ewes

In control animals, GnRHR protein was found at different levels in the tissue continuum throughout the POA, AH, VMH,

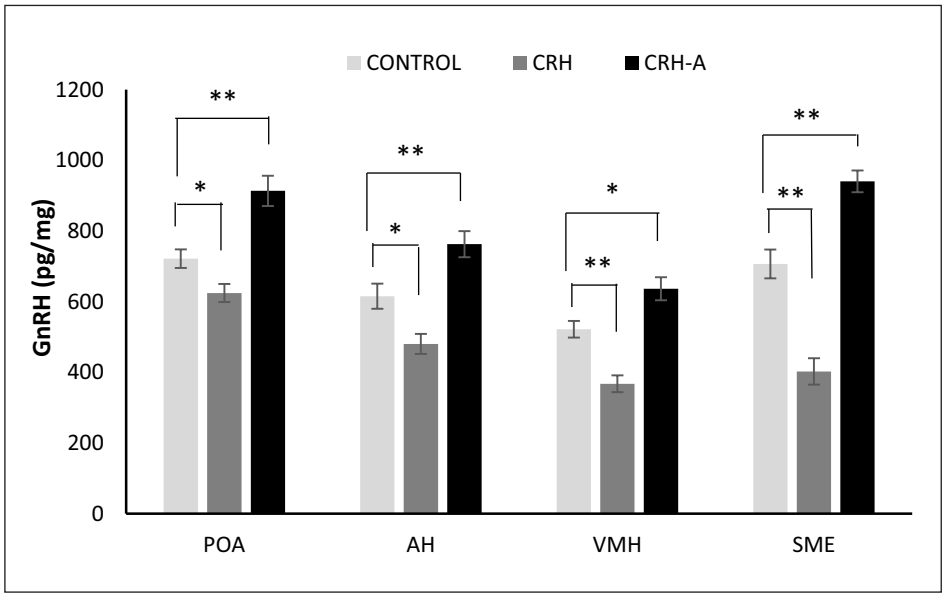

Fig. 1. Effect of CRH and CRH antagonist (CRH-A) on the level of GnRH in the preoptic area (POA), anterior hypothalamus (AH), ventromedial hypothalamus (VMH) and the stalk median eminence (SME) of follicular-phase ewes; *P < 0.05, **P < 0.01. Asterisks indicate values that differ significantly from the control group animals. Data are the mean ± SEM, n = 6 animals per group.

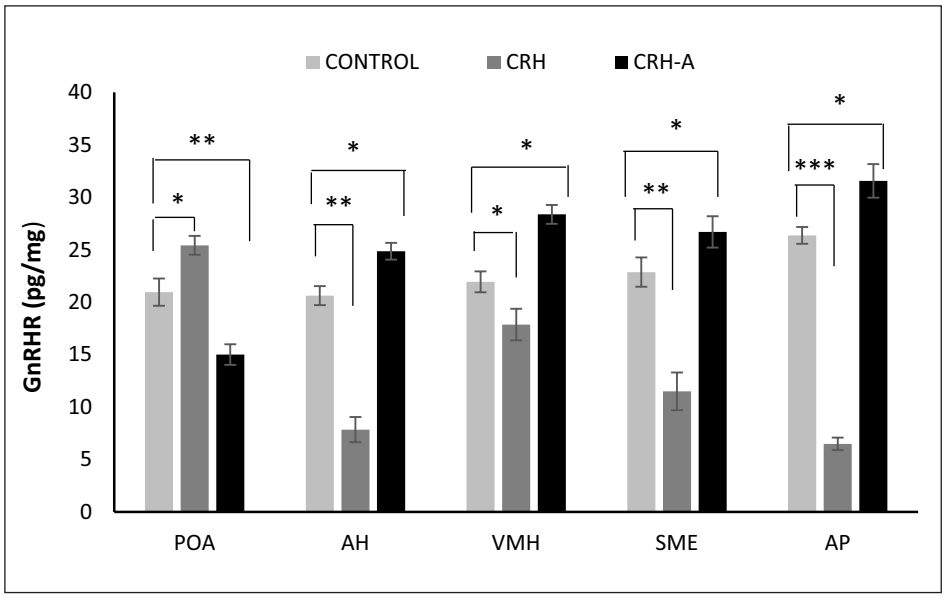

Fig. 2. Effect of CRH and CRH antagonist (CRH-A) on the level of GnRHR concentration in the preoptic area (POA), anterior hypothalamus (AH), ventromedial hypothalamus (VMH), stalk median eminence (SME) and in the anterior pituitary gland (AP) of follicular-phase ewes. Asterisks indicate values that differ significantly from the control group animals. *P < 0.05, **P < 0.01, ***P < 0.001. Data are the mean ± SEM, n = 6 animals per group.

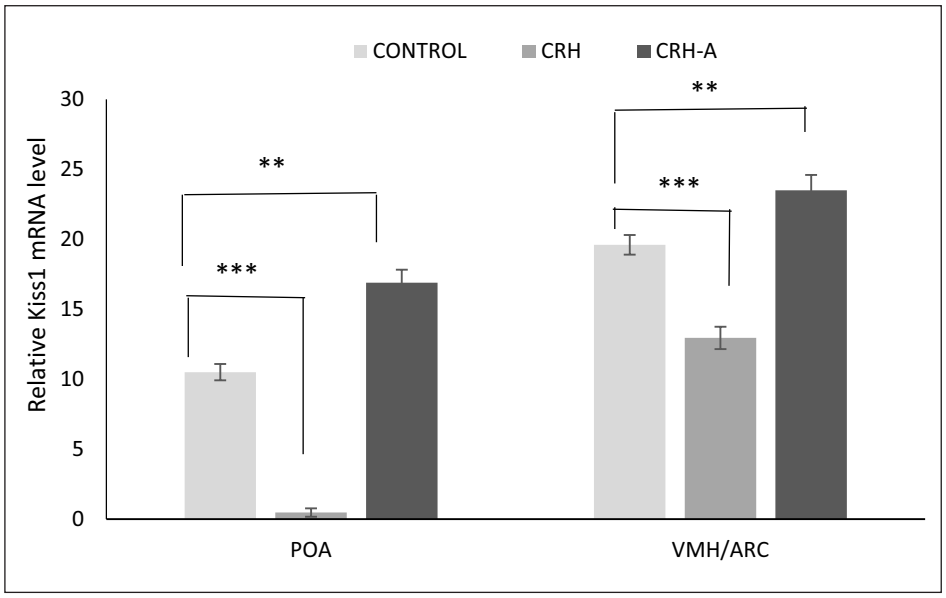

Fig. 3. Effect of CRH and CRH antagonist (CRH-A) on the level of Kiss1 mRNA in the preoptic area (POA) and the ventromedial hypothalamus including arcuate nucleus (VMH/ARC) of follicular-phase ewes. **P < 0.01, ***P < 0.001. Asterisks indicate values that differ significantly from the control group animals. Data are the mean ± SEM, n = 6 animals per group.

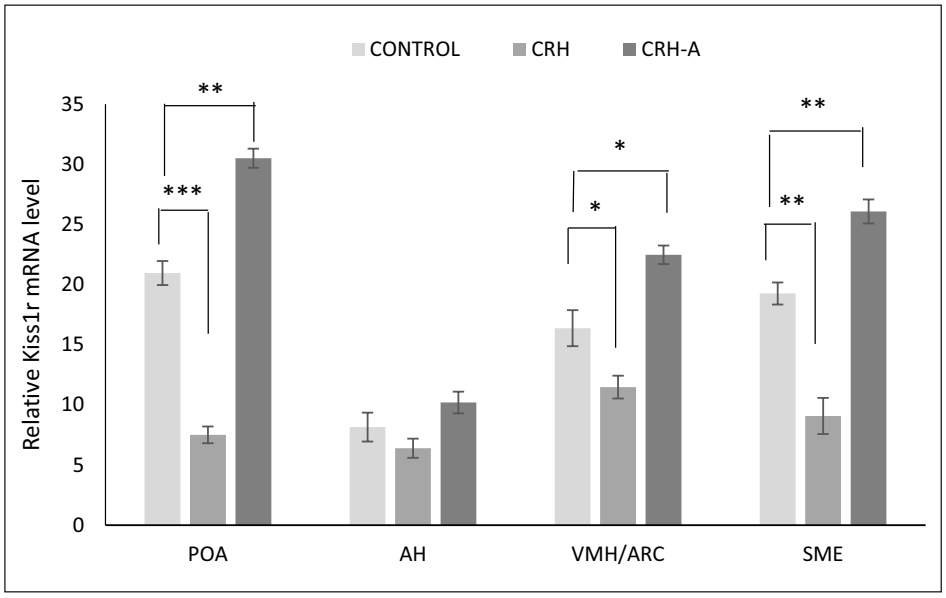

Fig. 4. Effect of CRH and CRH antagonist (CRH-A) on the level of Kiss1r mRNA in the preoptic area (POA), anterior hypothalamus (AH), ventromedial hypothalamus including arcuate nucleus (VMH/ARC) and the stalk median eminence (SME) of follicular-phase ewes. *P < 0.05 **P < 0.01,***P < 0.001. Asterisks indicate values that differ significantly from the control group animals. Data are the mean ± SEM, n = 6 animals per group.

SME, and in the AP (*Fig. 2*). Administration of CRH increased significantly the level of GnRHR in the POA (P < 0.05) and decreased it in the AH (P < 0.01), VMH (P < 0.05) and SME (P < 0.01). In CRH-treated ewes, a decreased in GnRHR quantity was also noted in the AP (P < 0.001). However, in CRH-A-treated animals, GnRHR levels decreased significantly in the POA (P < 0.01) and increased in the AH (P < 0.05), VMH (P < 0.05) and SME (P < 0.05) as well as in the AP (P < 0.05).

Effects of CRH and CRH-A on the expression of Kiss1 mRNA in preoptic area-hypthalamus of follicular-phase ewes

In control ewes, Kiss1 mRNA was detected in the POA and VMH/ARC (*Fig. 3*). Relative to the control values, levels of Kiss1 mRNA decreased significantly in analysed hypothalamic structures of CRH-treated ewes (POA: P < 0.001 and VMH/ARC: P < 0.001). Blockade of CRH receptors had an opposite effect to those induced by CRH; CRH-A increased significantly Kiss1 mRNA level in the POA (P < 0.01) and VMH/ARC (P < 0.01) versus control values.

Effects of CRH and CRH-A on the expression of Kiss1r mRNA in the preoptic area-hypothalamus of follicular-phase ewes

In control animals, Kiss1r mRNA was found at different concentrations in all POA/hypothalamus structures, *i.e*. POA, AH, VMH/ARC and in the SME (*Fig. 4*). Administration of CRH decreased significantly the expression of Kiss1r mRNA in the POA (P < 0.001), VMH/ARC (P < 0.05) and in the SME (P < 0.01). The level of Kiss1r mRNA in the AH of CRH-treated animals, despite its downward tendency, did not differ significantly from control. Infusion of CRH-A into the third cerebral ventricle resulted in a significant increase in Kiss1r mRNA level in the POA (P < 0.01), VMH/ARC (P < 0.05) and SME (P < 0.01) with the exception of AH, where Kiss1r mRNA level was in the range of the control value.

Effects of CRH and CRH-A on plasma luteinising hormone concentrations

Infusion of CRH into the third cerebral ventricle decreased significantly LH concentrations in the blood plasma compared with control level ($P < 0.05$; *Fig. 5A*). This down-regulation of LH release is probably due to a decrease in the LH pulse frequency ($P < 0.05$; *Fig. 5B*) because the amplitude of these pulses, despite its downward tendency, did not differ significantly from the controls.

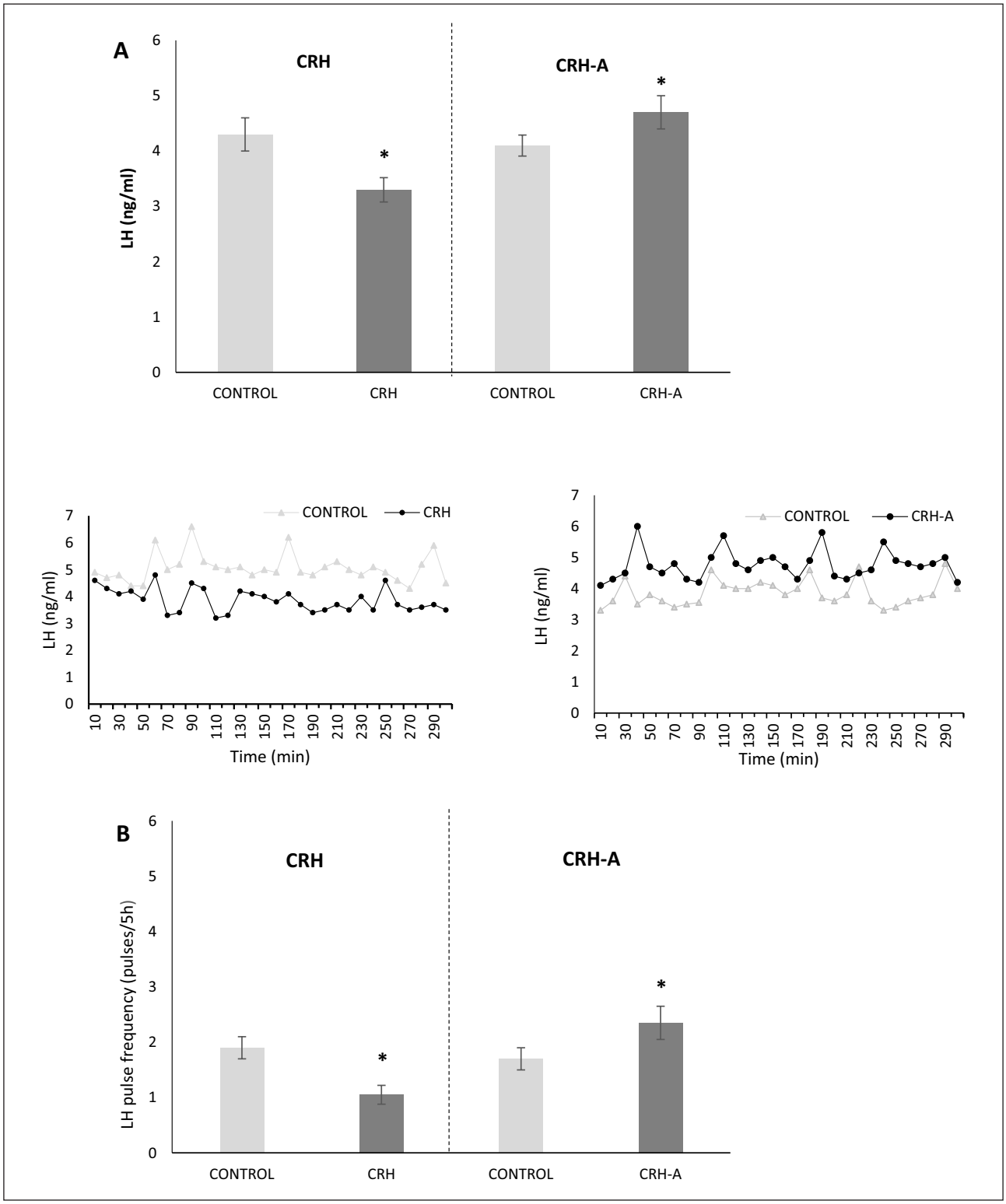

Fig. 5. Effect of CRH and CRH antagonist (CRH-A) on the level of LH with representative profiles of pulsatile hormone secretion (*A*) and LH pulse frequency (*B*) in blood plasma of follicular-phase ewes. Asterisks indicate values that differ significantly from the control group animals. *$P < 0.05$. Data are the mean ± SEM, n = 6 animals per group.

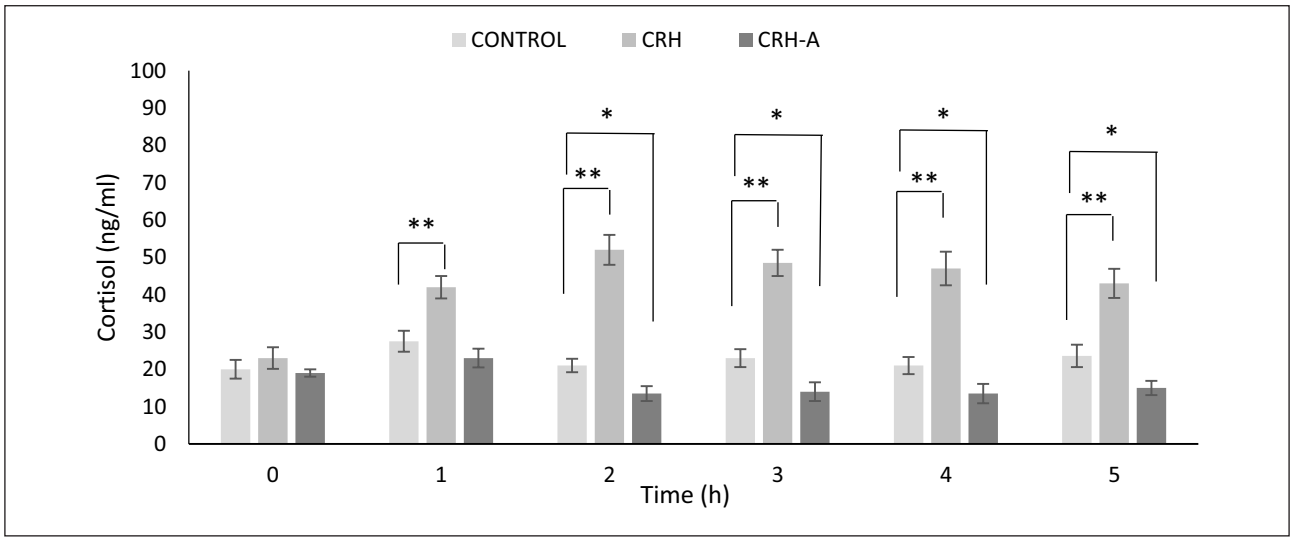

Fig. 6. Effect of CRH and CRH antagonist (CRH-A) on the level of cortisol in blood plasma of follicular-phase ewes. Asterisks indicate values that differ significantly from the control group animals. *P < 0.05, **P < 0.01. Data are the mean ± SEM, n = 6 animals per group.

In CRH-A-treated ewes, LH secretion (P < 0.05) and LH pulse frequency (P < 0.05) increased significantly in all animals (*Fig. 5*). The LH pulse amplitude did not differ significantly between control and CRH-A-treated groups (data not shown).

Additionally, *Fig. 5A* shows representative profiles of pulsatile LH secretion in individuals.

Effects of CRH and CRH-A on plasma cortisol concentrations

The infusion of Ringer's solution did not affect significantly the concentration of cortisol as compared with control intact group (data not shown). When CRH was administered to the third cerebral ventricle of sheep, the plasma cortisol concentration increased in the course of five hours and its values raised significantly (P < 0.01) at all analysed time points after beginning the infusion procedure (*Fig. 6*).

In ewes treated with CRH-A, the levels of cortisol decreased versus the pre-treatment values (P < 0.05). This decrease achieved statistical significance in the second hour of CRH-A infusion (*Fig. 6*). Changes in cortisol secretion after i.c.v. infusion of both drugs strongly suggest that either CRH and CRH-A had effects directly at the AP.

DISCUSSION

The results of the present study demonstrate, for the first time, that infusion of both CRH and CRH-A into the third cerebral ventricle of follicular-phase ewes affects the levels of post-translational product of gene encoding GnRH in the POA-hypothalamus region. The decrease in the level of GnRH induced by CRH administration as well as the increase of GnRH amount following delivery of CRH-A may suggest that down- or up-regulation of GnRH gene expression is strictly CRH-dependent. The current study supports our previous research, in this area in which an analogous, CRH-dependent relationship for the GnRH mRNA was established with the use of the same experimental schedule (22). Along with the down- or up-regulation of GnRH levels after treatment with CRH or CRH-A, respectively, a similar direction of changes in plasma LH concentrations were determined in the present study. Although we did not measure the level of GnRH in the hypophyseal portal blood, the diminished LH secretion following CRH treatment could indicate that infusion of CRH suppresses the level of GnRH in portal circulation. Thus, it cannot be excluded that the mechanisms of CRH-mediated modulation of GnRH gene expression are similar to the effects of CRH on GnRH release. However, this assumption should be treated with caution because such a point of view is in contrast with the earlier studies in sheep, suggesting that the neural processes governing GnRH release are distinct from those regulating GnRH gene expression (40).

Our results differ from those of a previous study on sheep in which peripheral or central administration of CRH during non-breeding or during the breeding season has generally no effect on GnRH and/or LH release (18-20). There is also disagreement between our data and study demonstrating increased LH pulse frequency after central delivery of CRH in sheep (1, 41). On the other hand, the current observations concur, at least partially, with those reported by Polkowska and Przekop (21) who showed that i.c.v. infusion of CRH during the preovulatory period suppressed the surge of LH and caused the lengthening of the oestrous cycle. It's worth pointing out that both of these studies were conducted on sheep in the late follicular phase of the oestrous cycle and it is thus probable that in this period administration of CRH decreases either GnRH biosynthesis and LH secretion. Overall, one probable reason for conflicting results may be due to the different physiological states of animals that were used in each experiment. Indeed, the sex steroid status of the organism appears to be important for interactions between the adrenal and gonadal axes and gonadal steroids seem to be obligatory for the expression of CRH-mediated effects (1). For example, central administration of CRH in rats resulted in an interruption of LH pulses in 17 beta oestradiol (E_2) treated animals, but had little or no effect in the absence of this gonadal steroid (42). Furthermore, stress-induced increase in the level of mRNA encoding CRH ligand and CRH receptor in rats (43, 44) were more pronounced on the morning of proestrus compared with diestrus. The up-regulation of CRH system activity occurred with an elevation of plasma E_2 concentrations suggesting that this gonadal steroid may stimulate the HPA axis by an effect predominantly on CRH. It stands to reason that CRH in the follicular-phase ewes is more probably to interfere with GnRH/LH activity than if this was to

occur in the luteal-phase animals, however, this proposition is not rooted in strong evidence. Secondly, the discrepancies across studies may result from different experimental conditions, in particular from different models of experimental treatment (prolonged, intermittent infusion schedule and higher CRH dose used in our study compared to those used by teams of both Caraty and Naylor). Data obtained in the immortalised GnRH neuronal cell line proved that the effects of CRH on the expression of GnRH *mRNA* depend on the duration of CRH exposure; GnRH transcription was facilitated during short-term incubation with CRH but decreased following an extended incubation time (30, 45).

The present results also confirm once more that administration of both CRH and CRH-A affects the expression of GnRHR. A significant increase in GnRHR level in the POA and the reduction of receptor amount in the entire hypothalamus following CRH treatment as well as the opposite effect induced by CRH-A are in agreement with our previous results, in which such long-term i.c.v. CRH infusion model affected the transcriptional activity of the GnRHR gene in the same way (22). Of particular interest is the inverse relationship between GnRHR level and GnRH concentration in the POA of CRH- and CRH-A-treated ewes. Recently, we do not have unequivocal explanation of neuroendocrine mechanism(s) of this phenomenon. Nevertheless, it cannot be excluded that GnRHRs in various hypothalamic areas belong to different neuronal systems. They might be innervated by different afferents that specifically act on regulatory pathways governing the GnRH biosynthesis. However, this surmise should be interpreted cautiously because physiological significance of hypothalamic GnRHR is still poorly recognised. Unfortunately, this issue has only been addressed in a very few studies on rats. From these studies, it may be suggested that GnRHR mRNA in the VMH are directly coupled with the control of GnRH secretion (46), but not with the regulation of GnRH gene expression (47). Since we used the i.c.v. infusion model, the decrease in the GnRHR level in the AP after treatment with CRH could result from reduced gonadotrophs stimulation by GnRH, which is the main factor controlling GnRHR gene expression (48), the GnRHR amount (49), and GnRHR activity (50). However, from a homeostatic perspective, one would predict an increase in GnRHR biosynthesis in the AP for the system to try to correct the lack of GnRH. Surprisingly, such a compensatory adjustment was not observed in the present study. This could suggest that i.c.v. infusion of CRH acts directly or *via* other intermediary mechanism upon the pituitary to inhibit the GnRHR biosynthesis. Our data suggest that this intermediary function may be performed by cortisol, as will be described in detail below.

In the present study, the down-regulation of post-translational product of GnRH gene induced by CRH occurred with a concomitant decrease in Kiss1 mRNA level in the POA and in VMH/ARC. We also observed similar relationship in the case of the transcriptional activity of Kiss1r gene, when CRH-related changes in the level of mRNA encoding Kiss1r where determined in the POA, VMH/ARC and in the SME. Thus, CRH-mediated decrease in stimulatory Kiss1 input to GnRH cells may be critical in the mechanisms by which i.c.v. administered CRH inhibits GnRH biosynthesis and consequently pulsatile release of GnRH. The diminished Kiss1 expression in POA of follicular-phase sheep after treatment with CRH as well as the opposite effect induced by CRH-A is not surprising because this neuronal formation is critical for the positive feedback of oestrogen that induces GnRH/LH surge (51). Since ARC Kiss1 neurons is implicated in both oestrogen-negative and -positive feedback regulation of GnRH/LH secretion (52), CRH-induced changes in Kiss1 mRNA in the VMH/ARC are less convincing. Because Kiss 1 cells in the POA as well as those in the ARC appear to project directly to GnRH neurons (53, 54), it is probable that GnRH cells are direct target for the action of POA-ARC kisspeptin. In physiological context, participation of Kiss 1 pathway in CRH-induced suppression of GnRH biosynthesis is fully justified based on the previous study indicating lack of input to the POA region of the sheep brain from CRH cells of the PVN (55). Furthermore, the median eminence, as a neural structure at the base of the hypothalamus, should also be considered to be a region of communication between Kiss1 and GnRH systems because contact between Kiss1 and GnRH neurons has been postulated in the SME of sheep (9). The question remains whether Kiss 1 cells in sheep, just as it is in rats (56), contain CRH receptors. However, the kisspeptin and CRH receptors Type 2 distributions overlap in ewes and, therefore, interactions between the two systems seem likely (57).

In the light of available data, it is indisputable that Kiss1 may directly stimulate GnRH secretion, considering that most of GnRH neurons express Kiss1r (51,58), and administration of Kiss1 profoundly increases Fos expression (59) and GnRH neurons excitability (60). Furthermore, Kiss1 stimulates GnRH release in a Kiss1r dependent manner from mouse VMH explants containing SME (61). Similar effect also appears in sheep, when i.c.v. administration of Kiss1 produces a dramatic release of GnRH into the cerebrospinal fluid, with a parallel rise in serum LH (62).

In the present study, central administration of CRH also released cortisol into the general circulation. This release was not observed with the control infusion, so it was not due to any stress that might have been associated with the infusion technique. The increase in cortisol level following CRH treatment strongly suggests that i.c.v. infused CRH could manage to access the AP, from where ACTH is released (63). Since CRH-A decreased the circulating cortisol concentration, it is probable that CRH-A reaching the AP could inhibit this gland from producing and releasing ACTH, which in turn suppressed cortisol release. It is highly likely, because study in hypothalamo-pituitary disconnected sheep suggests that pulsatile input from hypothalamic CRH is not necessary for the ultradian rhythm in cortisol secretion or for pulsatile cortisol release (64). Thus, cortisol seems to be important in mediating the effects of CRH and CRH-A infusions on LH release acting upon the AP to modulate the sensitivity of gonadotrophs to GnRH (26, 27).

Our findings also established the hypothesis that intermediary role of cortisol in CRH- and CRH-A induced modifications of GnRH/LH system activity can also occur at the level of hypothalamus. While the precise mechanism that mediates cortisol signals to GnRH cells are yet to be determined, we propose that changes in cortisol secretion induced by CRH and CRH-A affect the expression of GnRH and GnRHR genes by modulating Kiss1 inputs into GnRH system. This possibility is supported by the finding demonstrating that stress-induced glucocorticoid secretion, as well as glucocorticoid administration itself, inhibited Kiss1 mRNA level in mice, while conditions that did not change glucocorticoid secretion did not alter Kiss1 mRNA expression (65). There are also published data on rats to showing that Kiss 1 cells express glucocorticoid receptors (56), so this could be intermediary pathway by which CRH influences GnRH function in the sheep brain. This is likely because glucocorticoid receptors Type 2 are abundant in the POA and ARC of ewes (66) where kisspeptin cells are located. Furthermore, disruption of sexual behaviour and the LH surge induced by immunological stress is accompanied by inhibition of kisspeptin cells activity in both the POA and ARC (57).

Interestingly, we also observed the increase in RFamide related peptide-3 (RFRP-3) mRNA level in the hypothalamus of CRH-treated ewes (Ciechanowska, unpublished data). Indeed, recent work in the sheep indicates that stress and high cortisol levels activate RFRP-3 neurons in the hypothalamus (67), which have a direct input to GnRH cells (68). RFRP3, the mammalian ortholog of the avian gonadotropin inhibitory hormone (GnIH), inhibits LH secretion in the ewe *via* both central (GnRH) and peripheral (pituitary gonadotropes) sites of action (69, 70). This raises the possibility that elevated cortisol secretion could act on RFRP3 cells and this is how infusion of CRH inhibits GnRH biosynthesis. In this network of CRH-induced changes, one should also consider the existence of mutual communication between RFRP3 and Kiss 1 populations. Supporting this, in the rat, RFRP3 fibers are in contact with Kiss 1 neurons and the subpopulation of these kisspeptin cells expresses the receptor for RFRP-3 (71). Acceptance of this is also gained by the observation that in mice RFRP3 may modulate a small proportion of kisspeptin-producing neurons in the ARC (72). This may indicate the likely involvement of both these neuronal populations in CRH-induced changes of GnRH/GnRHR biosynthesis in the ewe. However, the precise means by which CRH signals are conveyed to GnRH system in sheep remain to be deeply elucidated.

Overall, the regulation of GnRH/LH release is associated with complex interplay among excitatory and inhibitory neural and hormonal signals that converge on hypothalamic neurons responsible for the pulsatile secretion of GnRH. In the light of recent literature data, apart from classic neuronal pathways involved in the regulation of GnRH/GnRHR system, other factors should also be considered to participate in the control of reproductive activity. For example, gaseous molecule such as carbon monoxide (CO) affected the expression levels of mRNA encoding both GnRH and GnRHR and helped to protect the reproductive processes by decreasing the exaggerated activation of the HPA axis (73). Furthermore, vascular endothelial growth factor (VEGF) as well as bone morphogenetic proteins (BMPs), which belong to the transforming growth factor (TGF)-β superfamily, were also identified as important regulatory components of reproductive function (74, 75). Both, the VEGF and BMP systems may play a key role in regulating not only ovarian functions but also in modulating hypothalamic GnRH secretion and/or gonadotropin secretion from the AP (76, 77).

In conclusion, the results of the present study strongly suggest that i.c.v. administration of CRH and CRH-A affects the activity of hypothalamic-pituitary GnRH/LH system in follicular-phase sheep involving different intermediary mechanisms. Central effect of CRH and CRH-A infusions include down- or up-regulation of the GnRH and GnRHR biosynthesis in the hypothalamus, respectively. Peripheral effects of both drugs are related with changes in LH secretion from the AP that similar to those observed in the case of GnRH. Changes in cortisol secretion induced by central delivery of CRH and CRH-A indicate that this adrenal steroid may be important in mediating the inhibitory effect of CRH on LH release acting upon the AP to modulate the sensitivity of gonadotrophs to GnRH. They also strongly suggest that CRH and CRH-A infusions had effects directly at the AP. Finally, the results reported here provide the basis for our next interpretation: kisspeptin neurons in the hypothalamus could play an important role in mediating effects of cortisol on GnRH/GnRHR genes expression. However, our data are still preliminary and need to be verified by further experiments.

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