

Changes in the GnRH mRNA and GnRH receptor (GnRH-R) mRNA levels in the hypothalamic-anterior pituitary unit of anestrus ewes after infusion of GnRH into the third cerebral ventricle

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Received: 9 April 2008; accepted: 30 June 2008

SUMMARY

In the present paper the role of GnRH in the ultrashort loop of the negative feedback action on GnRH secretion was evaluated on the molecular level by the Real-time PCR technique. Specifically, the effect of GnRH infused into the third cerebral ventricle on the expression of GnRH and GnRH receptor (GnRH-R) genes was analyzed in the hypothalamic-pituitary unit of anestrus ewes. GnRH did not significantly affect GnRH mRNA levels in the preoptic/anterior hypothalamic area but drastically increased its level in the ventromedial hypothalamus. In addition, GnRH infusion augmented GnRH-R mRNA level in the entire hypothalamus. In the GnRH-treated animals, anterior pituitary GnRH-R mRNA level and plasma LH concentration were also elevated. The changes in GnRH mRNA and

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GnRH-R mRNA levels in the hypothalamus in response to treatment with GnRH suggest that GnRH acts differently on the stability of these transcripts. On the basis of presented results it seems that GnRH may affect GnRH and GnRH-R biosynthesis and, consequently, GnRH/LH release. *Reproductive Biology* 2008, 8, 2:149-161.

Key words: GnRH, GnRH receptor, anestrus ewes, cerebral ventricle

INTRODUCTION

It is generally accepted that hypothalamic GnRH, among other neurohormones and/or neurotransmitters, is involved in the control of GnRH release. The regulation of GnRH release through the ultrashort loop feedback mechanism is supported by the results based on changes in GnRH release after *in vivo* and *in vitro* treatment with GnRH or its agonists. It has been shown that the activation of GnRH receptors in the ventromedial hypothalamus with GnRH exerts an inhibitory effect on GnRH release from the nerve terminals in this structure [4, 14]. The analysis of the relationship between the GnRH receptor (GnRH-R) mRNA levels in the ventromedial hypothalamus and LH secretion in rats and sheep strongly suggests the suppressive effect of GnRH-R mRNA on GnRH/LH release. Indeed, the increase in GnRH-R mRNA in the ventromedial hypothalamus during the morning of proestrus in rats [7] occurs concomitantly with the decrease in LH secretion. In the non-breeding season, dopamine (DA) displays the most important inhibitory effect on GnRH secretion, acting on dopaminergic DA-2 receptors [13]. Administration of DA-2 antagonists during anestrus decreases GnRH-R mRNA in the ventromedial hypothalamus/stalk median eminence and increases GnRH/LH release [3]. However, there is still a lack of evidence how exogenous GnRH affects the expression of GnRH and GnRH-R genes in the hypothalamic-anterior pituitary unit. In this paper, we analyzed the effect of small doses of GnRH infused into the third cerebral ventricle on 1/ the expression of GnRH and GnRH-R genes in the hypothalamus, 2/ the expression of GnRH-R gene in the anterior pituitary gland and 3/ LH secretion.

MATERIAL AND METHODS

Animals and surgical procedure

The studies were performed on three-to-four year old Polish Merino ewes during the middle of the anestrus season (April-May). The animals were maintained indoors in individual pens and exposed to natural lighting. Each group - control and experimental - consisted of six animals. The ewes were well adapted to the experimental conditions; they had constant visual contact with their neighbors to prevent the stress of social isolation. Food and water were available *ad libitum*.

Infusion cannulae were implanted into the third cerebral ventricle in ewes under general anesthesia with Vetbutal (pentobarbitalum: 10 mg/ml and pentobarbitalum natricum: 50 mg/ml; Biovet, Puławy, Poland). Using a stereotaxic procedure [16, 20] the stainless steel guide cannulae were directed toward the third ventricle and secured to the skull with screws and dental cement. Each guide cannula was fitted with an indwelling stylette to prevent backflow of cerebrospinal fluid. After surgery, antibiotics were provided to all ewes for four consecutive days.

Infusion and tissue collection

One hour prior to infusion, the cannulae were inserted through the guide cannulae and locked in position with tips placed approximately 2.0-2.5 mm above the base of brain; when the cannulae tips were in the third ventricle, the cerebrospinal fluid flowed into the cannulae. Both control (Ringers solution) and GnRH (2 µg GnRH/ml Ringers solution; Sigma-Aldrich, St Louis, USA, L-7134) infusions were performed at a flow rate of 2 µl/min for 20 min every one hour, for 5 hours daily, during three consecutive days. Such infusion yielded a total dose of 0.4 µg GnRH per animal daily.

The infusions were applied by calibrated one ml glass-tight syringes and microinjection pump (CMA/100/CAA microdialysis AB, Stockholm, Sweden). To determine the LH concentration in control and GnRH treated ewes, the series of blood samples were collected on the last day of infusion for 5 hours at 10 min intervals, through a catheter inserted into

the jugular vein a day prior the collection. Blood samples were taken into tubes containing 100 μ l of heparine (100 U/ml) and centrifuged within 1h after collection. Plasma was stored at -20°C until assay. Immediately after blood collection the ewes were euthanized with a barbiturate overdose. The procedures were performed with the consent of the Local Ethics Committee of the Warsaw Agricultural University.

The brains were rapidly removed from the skulls and the following parts of hypothalamus were dissected: the stalk/median eminence, ventromedial hypothalamus, anterior hypothalamus and preoptic area (fig. 1)

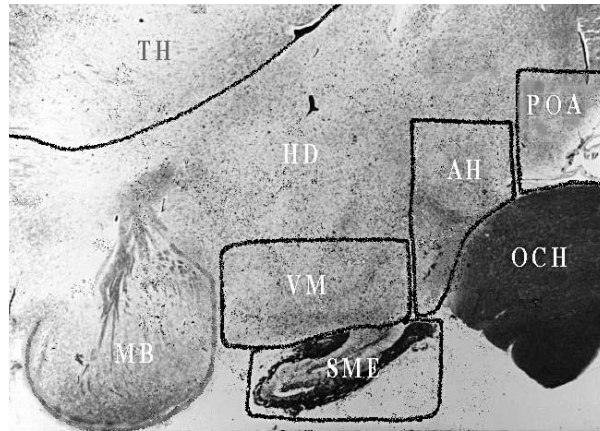


Figure.1. Sagittal section through ovine brain; outlined areas indicate structures taken for analysis of GnRH mRNA and GnRH-R mRNA. AH-anterior hypothalamus, OCH-optic chiasm, POA-preoptic area, SME-stalk/median eminence, MB-mammillary body, TH-thalamus, VM-ventromedial hypothalamus, HD-dorsal hypothalamus.

to determine GnRH mRNA and GnRH-R mRNA expression. Tissue from the anterior pituitary gland (the ventral parts of pars distalis) was also taken to determine GnRH-R mRNA expression. All samples were frozen in liquid nitrogen and stored at -80°C until assay.

Measurement of gene expression

RNA extraction. Total RNA was extracted from frozen tissue with a GenElute Mammalian Total RNA Kit (Sigma-Aldrich, St Louis, USA)

according to the manufacturer's instructions. Briefly, up to 40 mg of frozen tissue was homogenized in lysis solution. 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 μ l of TE buffer. In order to quantify the amount of total RNA extracted, the optical density was determined with a ultraspectrophotometer (Ultraspec 3000) and RNA integrity was electrophoretically verified in a 1.5% agarose gel. Bands were visualized by UV after the gel was stained for 20 minutes in a 0.5 mg/ml ethidium bromide and the amount of 18s and 28s RNA was measured by densitometry on Molecular Imager (BioRad, CA, USA). This information was additionally used for normalization of the amount of RNA in probes.

Real-time PCR. To eliminate probe contamination by genomic DNA, the total RNA was treated with RNase-free DNase I (Sigma-Aldrich, St Louis, USA), 1 μ g of RNA was treated with 1 U of DNase I for 15 min at room temperature. The reaction was blocked by adding 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, St Louis, USA) according to the manufacturer's instructions and 1 μ g of DNase-treated RNA. Following 10 min of 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 for PCR or stored at -20°C.

The primers for GnRH and GnRH-R were based on the sheep sequences (GenBank Acc. No. U02517 and L22215; tab. 1). Amplification of the cDNAs produced a 152 bp product for GnRH and a 200 bp product for GnRH-R. Samples were normalized using a reference gene, ovine glyceraldehydes 3-phosphate dehydrogenase (GAPDH), as described previously [2, 11]. The PCR products was visualized on agarose gel and sequenced in both directions (Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland). The real-time PCR amplification mix consisted of 2 μ l RT product, 5 μ l SYBR Green JumpStart Taq Ready Mix Capillary Formulation (Sigma-Aldrich, St Louis, USA) and 3 μ l of each primer (5 mM) in a final volume of 20 μ l. The optimal conditions of amplification for GnRH, GnRH-R and GAPDH consisted of an initial 5-min hold at 95°C, 40 cycles of 95°C for 10 s, 55°C for 10 s and 72°C for 15 s in a LightCycler

(Roche, Basel, Switzerland). Melting curves were run with each series to confirm the specificity of the amplified products. The $2^{-\Delta\Delta C_t}$ method [10] was used to calculate the relative ratio.

LH radioimmunoassay

The plasma LH concentration was analyzed by a double-antibody radioimmunoassay [15] using anti-ovine LH and anti-rabbit gamma globuline antisera and ovine LH standard (NIH-LH-SO18). The assay sensitivity was 0.06 ng/ml and intra- and interassay coefficients of variation were 4% and 10% respectively.

Statistical analysis

GnRH mRNA and GnRH-R mRNA levels as well as plasma LH concentration were expressed as mean \pm SEM and the differences between control and drug-infused animals were analyzed by a two-tailed t-test (Excel 2003; Microsoft, WA, USA). The frequency and amplitude of LH pulses were determined by the PC-PULSAR computer program [12] with cut-off G parameters: G1=3.98, G2=2.40, G3=1.68, G4=1.24, G5=0.93. Analysis was performed individually for every ewe and encompassed the entire sampling period. The frequency of LH pulses was defined as the number of identified pulses per collecting period (5 hours). The significance of differences in LH pulse frequency within groups was assayed by the Wilcoxon test and between groups by the Mann-Whitney test. The differences in the amplitude of LH pulses within and between groups were analyzed by nonparametric ANOVA rank Kruskal-Wallis test.

RESULTS

GnRH mRNA level in the hypothalamus of control and GnRH-treated anestrus ewes

In control and experimental ewes, the GnRH mRNA was expressed in the entire hypothalamus. In the GnRH-treated ewes, the GnRH mRNA level

was distinctly higher ($p < 0.001$) only in the ventromedial hypothalamus as compared to control values (fig. 2). The GnRH mRNA levels in the preoptic area and anterior hypothalamus were comparable in both control and experimental groups.

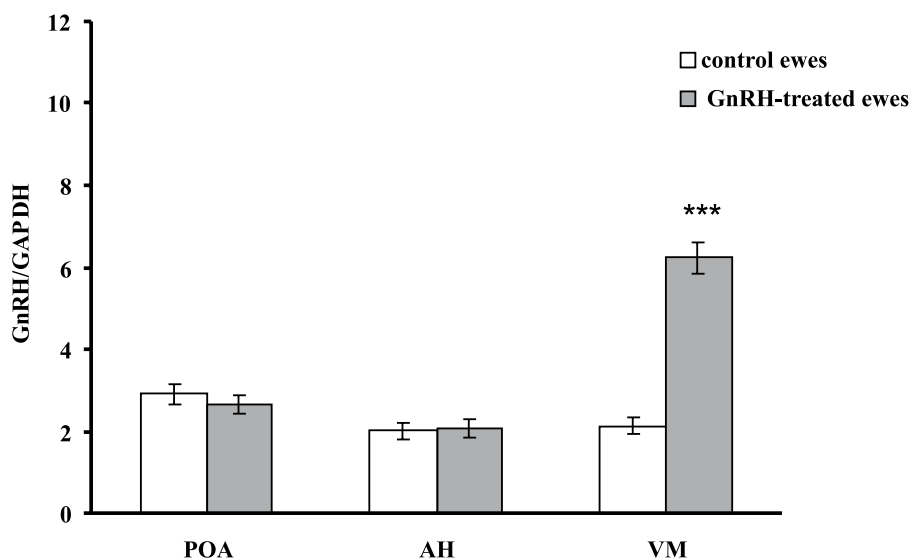


Figure 2. The effects of GnRH infusion into the third cerebral ventricle on GnRH mRNA level (mean \pm SEM) in selected areas of hypothalamus in anestrus ewes (n=6 per group). The applied abbreviations are explained in the fig. 1; GnRH/GAPDH mRNA ratio (arbitrary unit); *** $p \leq 0.001$

GnRH-R mRNA level in the hypothalamus and anterior pituitary gland of control and GnRH-treated anestrus ewes

Expression level of GnRH-R mRNA in the preoptic area, anterior and ventromedial hypothalamus, as well as stalk/median eminence in both groups was differentiated. The highest levels of GnRH-R mRNA were found in the stalk/median eminence and the anterior pituitary gland (fig. 3). GnRH infusion caused a significant increase in GnRH-R mRNA levels in all examined structures of the hypothalamus and in the anterior pituitary gland.

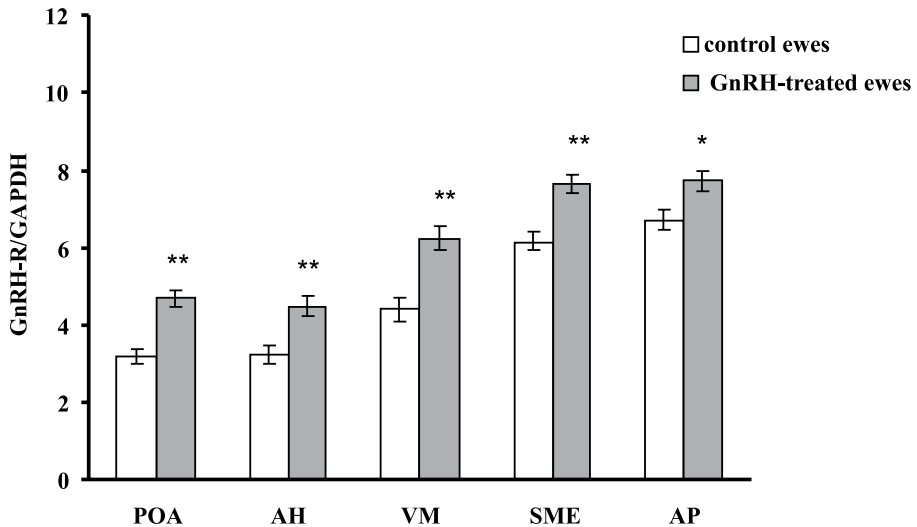


Figure 3. The effects of GnRH infusion into the third cerebral ventricle on GnRH-R mRNA level (mean±SEM) in selected areas of hypothalamus and anterior pituitary gland of anestrus ewes (n=6 per group). The applied abbreviations are explained in the fig. 1; GnRH-R/GAPDH mRNA ratio (arbitrary unit) * $p \leq 0,05$ ** $p \leq 0.01$

LH levels in blood plasma of GnRH-treated animals

The mean LH concentration (fig. 4A) and LH pulse amplitude (fig. 4B) increased significantly in the GnRH-treated ewes compared to controls. The frequency of LH pulses did not differ significantly between both groups (data not shown). Figure 5 represents exemplary plasma concentrations of LH in control and GnRH-treated ewes.

DISCUSSION

The study demonstrated that small doses of GnRH infused into the third cerebral ventricle affected the GnRH mRNA and GnRH-R mRNA levels in the hypothalamic-anterior pituitary gland unit in anestrus ewes. Specifically, GnRH significantly increased the GnRH mRNA level in the ventromedial hypothalamus, but had no evident influence on GnRH

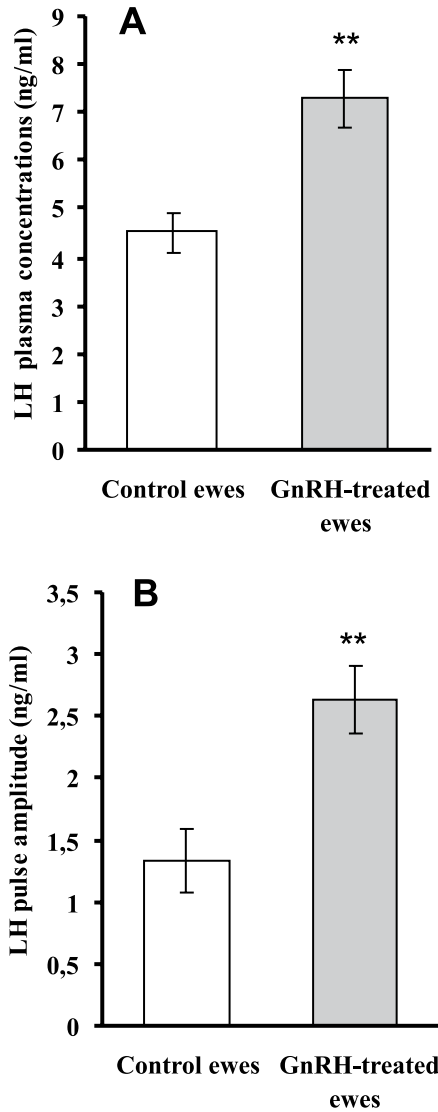


Figure. 4. The effects of GnRH infusion into the third cerebral ventricle on A/ LH plasma concentration (mean±SEM) and B/ LH pulse amplitude of anestrus ewes (n=6 per group): **p<0.01

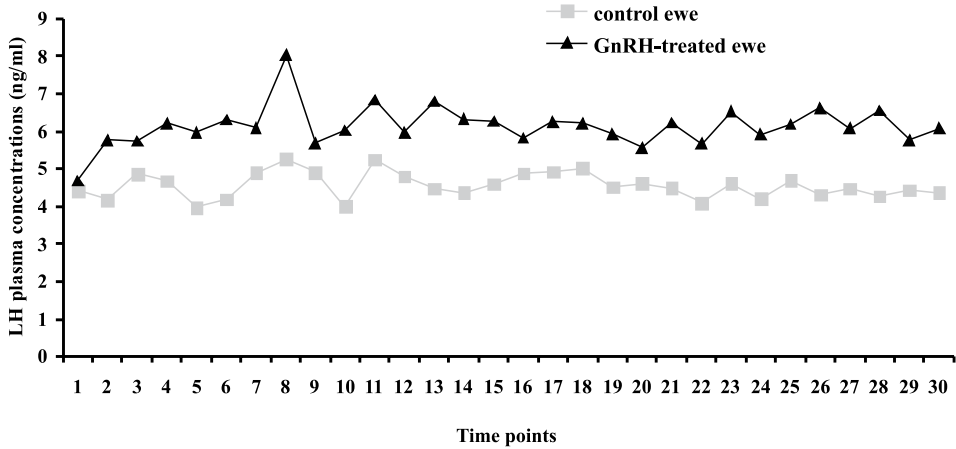


Figure. 5. Exemplary plasma concentration of LH in representative control and GnRH-treated ewes. Blood plasma was collected every 10 min from 8:00 A.M. (Time 0) to 1 pm

amount in the preoptic/anterior hypothalamic area. These results suggest that exogenous GnRH does not significantly affect GnRH gene expression in the preoptic/anterior hypothalamic region and acts on the transcriptional activity of this gene in the ventromedial hypothalamus. However, the increase in GnRH mRNA in this structure may also result from an increase of transcript stability. Because the levels of GnRH mRNA and GnRH protein are not strictly coupled, further research is needed to establish a relationship between actual transcriptional activity of GnRH gene and biosynthesis of GnRH in different physiological states of animals.

The significant increase in GnRH-R mRNA level in the entire hypothalamus in GnRH-treated ewes suggest that the GnRH stimulates GnRH-R gene expression and/or increases GnRH-R mRNA stabilization in this structure. On the basis of these results and literature reports it is impossible to define a clear relationship between GnRH-R mRNA level and GnRH-R activity. However, the decrease in GnRH-R mRNA level in the ventromedial hypothalamus/stalk median eminence in anestrus ewes after blockade of dopaminergic DA-2 receptors with a concomitant increase in the pituitary GnRH-R mRNA level and LH secretion suggest the reversed relationship between hypothalamic GnRH-R mRNA level

and GnRH release [3]. It is likely that the increased GnRH-R mRNA level in the hypothalamus/stalk median eminence exerts an inhibitory effect on GnRH release.

The regulation of GnRH release *via* the hypothalamic GnRH-R was suggested in several publications reporting changes in GnRH release after *in vivo* and *in vitro* treatment with GnRH. In those studies, the activation of GnRH-R in the ventromedial hypothalamus with GnRH or its analogues exerted an inhibitory effect on GnRH release from the hypothalamus as well as LH secretion from the anterior pituitary gland [4, 14]. In view of the presented results and literature data, it is suggested that the GnRH in the hypothalamus is involved in the regulation of GnRH and GnRH-R genes expression in a complex fashion leading to suppression in GnRH secretion.

The increase in GnRH-R gene expression in the anterior pituitary gland and in LH secretion may result from leaking of intracerebroventricularly infused GnRH into this gland. It is known that small doses of GnRH applied in a physiological pulsatile manner into the pituitary gland have a stimulatory effect on GnRH-R gene expression [1, 18, 22], GnRH receptor activity [9, 17, 21], biosynthesis of LH β subunit [5, 8], and LH synthesis [6].

In conclusion, the changes in levels of GnRH mRNA and GnRH-R mRNA in the various regions of hypothalamus in GnRH treated-ewes suggest that the hormone acts on the transcriptional activity of both examined genes and/or of mRNA transcript stability. On the basis of presented results, it seems that GnRH may affect GnRH and GnRH-R biosynthesis and, consequently, GnRH/LH release.

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