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Effect of stress on the expression of GnRH and GnRH receptor (GnRH-R) genes in the preoptic area-hypothalamus and GnRH-R gene in the stalk/median eminence and anterior pituitary gland in ewes during follicular phase of the estrous cycle

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Abstract. The RT-PCR (reverse transcription polymerase chain reaction) technique was used to analyze GnRH mRNA and GnRH-R mRNA in the preoptic area, anterior and ventromedial hypothalamus, and GnRH-R mRNA in the stalk/median eminence and anterior pituitary gland of follicular ewes subjected to short (3 h during one day) or prolonged (5 h daily during four consecutive days) footshock stimulation. To analyze relationship between expression of GnRH and GnRH-R genes with LH secretion the blood samples were collected at 10 min intervals to determine LH levels in control and stressed animals. The concentration of GnRH mRNA increased significantly in the preoptic area, anterior and ventromedial hypothalamus of ewes subjected to short stress. The prolonged stressful stimuli significantly decreased GnRH mRNA levels in all analyzed structures. In short stressed ewes the significant augmentation of mRNA encoding GnRH-R was detected in the preoptic area, entire hypothalamus, stalk/median eminence and anterior pituitary gland. The GnRH-R mRNA was significantly reduced in all tested structures of animals subjected to prolonged footshocking except for the preoptic area, where GnRH-R mRNA did not differ from control values. The changes in GnRH mRNA and GnRH-R mRNA levels under short or prolonged stress were associated with an increase or decrease of LH concentration in blood plasma, suggesting the existence of a direct relationship between GnRH mRNA and GnRH-R mRNA expression with LH secretion. The results indicate that the expression of both GnRH gene and GnRH-R gene, as well as LH secretion in ewes during the follicular phase of the estrous cycle, are dependent upon the kind of stress.

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INTRODUCTION

An inverse relationship between prolonged and chronic stress and normal reproductive efficiency has frequently been observed in domesticated animals (Armstrong 1986, Collu et al. 1984, Dantzer and Hermide 1995). The psycho-emotional state evoked by prolonged intermittent footshock stimulation of cyclic ewes suppresses the preovulatory LH surge and, consequently, causes long-lasting disturbances in the course of the estrous cycle in most animals (Przekop et al. 1984). Premating stress has also been linked with lowering of the ovulation rate in ewes (Domey et al. 1973) and inhibition of the preovulatory LH release in heifers (Moberg 1976, Stoebel and Moberg 1982). Confinement-stress stimuli has been documented to affect episodic secretion of LH in ovariectomized sheep (Rasmussen and Malven 1983). Diestrous rats exposed to footshocks display marked changes in the course of the estrous cycles in the post-stress period (Chomicka 1984).

In spite of the considerable body of data showing that prolonged or chronic stress inhibits gonadotropin secretion, there is little doubt that the early effect of stress may, at least in some circumstances, stimulate GnRH/LH secretion. Intact rodents (Briski and Sylvester 1988, Euker et al. 1975, Mann et al. 1986, Rivier and Rivest 1991, Tanebe et al. 2000) exposed to acute stress respond with a small and often short-lived increase in plasma LH.

The precise central mechanisms that media te GnRH secretion are still far from being understood. Current available data suggest that the immediate response of GnRH to stressful stimuli depends primarily on the neural mechanism that activates excitatory neurotransmitters, neurohormones, and amino acids, and that all compounds released from the hypothalamic-pituitary-gonadal and hypothalamic-pituitary-adrenal axes during prolonged stress act mainly within the preoptic area, hypothalamus and anterior pituitary gland to mediate the inhibitory influence on GnRH/LH release (Rivier and Rivest 1991).

Despite a number studies that have been concerned with the mechanism of GnRH release under stress, there is as yet no coherent understanding of how the stressful stimuli affect intraneural events associated with the biosynthesis of GnRH and GnRH-R in the hypothalamus and the anterior pituitary gland, respectively.

Lack of clarity in this area is exemplified by the debate on the effect of different kinds of stress on GnRH mRNA in the preoptic area-hypothalamus and GnRH-R mRNA in the anterior pituitary gland. Indeed, it has been reported that stress in rats generally has a suppressive influence on GnRH mRNA (Gruenewald and Matsumoto 1993, Nappi and Rivest 1997, Tanebe et al. 2000) as well as GnRH-R mRNA (Nappi and Rivest 1997) expression but the responses may be different in the various structures of the central nervous system and, to a high degree, are dependent upon the kind of stress, physiological state of the animals and gender. For example, prolonged cold stress in female rats suppresses GnRH gene expression in the preoptic area (Tanebe et al. 2000) while prolonged food deprivation stress does not affect GnRH gene transcription in the hypothalamus in cycling female rats, but provokes deep inhibition of GnRH-R mRNA in the pituitary gland (Nappi and Rivest 1997). On the other hand, a decreased number of neurons expressing GnRH mRNA in the hypothalamus was found in male rats after prolonged fasting stress (Gruenewald and Matsumoto 1993). Acute stress induced by lipopolysaccharides causes similar profound down-regulation of GnRH-R gene expression in the anterior pituitary gland throughout the entire estrous cycle, but attenuates the GnRH mRNA concentration in the hypothalamus of cycling female rats in a relatively short period at proestrus (Nappi and Rivest 1997). There is no information on the response of GnRH mRNA in the preoptic area, hypothalamus and GnRH-R mRNA in the hypothalamus and anterior pituitary gland to stress in ewes. We have recently presented data showing the existence of GnRH-R mRNA in the preoptic area and hypothalamus in sheep (Ciechanowska et al. 2004). On the basis of temporal analysis of the relationship between changes in LH secretion and GnRH mRNA expression during an estradiol-induced LH surge and on the negative feedback action of progesterone on this hormone secretion, it has been suggested that the neural system controlling GnRH biosynthesis may be distinct from that regulating GnRH release in this species (Harris et al. 1998, Robinson et al. 2000). A similar view is also supported by results obtained on rats (Petersen et al. 1995). Since expression of the GnRH gene and GnRH-R gene is highly regulated by estradiol (Dorling et al. 2003, Petersen et al. 1995, Seong et al. 1998, Thanky et al. 2003) and a variety of neurohor-

mones and neurotransmitters, it is reasonable to suggest that changes in steroid hormones and some neurochemical compounds (Kang et al. 1995, Kim et al. 1993, 1994, Tellam et al. 1998) released from the hypothalamic-pituitary-gonadal and hypothalamic-pituitary-adrenal axis under stress condition may affect the transcription of GnRH mRNA and GnRH-R mRNA. Therefore, the objective of this study was to analyze the effect of short and prolonged intermittent footshock stimulation on the levels of GnRH mRNA and GnRH-R mRNA in the preoptic area, anterior hypothalamus, ventromedial hypothalamus, and on the GnRH-R mRNA in the stalk/median eminence and in the anterior pituitary gland of ewes during the follicular phase of the estrous cycle to better understand the mechanisms through which stress induces reproductive dysfunction.

METHODS

Animal procedures and stress application

The studies were performed on 3–4-year-old Polish Merino ewes during the middle of the breeding season (October–November). The animals were maintained indoors in individual pens and exposed to natural lighting. They were well adapted to the experimental conditions: they had constant visual contact with their neighbors even during blood collection to prevent the stress of social isolation. Food and water were available *ad libitum*.

The onset of estrus was checked twice daily using a vasectomized ram. The day of onset of estrus is referred to as Day 0. Only animals which showed two consecutive normal estrous cycles were chosen for experiments. Six ewes were used in each group.

The state of stress was induced by applying repetitive trains of 3 mA alternative current of 0.5 s on and 1 s off arranged in a series of 10 during a 20-minute period of every hour. The pulses were delivered in a programmed schedule to animals by electrodes on the legs of ewes at the level of the metacarpus over a period of 3 h (from 08:00 A.M. to 11:00 A.M.) on the sixteenth day of the estrous cycle (for the short-stressed ewes) and 5 hours daily (from 08:00 A.M. to 01:00 P.M.) over four days (from the 13th to 16th day of the estrous cycle) for the prolonged-stressed animals. This procedure was described in detail in an earlier paper (Przekop et al. 1985).

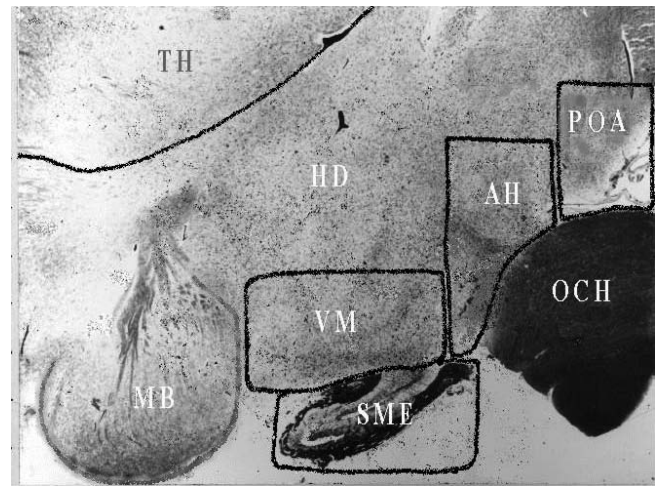

Fig. 1 Sagittal section through ovine brain: outlined areas indicate structures taken for analysis of GnRH mRNA and GnRH-R mRNA. Legend: (AH) anterior hypothalamus; (OCH) optic chiasm; (POA) preoptic area; (SME) stalk/median eminence; (MB) mammillary body; (TH) thalamus; (VM) ventromedial hypothalamus; (HD) dorsal hypothalamus.

To determine the concentration of plasma LH and the secretion profile of this hormone, a series of blood samples was collected twice from each animal at 10 min intervals during 5 h *via* an indwelling jugular catheter: first, on the 16th day of the estrous cycle prior to stressing and the second time on the 16th day of the next estrous cycle during the last day of stimulation. The 16th day of the estrous cycle is the last day prior to appearance of behavioral estrus in ewes. The effect of stress on LH secretion was evaluated on the basis of LH level in the blood plasma of non-stressed and stressed animals. Immediately after stimulation and blood collection the ewes were euthanized with barbiturate overdose. Each animal served as a control. The procedures were done in compliance with regulations of the Local Ethics Committee of the Warsaw Agricultural School.

The brains were immediately removed from the skull; the stalk/median eminences were isolated and frozen in liquid nitrogen. Blocks of brain encompassing the preoptic area-hypothalamus were sectioned sagittally and dissected from both sides on three parts, i.e., the preoptic area, anterior hypothalamus, ventromedial hypothalamus, according to the stereotaxic atlas of the ovine brain (Welento et al. 1969) (Fig.1). Tissue from the anterior pituitary gland was also taken for GnRH-R mRNA.

Measurement of relative genes expression

RNA EXTRACTION

Total RNA from frozen tissue was extracted with a GenElute Mammalian Total RNA Kit (Sigma-Aldrich, Inc.) according to the manufacturer's instructions. Briefly, up to 40 mg frozen tissue was homogenized in the lysis solution. In the following steps, the lysate was filtered and loaded onto an RNA-binding column. The column was washed with Wash 1 and Wash 2 solutions and RNA was eluted by 50 µl of TE buffer. In order to quantify the amount of total RNA extracted, the optical density was determined with an (Ultraspec 3000) ultraspectrophotometer, RNA integrity was electrophoretically verified in a 1.5% agarose gel stained with ethidium bromide.

RT-PCR

For elimination of probe contamination by genomic DNA, the total RNA was treated with Rnase-free Dnase 1 (Sigma-Aldrich, Inc.). One microgram of RNA was treated with 1 U of Dnase 1 for 15 min at room temperature. The reaction was stopped by addition of stop solution and Dnase was inactivated at 70°C for 10 min. Reverse transcription was carried out using an Enhanced Avian HS RT-PCR Kit (Sigma-Aldrich, Inc.) according to the manufacturer's instructions. One microgram of anchored oligo$(dT)_{23}$ primer was annealed to a mixture containing 1 µg Dnase-treated RNA. Following 10 min incubation at 70°C, all of the remaining components were added and the reaction was carried out at 45°C for 50 minutes. The cDNA was used immediately in the PCR or stored at –20°C.

The primers for gonadotropin-releasing hormone and gonadotropin-releasing hormone receptor cDNAs were derived from sheep sequences (GenBank Acc. No. U02517 and L22215), (Table I). Ovine reference cDNA, glyceraldehyde 3-phosphate dehydrogenase GAPDH, is successfully amplified with primers designed by us previously for bovine GAPDH cDNA. Primer design was done using Primer 3 software.

On cDNA for GnRH, amplification produced a 152 bp product, and a 200 bp product for GnRH-R. Conditions for PCR were optimized in a gradient cycler (MJ Research Inc.) with regard to various annealing temperatures, amount of RT product, and number of cycles. The optimal conditions for GnRH amplification were 3 µl, for GnRH-R, 1 ml of RT product, 35 cycles of amplification consisting of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and 72°C for 10 min in an MJ DNA engine Tetrad (MJ Research, Inc.). Expression of GnRH and GnRH-R genes was normalized to expression of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. Optimal conditions used for GAPDH amplification were 1 µl RT product, 35 cycles of amplification. PCR was performed in a 25 µl reaction mixture using the Sigma ReadyMix RedTag PCR Reaction Mix with a forward and reverse primer concentration of 10 pM each. Products were run on 1.5% agarose gel in 1 × Tris-Borate-EDTA buffer at 100 V. Bands were visualized by UV after staining the gel for 20 minutes in a 0.5 µg/ml ethidium bromide solution and the amount of PCR product was measured by densitometry on a Molecular Imager (BioRad, Inc.).

Table I

Primers for gonadotropin-releasing hormone and gonadotropin-releasing hormone receptor and glyceraldehyde 3-phosphate dehydrogenase genes amplification

Gene, GenBank Acc. No	Primer	Sequence
GnRH, U02517	Forward	TGGAGGAAAGAGAAATGCTAAGA
	Reverse	AGACTTTCCAGAGCTGCCTTC
GnRH-R, L22215	Forward	AGCAAGCTGGGACAGTTCAT
	Reverse	AGGCAGCTGAAGGTGAAAAA
GAPDH; BvGAPDH	Forward	CACTCCCAACGTGTCTGTTG
	Reverse	CCCAGCATCGAAGGTAGAAG

Radioimmunoassay for LH

The plasma LH concentration was assayed by a double-antibody radioimmunoassay using antiovine LH and rabbit gamma globulin antisera and ovine LH standard (NIH-LH-S018) according to Stupnicki and Madej (1976).

The assay sensitivity was 0.6 ng/ml and intra- and interassay coefficients of variation were 9% to 12%, respectively.

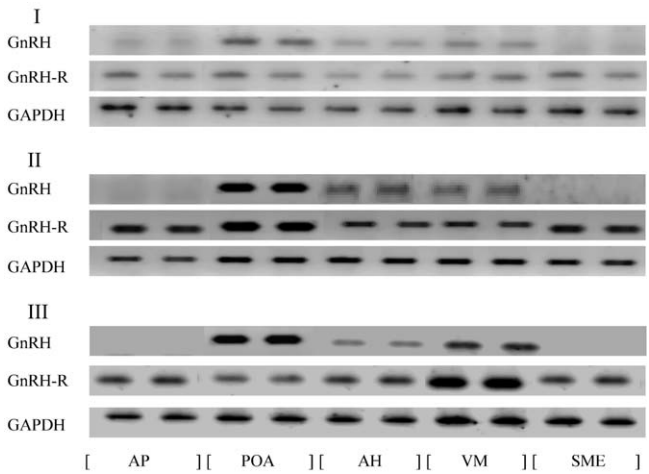

Fig. 2 Representative composite diagrams of GnRH mRNA and GnRH-R mRNA in an individual ewe from the preoptic area (POA), anterior hypothalamus (AH), ventromedial hypothalamus (VM), and GnRH-R mRNA from the anterior pituitary gland (AP), preoptic area (POA), anterior hypothalamus (AH), ventromedial hypothalamus (VM) and stalk/median eminence (SME) of control (I), short stressed (II) and prolonged stressed ewes (III).

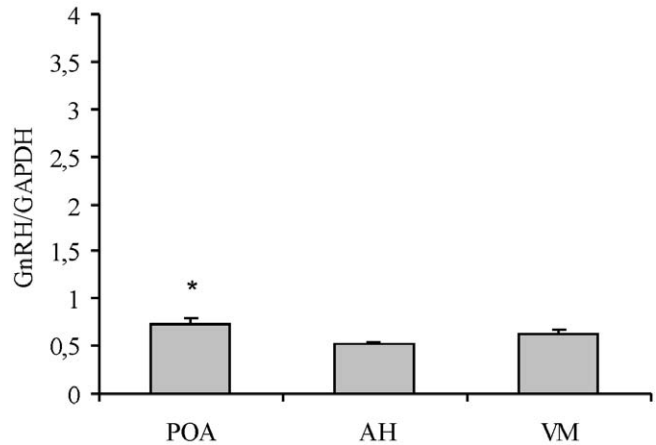

Fig. 3 GnRH mRNA concentration in the preoptic area (POA), anterior hypothalamus (AH) and ventromedial hypothalamus (VM) of control ewes (n=6), mean ± SEM, * $P \leq 0.05$.

Statistical analysis

GnRH mRNA and GnRH-R mRNA concentrations are expressed as a mean ± SEM. One-way ANOVA was used followed by Tukey's test to evaluate differences between the concentrations of these compounds in control vs. stressed animals. Differences in the concentration of GnRH mRNA and GnRH-R mRNA in various regions of hypothalamus in control ewes were also statistical evaluated.

Plasma LH concentrations are expressed as mean ± SEM. The significance of differences in the LH concentration between control and stressed animals was assessed by one-way ANOVA followed by the least significant difference (LSD) test (STATISTICA).

The number and amplitude of LH pulses were determined by the PC-PULSAR computer program according to the method of Marriam and Wachter (1982) with G parameters: G1=3.98; G2=2.40; G3=1.68; G4=1.24; G5=0.96. The frequency of LH pulses was defined as the number of identified pulses per collecting period (Viguie and coauthors 1995).

Amplitude was defined as the difference between peak and nadir values. Differences in LH pulse frequency and amplitude between groups were analyzed by the unilateral Wilcoxon test.

RESULTS

Representative composite diagrams of cytoplasmic GnRH mRNA and GnRH-R mRNA in the preoptic area, anterior hypothalamus, ventromedial hypothalamus, and GnRH-R mRNA in the stalk/median eminence and in the anterior pituitary gland in control and stressed animals are presented in Fig. 2.

The effects of short and prolonged intermittent footshock stimulation on GnRH mRNA expression in the preoptic area, anterior hypothalamus, and ventromedial hypothalamus

GnRH mRNA was found in structures throughout the preoptic area, anterior and ventromedial part of the hypothalamus. The highest concentrations of GnRH mRNA were found in the preoptic area (Fig. 3).

Relative to control values, the amounts of GnRH mRNA increased significantly in these structures of ewes subjected to short stress. Prolonged intermittent footshock stimulation significantly decreased GnRH mRNA levels in all analyzed structures (Fig. 4).

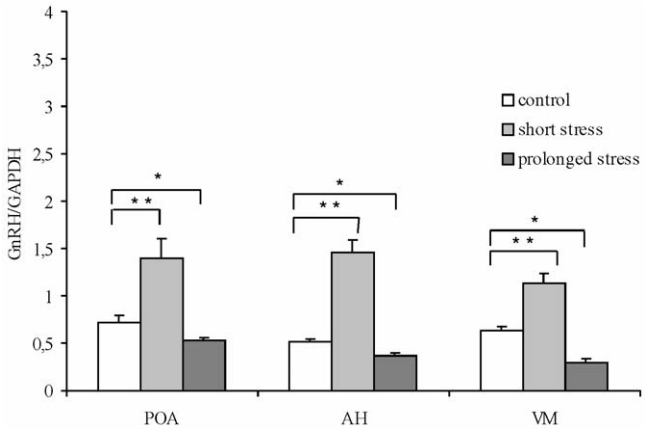

Fig. 4 Effects of short and prolonged stress on GnRH mRNA concentration in the preoptic area (POA), anterior hypothalamus (AH) and ventromedial hypothalamus (VM) of ewes (n=6), mean ± SEM, * $P \leq 0.05$, ** $P \leq 0.01$.

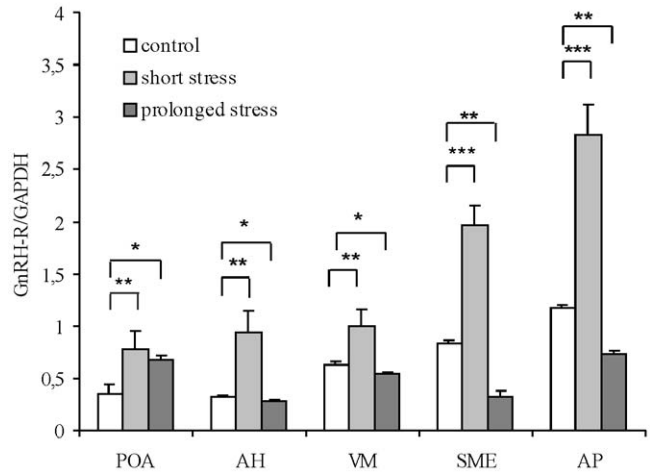

Fig. 6 Effects of the short and prolonged stress on GnRH-R mRNA concentration in the preoptic area (POA), anterior hypothalamus (AH), ventromedial hypothalamus (VM), stalk/median eminence (SME) and anterior pituitary gland (AP) of ewes (n=6), mean ± SEM, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

The effects of short and prolonged intermittent footshock stimulation on GnRH-R mRNA in the preoptic area, anterior hypothalamus, ventromedial hypothalamus, stalk/median eminence, and in the anterior pituitary gland

The GnRH-R gene was expressed at different levels in the analyzed tissue of control ewes; the highest concentrations of GnRH-R mRNA were found in the anterior pituitary gland and the stalk/median eminence. In the ventromedial hypothalamus the GnRH-R mRNA concentration was significantly higher than in the preoptic area and anterior hypothalamus (Fig. 5).

Marked augmentation of mRNA encoding GnRH-R was detected in these tissues after short stress stimulation; the lowest responses were found in the ventromedial hypothalamus.

With the exception of GnRH-R mRNA in the preoptic area, the GnRH-R mRNA was significantly reduced by prolonged footshocking. The concentrations of GnRH-R mRNA in the preoptic area in animals subjected to prolonged stimulation did not differ significantly as compared with controls (Fig. 6).

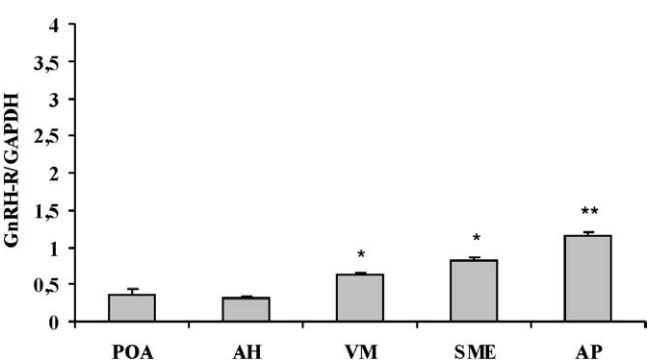

Fig. 5 GnRH-R mRNA concentration in the preoptic area (POA), anterior hypothalamus (AH), ventromedial hypothalamus (VM), stalk/median eminence (SME) and anterior pituitary gland (AP) of control ewes (n=6), mean ± SEM, * $P \leq 0.05$, ** $P \leq 0.01$.

The effects of short and prolonged footshock stimulation on LH secretion

In ewes subjected to short footshock stimulation, an increase in the LH concentration was observed as compared with controls (Fig. 7A). The frequency and amplitude of LH pulses showed a tendency to increase, but their differences as compared with control values did not attain statistical significance. However LH levels in blood plasma in individual ewes collected during the stressing and post-stressing periods did not differ significantly.

In prolonged-stressed ewes the LH concentration decreased significantly compared with pre-stimulation values; the frequency and amplitude of LH pulses were also in the range of control value (Fig. 8A).

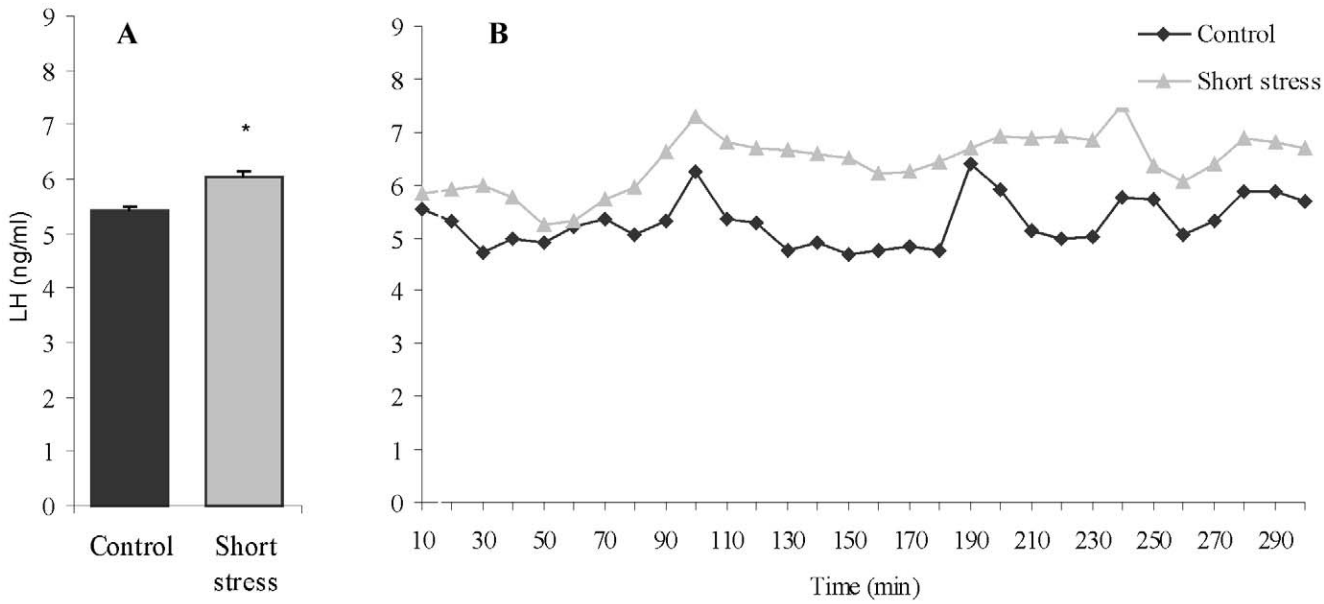

Fig. 7 Effect of the short stress on LH concentration in blood plasma of ewes (n=6), mean ± SEM, * $P \leq 0.01$ (A) and profile of LH in blood plasma in one representative ewe (B)

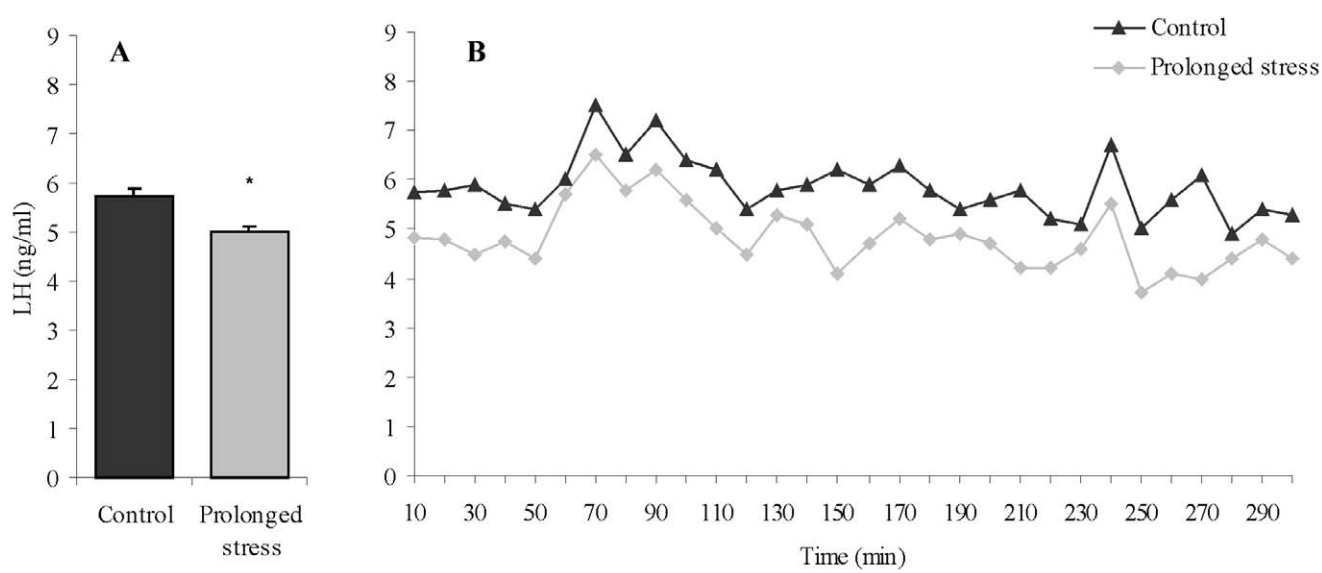

Fig. 8 Effect of the prolonged stress on LH concentration in blood plasma of ewes (n=6), mean ± SEM, * $P \leq 0.01$ (A) and profile of LH in blood plasma in one representative ewe (B)

DISCUSSION

The results of this study provide new insight into the expression of GnRH mRNA and GnRH-R mRNA in the preoptic area-hypothalamus and of GnRH-R mRNA in the stalk/median eminence and anterior pituitary gland during the follicular phase of the estrous cycle of control ewes and those subjected to stress. They indicate that GnRH mRNA occurs in the structure continuum throughout the preoptic area, anterior and ventromedial hypothalamus. The highest concentrations of GnRH mRNA were found in the preoptic area. No GnRH gene expression was observed in the stalk/median eminence. Since most GnRH neurons (above 50%) are located in the preoptic area in sheep (Caldani et al. 1995), the highest concentration of GnRH mRNA in these structures indicates that cytoplasmic GnRH mRNA is directly coupled with GnRH cell number in the neural structures. Because there is no information about GnRH mRNA degradation and translation in cellular biosynthetic processes in various structures, it is not possible to establish a clear connection between GnRH mRNA levels and actual transcriptional activity.

Analysis of the temporal relationship between changes in GnRH mRNA levels and LH release in ovariectomized-estradiol treated ewes shows that the LH surge is associated with a decrease in GnRH mRNA expression, thus suggesting that the neural mechanism controlling GnRH biosynthesis may be distinct from that regulating GnRH release (Harris et al. 1998). This is further supported by the finding that inhibitory action of progesterone on GnRH release is not linked with cellular content of GnRH mRNA in the hypothalamus (Robinson et al. 2000). Similarly, the results obtained on rats support the hypothesis that the biochemical events by which estradiol increases the GnRH mRNA level in the preoptic area prior to the preovulatory surge of gonadotropin is separate from the progesterone-amplified mechanism that induces GnRH release (Park et al. 1990, Petersen et al. 1995).

The simultaneously performed analysis of GnRH-R mRNA in these structures presents the first data set showing that the GnRH-R gene is also expressed in the preoptic area and hypothalamus. The large quantities of GnRH-R mRNA were found in the stalk/median eminence; only in the anterior pituitary gland the GnRH-R mRNA concentrations were significantly higher than those detected there. In the ventromedial hypothalamus the GnRH-R mRNA concentration was significantly higher than in the preoptic area and anterior hypothalamus. In rats the GnRH-R mRNA concentration in the hypothalamus and anterior pituitary gland is highly dependent on estradiol and progesterone concentrations, but their action in this aspect is different at the level of the mediobasal hypothalamus and pituitary (Seong et al. 1998). Indeed, in the rat medial basal hypothalamus estrogen increases and progesterone decreases GnRH-R mRNA, but in the anterior pituitary gland estrogen decreases GnRH-R gene expression and progesterone reinstates the estradiol-induced decrease in the GnRH-R mRNA concentration. Other studies indicate that the stimulatory effect of GnRH on GnRH-R mRNA in the anterior pituitary gland of female rats is markedly enhanced by estradiol (Yasin et al. 1995).

In the sheep pituitary, progesterone has a suppressive influence on GnRH-R mRNA expression (Turzillo et al. 1994, 1995), while estradiol is stimulatory (Adams et al. 1996, Brooks and McNeilly 1994, Kirkpatrick et al. 1998, Turzillo et al. 1998), thus suggesting that the action of these steroids on GnRH-R gene expression may be species-specific. It is also of interest that GnRH affects the GnRH-R mRNA concentration in the anterior pituitary gland. Although the results are still not univocal it is generally believed that homologous up- (Bauer et al. 1995, Kaiser et al. 1997, Lin and Conn 1999, Turzillo et al. 1995, Yasin et al. 1995) or down-regulation (Adams et al. 1996, Cheng et al. 2000, Sakakibara et al. 1996, Turzillo et al. 1998, Vizcarra et al. 1997) of GnRH-R gene expression depends to a high degree on the mode of treatment (continuous vs. pulsatile) and concentration (high vs. low) of GnRH.

The changes in the GnRH mRNA and GnRH-R mRNA concentration under short and prolonged footshock stimulation in ewes support the view that different kinds of stress act in specific ways on the expression of the GnRH gene as well as on GnRH-R.

Indeed, the amounts of GnRH mRNA increased significantly in the preoptic area, anterior hypothalamus, and ventromedial hypothalamus in ewes after short footshock stimulation, thus suggesting that short stress activates GnRH gene transcription. In contrast, the decreased GnRH mRNA concentration in these structures in ewes subjected to prolonged stimulation indicates that such stressful stimuli suppress GnRH gene expression. Interestingly, metabolic stress induced by

prolonged food restriction in lambs significantly inhibits LH secretion, but has no influence on GnRH biosynthesis (McShane et al. 1993). The results of metabolic and neurogenic stressful conditions on GnRH mRNA in rats also indicate that various kinds of stress affect GnRH gene expression in different ways. In this species the acute stress induced by lipopolysaccharide (Nappi and Rivest 1997), but not cold stress (Tanebe et al. 2000), attenuates the GnRH mRNA concentration in the hypothalamus of females in a relatively short period at proestrus, while prolonged food deprivation stress does not affect GnRH gene transcription in female cycling rats (Nappi and Rivest 1997), but decreases the number of neurons expressing GnRH mRNA in male rats (Gruenewald and Matsumoto 1993).

Concomitantly with the increase of GnRH gene expression in ewes under short episodes of footshock, and its decrease under prolonged footshock stimulation, respectively, a parall alternation in GnRH-R mRNA concentrations were also observed in the hypothalamus, stalk/median eminence and in the anterior pituitary gland. However, in the preoptic area of ewes subjected to prolonged stressful stimuli the expression of GnRH-R mRNA did not differ significantly from that of controls. Generally, these results show that the reactivity of GnRH-R gene expression in the hypothalamus, stalk/median eminence and in the anterior pituitary gland to stressful stimuli varies as a function of time, being facilitatory at the initial phase of stimulation and inhibitory under prolonged footshock application. The lack of changes in the GnRH-R mRNA concentration in the preoptic area of ewes subjected to short and prolonged stimulation is far from being clearly understood. This phenomenon suggests the existence of region-specific reaction in GnRH-R gene expression under prolonged stress conditions. Similarly, in rats, neurogenic stress causes profound down-regulation of GnRH-R gene expression in the anterior pituitary gland throughout the entire estrous cycle, but metabolic stress induced by food deprivation decreases GnRH-R mRNA only during particular phase of estrous cycle (Nappi and Rivest 1997). The increase of GnRH-R gene expression in the preoptic area, hypothalamus and stalk/median eminence under short footshock stimulation and down-regulation in the entire hypothalamus, stalk/median eminence of ewes under prolonged stressful stimuli suggests that GnRH mRNA in these structures may play a role in the intracellular events that control GnRH biosynthesis or release. Further studies are necessary to determine the precise functional link that exists between GnRH-R mRNA and GnRH-R mRNA with the biosynthesis of GnRH and its receptor protein. It is of interest to determine how long these changes of both GnRH and GnRH-R expression persists after termination of stress, and to what extent the down-regulation of GnRH mRNA and GnRH-R mRNA induced by prolonged intermittent stress is responsible for disturbances of ovulatory cycles observed in the long-term stressed ewes (Przekop et al. 1984).

On the basis of these results it could hypothesized that stress may have the capacity to modulate the sensitivity of both GnRH gene and GnRH-R gene expression mainly by different hormones and neurochemical compounds released in the central nervous system during stressful stimuli. Currently available data strongly suggest that the noradrenergic, corticoliberinergic, GABA-ergic systems may affect GnRH mRNA and GnRH-R mRNA levels in the hypothalamus. Indeed, in rats blockade of noradrenergic neurotransmission decreases the GnRH mRNA level in the hypothalamus (Kim et al. 1993, 1994), thus suggesting that the noradrenergic system has a stimulatory effect on the transcriptional activity of GnRH mRNA. Similarly GABA-A receptor activation increases the expression of the GnRH gene in the preoptic area-hypothalamus (Kang et al. 1995) but has an inhibitory effect on GnRH-R mRNA concentrations in these structures; activation of these receptors has no influence on the GnRH-R mRNA concentration in the anterior pituitary gland (Seong et al. 1995).

An *in vitro* analysis of the influence of CRF-like peptide on GnRH gene expression shows that CRF may alter in a specific way the transcription of GnRH mRNA in the hypothalamus over time, the course of incubation being facilitatory during the short-term incubation, followed by developing inhibitory components under a prolonged procedures (Tellam et al. 1998). Such a mode of action of all of these neurochemical components on GnRH mRNA and GnRH-R mRNA must be viewed with caution, since their effects in the normal state are by themselves inadequate proof for action under conditions of stress. There is evidence that stress may alter the functional activity of many neural pathways including GnRH (Roozendaal et al. 1995, 1997), opioidergic neurons (Przekop et al. 1990), and noradrenergic and serotoninergic systems (Przekop and Tomaszewska 1996).

The increase or decrease of expression of genes encoding GnRH and GnRH-R under short or prolonged intermittent stimulation are related in a parallel way with LH secretion, thus suggesting that this phenomenon is probably mediated by both changes of GnRH release from nerve terminals (Tomaszewska et al. 1999, 2002) as well as GnRH-R activity in the anterior pituitary gland.

The increase or decrease of LH concentrations in animals subjected to short or prolonged stress, respectively, is probably due to changes in the interpulses of LH release because the frequency of LH pulses and LH pulse amplitude did not differ significantly from the controls.

CONCLUSIONS

Footshock stimulation affects both genes encoding GnRH and GnRH-R in the preoptic area, anterior and ventromedial hypothalamus, and GnRH-R mRNA in the stalk/median eminence and the anterior pituitary gland in ewes during the follicular phase of the estrous cycle. Their responses are dependent on the kind of stress. Short stress increases GnRH mRNA and GnRH-R mRNA in the preoptic area, anterior and ventromedial hypothalamus, and GnRH-R mRNA in the stalk/median eminence and in the anterior pituitary gland. Prolonged stressful stimuli, on the other hand, significantly decreased GnRH mRNA level in all analyzed structures. With the exception of GnRH-R mRNA in the preoptic area, GnRH-R mRNA was significantly reduced in all structures of animals subjected to prolonged footshocking; GnRH-R mRNA in the preoptic area did not differ from control values. These changes in GnRH mRNA and GnRH-R mRNA levels under short or prolonged stress were associated with an increase or decrease of LH concentration in the blood plasma, thus suggesting the existence of a direct relationship between GnRH mRNA and GnRH-R mRNA with GnRH and GnRH-R biosynthesis.

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