To examine the role of GABA_α receptors mediating systems in the control of gonadotropin-releasing hormone (GnRH) release from the ventromedial-infundibular region (VEN/NI) in ewes during luteal phase, the extracellular concentrations of GnRH, β-endorphin, noradrenaline (NE), dopamine (DA), and their metabolites: MHPG and DOPAC were quantified by local stimulation or blockade of GABA_α receptors with muscimol or bicuculline, respectively. Stimulation of GABA_α receptors in the VEN/NI did not affect GnRH, β-endorphin release or catecholaminergic system activity. Blockade of GABA_α receptors decreased β-endorphinergic and dopaminergic activity, and lowered the extracellular concentration of MHPG. It did not affect GnRH release or luteinizing hormone (LH) secretion. It is suggested that progesterone-induced GABAergic activity during the luteal phase may desensitize GABA_α receptors to muscimol. Lack of changes in GnRH/LH secretion with concomitant depressed β-endorphinergic activity corroborated the conclusion that β-endorphin does not inhibit GnRH release from the VEN/NI during the luteal phase. The physiological significance of changes in the catecholaminergic system activity under GABA_α receptor blockade in the control of GnRH secretion awaits to be established.

**Key words:** ewe, hypothalamus, gonadotropin-releasing hormone (GnRH), β-endorphin (B-END), γ-aminobutyric acid (GABA), catecholamines
INTRODUCTION

The regulation of hypothalamic GnRH secretion in the hypothalamus is provided by both stimulatory and inhibitory neurotransmitters/neurohormones. Among these transsynaptic regulatory systems, γ-aminobutyric acid (GABA), the dominant inhibitory neurotransmitter in the hypothalamus and preoptic area, affects GnRH secretion by two different classes of membrane receptor molecules: GABA_A receptors (1-3) and GABA_B receptors (4-6). Numerous experiments indicate, however, that GABA may mediate both inhibitory (7-9) as well as stimulatory (10-13) effects on GnRH release through a GABA_A receptor mechanism. The dual inhibitory-stimulatory action of GABA on GnRH release is not clearly understood. From literature results it appears that both the inhibitory and stimulatory influence of GABA on GnRH release may occur directly through a GABA_A receptor mechanism located on the GnRH neurons (14, 15) as well as indirectly through inhibitory or stimulatory interneurons that impinge on GnRH cells (16-20). The final effect of GABA on GnRH release is determined by the net result of inhibition and disinhibition of these neurotransmitter systems. The presented data strongly suggest that functional interconnections of the GABAergic system with the GnRHergic, opioidergic and catecholaminergic systems in the ventromedial-infundibular region of the hypothalamus (VEN/NI) play an important role in the control of GnRH secretion. Indeed, our results obtained from experiments on ewes are consistent with this concept, i.e. that GABA may suppress GnRH release, at least in part, through activation of a GABA_A receptor mechanism, β-endorphinergic and dopaminergic neurons in the VEN/NI (21) of anestrous ewes. According to literature reports, the mode of action of GABA on GnRH secretion appears to be highly dependent upon gonadal steroid activity (22-24) and site of interplay with the neuronal systems involved in the control of GnRH activity (23, 25). To address this issue we extended our studies on the role of GABA_A receptors in the control of GnRH release and β-endorphinergic and catecholaminergic system activity in the VEN/NI to the luteal phase of the estrous cycle.

MATERIAL AND METHODS

Animals

The studies were performed on four-year-old Polish Merino ewes (6 ewes in each group), well adapted to the experimental conditions. Using a stereotaxic procedure (26, 27), permanent guide cannulae were positioned on the skull at least three weeks prior to the perfusion procedures. The surgery was carried out under Vetbutal (Biovet, Pu³awy, Poland) anesthesia. The guide cannulae were directed towards VEN/NI and were secured to the skull with screws and dental cement. The animals were maintained indoors in individual pens throughout the study and exposed to natural daylight. They always had visual contact with their neighbors, even during the perfusion-sampling period, to prevent the stress of social isolation.
Food and water were available ad libitum. On the day of experiments two hours prior to perfusion, push-pull cannulae were introduced through the guide cannulae and placed in the proper position according to coordinates for the ovine hypothalamus. Each animal received two perfusions: first, a control perfusion with Ringer solution on the 7-th day of the estrous cycle; second, a perfusion with muscimol (10 µg muscimol/ml Ringer solution) or bicuculline (30 µg bicuculline/ml Ringer solution) on the 7-th day of the next estrous cycle. Each ewe served as a control. After the experiments the animals were euthanized with a barbiturate overdose. The procedures were done with the consent of the Local Ethical Committee at Warsaw University, Agriculture School. All experiments were performed during September-November.

Perfusate collection

The perfusions were performed at a flow rate of 5 µl/min and perfusates were collected continuously during 5 h (9:00 - 14:00) in 30-min fractions into tubes containing 50 µl 0.1 mM ascorbic acid. Every other sample was designed for catecholamine and β-endorphin and GnRH analysis, respectively. The perfusates were kept in an ice bath during sampling and stored at -80°C until assay. To determine the site of perfusion and to localize the place from which the perfusates were sampled, the brain of each animal was infused with 20 µl Prussian blue for 10 min. Then the brains were removed and sectioned sagitally under a stereoscopic binocular. Stained tissue was established in a spherical fraction about 2.0 - 2.5 mm around the tip of the cannula. The perfusates of animals with misplaced cannulae were excluded from analysis.

Analysis of catecholamines and their metabolites

The catecholaminergic system activity was evaluated on the basis of the extracellular concentration of noradrenaline (NE), dopamine (DA), and their main metabolites: 4-hydroxy-3-methoxy-phenylglycol (MHPG), 3,4-dihydroxy-phenylacetic acid (DOPAC). The concentrations of these compounds were analyzed using high performance liquid chromatography with electrochemical detection. Perfusates were centrifuged for 15 min and filtered through a 0.22 µm GVWP membrane; 50 µl of aliquots of each filtrate were injected into a LC-18-DB (15 cm x 4.6 mm ID, 5 µm) Supelco column protected by a superguard LC-18-DB 5 µm Supercosil, 2 cm precolumn. The column was coupled with an electrochemical detector (Hewlett Packard HP 1049 A Programmable Electrochemical Detector) equipped with a glassy carbon working electrode and Ag/AgCl reference electrode. The electrochemical detector was set at an oxidative potential of 0.650 V. Samples were eluted isocratically with a mobile phase consisting of 0.01 mol/L NaCl, 0.001 mol/L EDTA, 47-50 mg/L octyl sulfate sodium salt, 10 % CH3OH, the pH of mobile phase was 3.6. The mobile phase was filtered through a 0.22 µm GVWP membrane and degassed under vacuum with ultrasonic agitation. The flow rate was set at 0.8 ml/min. Stock solutions of standards were stored at -20°C. The limit of detection was 4 pg/50 µl for DA and NE, 3 pg/50 µl for MHPG and 3 pg/50 µl for DOPAC.

Analysis of β-endorphin-like immunoreactivity in perfusates

Extraction of β-endorphin-like immunoreactive compound(s) was performed by a method similar to the procedure described by Leshin and Malven (28). Briefly, 50 µl of perfusate were added to plastic tubes containing 100 mg of silic acid (70 - 325 Mesh, Merck), incubated for 20 min at 4°C with vortexing every 5 min. The suspension was centrifuged at 1000 g for 5 min and the supernatant decanted. The pellets were washed three times with 1 ml precooled distilled water, β-endorphin-like immunoreactive compound(s) was eluted from silic acid by three 1 ml rinses with a solution of 0.1 N HCl containing 80% acetone. The extracts were pooled and evaporated to dryness.
under a gentle stream of nitrogen at 60°C and stored at -80°C until assay. B-endorphin-like immunoreactivity was measured by a specific RIA according to a previously described method. The detection limit was 6 pg/100 µl. The recovery of β-endorphin-like immunoreactivity calculated on the basis of extraction using 125Iβ-endorphin added to plasma samples was 85-87%. Intra- and interassay coefficients of variation were 11% and 16%, respectively.

Radioimmunoassay of GnRH

The RIA procedure for GnRH was similar to that previously reported (29). The reference GnRH was obtained from Sigma. GnRH was radioiodinated using the chloramine-T method. GnRH antibodies (kindly provided by J. Kosowicz, Medical School, Poznań) at a final dilution of 1:16000 were used for determination. The detection limit was 1.9 pg/100 µl. The intra- and interassay coefficients were 7% and 11%, respectively.

Radioimmunoassay of LH

The plasma LH concentration was assayed by a double-antibody RIA using antiovine-LH and antirabbit gammaglobulin antisera and ovine LH standard (NIH-LH-SO18) according to Stupnicki and Madej (1976). The assay sensitivity was 0.06 ng/ml and intra- and interassay coefficients of variation were 4% and 10%, respectively.

Data analysis

Control levels of NE, DA, MHPG and DOPAC in the VEN/NI were evaluated in perfusates collected from Ringer solution-treated animals. The effect of perfusion of muscimol or bicuculline into the VEN/NI on the extracellular concentration of this compound(s) was expressed as a percentage change in the respective fraction pairs of muscimol/ or bicuculline to control treated animals, respectively. All results are presented as mean ±SEM. One-way ANOVA was used.
followed by Tukey's test to evaluate differences between means of concentrations in the respective pair fractions in perfusates muscimol/ or bicuculline treated animals to controls.

GnRH concentrations are expressed as means ± SEM and were assessed by one-way ANOVA. B-END concentrations are expressed as means ± SEM and were assessed by one-way ANOVA. Differences between muscimol/ or bicuculline and the control treatment in particular pair fractions were evaluated by Tukey's test.

Plasma LH concentrations are expressed as a mean ± SEM. The significance of differences between the results of control and muscimol/ or bicuculline treatment was assessed by ANOVA followed by the least significant differences (LSD) test (STATISTICA). The number and amplitude of LH pulses was determined by the PC-PULSAR computer program according to the method of Merriam and Wachter (31) with G parameters: G1 = 3.98; G2 = 2.40; G3 = 1.68, G4 = 1.24 and G5 = 0.93. The frequency in controls and muscimol-/ or bicuculline- treated animals was defined as the number of identified pulses per collecting period (32) and is expressed as a mean ± SEM. The amplitude was defined as the difference between peak and nadir values. Differences in LH pulse frequency and amplitude in the vehicle and muscimol/ or bicuculline perfused groups were analyzed by the Wilcoxon test.

RESULTS

Effects of muscimol perfusion in the VEN/NI on the extracellular concentration of GnRH in this structure and level of LH in the blood plasma

Schematic diagram showing location of the perfusion sites of muscimol in 6 ewes in the VEN/NI region of hypothalamus is illustrated in fig.1. The GnRH concentration in perfusates was low in the control perfusates, and in some samples the levels were at or near the assay sensitivity limit. Muscimol perfusion did not change either the GnRH (Fig.2a) concentration in perfusates or LH in the blood plasma. In all animals the estrous cycles occurred at the physiological time.

Effects of bicuculline perfusion in the VEN/NI on the extracellular concentration of GnRH

In all animals the perfusion of bicuculline in the VEN/NI caused no evident changes in the extracellular concentration of GnRH (Fig.2b) or in the blood LH level. In bicuculline-treated ewes the estrous cycles occurred at the normal physiological time.

Effects of muscimol perfusion in the VEN/NI on the extracellular concentrations of NE, DA, MHPG, DOPAC

In the control perfusates NE was found in a range from 8 to 23 pg/50 µl in 6 out 10 ewes. In the remaining animals, the NE value was essentially below the limit detection in most samples. The animals in which NE was undetectable were not taken for muscimol perfusion. In control treatment of ewes, DA was in a concentration ranging from 42 to 246 pg/50µl during whole period of perfusate collection. The concentration of MHPG in perfusates ranged from 32 to 287 pg
/50 µl and DOPAC from 75 to 875 pg /50 µl depending on the individual ewes. Perfusion of muscimol did not change the extracellular concentrations of NE DA, MHPG, DOPAC (Fig.3).

**Effects of bicuculline perfusion in the VEN/NI on the extracellular concentrations of NE, DA, MHPG, DOPAC**

The animals for bicuculline treatment were selected similarly as for muscimol perfusion. In all 6 selected ewes bicuculline treatment significantly depressed the extracellular concentration of DA and MHPG and DOPAC. NE in most perfusates remained at a low concentration and did not differ significantly from the control value (Fig.3).

**Effects of muscimol perfusion in the VEN/NI on the extracellular concentration of β-END-LI**

During the luteal phase of the estrous cycle of the ewes the extracellular concentration was at rather stable level and ranged from 81 to 114 pg /50 µl depending on the individual animal. Muscimol did not cause any evident changes in the concentration of β-endorphin in any of the animals (Fig.4a).

**Effects of bicuculline perfusion on the concentrations of B-END-LI in the VEN/NI of ewes**

Bicuculline perfusion caused a significant decrease of the B-END-LI concentration in all of the animals (Fig.4b).
The presented results demonstrate that activation of GABA_A receptors near GnRH nerve terminals in the VEN/NI during the luteal phase of the estrous cycle did not clearly affect GnRH, \( \beta \)-endorphin release or catecholaminergic activity. The failure of muscimol to alter the activity of these systems is not likely to reflect either a minor or no role for GABA_A receptors at this site in modulating...
GnRH, β-endorphin, NE and DA release: a similarly administered GABA<sub>A</sub> receptor antagonist, bicuculline, decreased β-endorphinergic and catecholaminergic activity. In this setting one plausible explanation for lack of responses of these systems to a GABA<sub>A</sub> receptor agonist would be that the progesterone-induced GABAergic activity during the luteal phase may desensitize GABA<sub>A</sub> receptors and make them resistant to muscimol treatment. Indeed, recent work revealed a clear stimulatory effect of progesterone on GABA<sub>A</sub> release in the medial preoptic area (33, 22). In the light of these results it is reasonable to suggest that progesterone secreted during the luteal phase of the estrous cycle may affect the dynamics of GnRH release through GABA<sub>A</sub> receptor mechanisms on GnRH nerve terminals or indirectly by changes in β-endorphinergic and dopaminergic system activities. In such multifactorial actions of GABA on gonadotropin secretion the ineffectiveness of bicuculline on GnRH release in the luteal phase of the estrous cycle could not be adequately interpreted. Bicuculline reduced the tone of β-endorphin activity but could not change GnRH release. Such results are not surprised, because it is well documented that the inhibitory effect of β-endorphin on GnRH release is only marginally apparent in the luteal phase of the estrous cycle in ewes (34). On the other hand, at the MPOA the decrease of opioidergic activity suppresses the stimulatory effect of progesterone on GABA release and enhanced LH secretion. Such observations suggest the existence of a regional specific action of β-endorphin on GnRH release during the luteal phase of the estrous cycle.

Temporal analysis of the relationship between bicuculline treatment and extracellular concentration of DA and DOPAC indicates that the GABA<sub>A</sub> receptor antagonist decreased the dopaminergic activity in the VEN/NI and that during the

![Fig. 4](image-url). The effect of muscimol (a) and bicuculline (b) perfusion into the VEN/NI on the β-endorphin concentrations in perfusates. Data presented are mean value ± SEM (*P ≤ 0.05, **P ≤ 0.01).
luteal phase of the estrous cycle dopamine does not play an important role on GnRH release in this structure.

The involvement of the dopaminergic system in the control of GnRH release in the VEN/NI of ewes is still not sufficiently consistent to provide a clear understanding of its action in this aspect. The increased dopaminergic activity in the VEN/NI during the luteal phase suggests an inhibitory effect of DA on GnRH release (35), the other results raise the possibility that the dopaminergic system in this structure is part of neural pathways mediating the positive feedback effects of estradiol on GnRH secretion (36). On the other hand in the MPOA the progesterone-induced increase of GABA secretion did not change DA release (33) suggesting that GABA-induced suppression of LH release is not mediated by the dopaminergic system in this structure.

The lack of changes in the extracellular concentration of NE with a concomitant decrease of MHPG during bicuculline treatment suggests that blockade of GABA<sub>α</sub> receptors leads mainly to a decrease of metabolic activity in the noradrenergic system in the VEN/NI. The physiological significance of this phenomenon in the control of GnRH release is unknown. Most of the results implicating noradrenaline in the control of GnRH release suggest the preoptic area as a main site of action of this amine in the control of GnRH secretion (33, 22).

In conclusion, these results indicate that stimulation of GABA<sub>α</sub> receptors in VEN/NI during luteal phase of the estrous cycle in ewes did not affect GnRH, LH, β-endorphin release and catecholaminergic activity, but blockade of these receptors decreased β-endorphin release and catecholaminergic activity but did not affect GnRH and LH release. It is suggested that progesterone-induced GABA release during luteal phase may desensitize GABA<sub>α</sub> receptors to muscimol. No changes in the GnRH/LH release with concomitant depressed β-endorphinergic activity during GABA<sub>α</sub> receptor blockade confirmed the suggestion that β-endorphin does not inhibit GnRH release from VEN/NI during luteal phase.

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Received: July 1, 2002
Accepted: October 29, 2002

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