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THE INVOLVEMENT OF $GABA_A$ RECEPTORS IN THE CONTROL OF GnRH AND β-ENDORPHIN RELEASE, AND CATECHOLAMINERGIC ACTIVITY IN THE VENTROMEDIAL-INFUNDIBULAR REGION OF HYPOTHALAMUS IN ANESTROUS EWES

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To examine the role of the $GABA_A$ receptor mediating systems in the control of gonadotropin-releasing hormone (GnRH) release from the ventromedial-infundibular region (VEN/IN) of anestrous ewes, the extracellular concentrations of GnRH, β-endorphin, noradrenaline (NE), dopamine (DA), 4-hydroxy-3-methoxy-phenylglycol (MHPG) and 3,4-dihydroxy-phenylacetic acid (DOPAC) were quantified during local stimulation or blockade of $GABA_A$ receptors with muscimol or bicuculline respectively. In most animals stimulation of $GABA_A$ receptors significantly attenuates GnRH release with concomitant increase of β-endorphin and DA release, and MHPG and DOPAC levels. Blockade of the $GABA_A$ receptors generally did not affect GnRH and NE release but inhibited in most animals β-endorphin release and decreased dopaminergic activity. These results suggest, that GABA may suppress GnRH release directly by $GABA_A$ receptor mechanism on the axon terminal of GnRH neurons or indirectly by $GABA_A$ receptor processes activating β-endorphin-ergic and dopaminergic neurons in the VEN/NI. On the basis of these results in could not be distinguish between these two events. The decrease in extracellular β-endorphin and dopamine concentration without evident changes in the GnRH level during $GABA_A$ receptor blockade may suggest that other neuronal systems are involved in this effect.

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

INTRODUCTON

A large body of evidence indicates that GABAergic neurons in the hypothalamus are involved in the regulation of GnRH/LH secretion. Most *in vivo* (1, 2) and *in vitro* (3) studies strongly suggest that GABA inhibits (4, 5) or stimulates (1, 6, 7) GnRH/LH release and biosynthesis of mRNA GnRH

(8, 9, 10). Immunohistochemical analyses and pharmacological manipulation with GABA receptors in the rat led to the conclusion, that a functional interconnection between GABA — GnRH (11, 12) GABAergic-catecholaminergic (13, 14, 15) and GABAergic — opioidergic neuronal systems (14, 15) in the preoptic area (MPOA) and medial basal hypothalamus (MBH) (1, 2, 16) plays an important role in the GnRH release. Studies indicate that GABAergic neurotransmission may differentially regulate GnRH secretion depending on GABA receptor subtypes encoded in these neuronal systems in these structures. In the case of an inhibitory effect, GABA could operate in at least in two ways to decrease GnRH release. One, by activating GABA receptors presented on GnRH perikary'a in the MPOA (17) or by these receptors on the excitatory neurons that impinge on GnRH neurons. At now, we cannot distinguish between these two possibilities.

On the other hand the stimulatory effect of GABA on LH release could be due to indirect effects via inhibitory neurons to GnRH. It is suggested that GABA may decrease the release of inhibitory neurotransmitter(s) and thereby remove inhibitory clamps on GnRH release. Still it is unknown what inhibitory neurotransmitter(s) or neurohormone(s) might be localized within these receptive neurons. It has been postulated that such facilitatory action of GABA neurons may occur by opioid-GnRH (15), catecholaminergic-GnRH (13, 14, 18). All of these stimulatory and inhibitory action of GABA on GnRH release are highly dependent upon the physiological state of animals (1). Howeer, most of these studies have dealt primarily with putative interaction of GABA with GnRH perikary'a in the MPOA of ovariectomized and steroid-treated animals. On the other hand limited observations suggest that such interaction between GABA neurons and the neuronal network that participates in the GnRH release may occur in the VEN/IN. Indeed the presence of a dense plexus of the GABA (19), opioid and dopaminergic neurons (20, 21) and noradrenergic nerve terminals (22) is closely associated with GnRH release. This consideration prompted us to investigate as a part of series studies in sheep the effect of local application of a $GABA_A$ receptor agonist (muscimol) and antagonist (bicuculline) into the ventromedial-infundibular region of the hypothalamus on GnRH release, β-endorphin and catecholaminergic systems activity in this structure.

MATERIALS AND METHODS

Animals

The studies were performed on the four year old Polish Merino ewes (8 animals in each group) weel adapted to the experimental condition. Using the stereotaxic procedure (23, 24) the permanent guide cannulae were positioned on the skull at tleast three weeks prior γ-aminobutyric acid

A-receptor agonist, muscimol, or $GABA_A$-receptor antagonist, bicuculline, perfusion. The surgical operation of ewes was carried out under Vetbutal (Biovet, Puławy, Poland) anesthesia. The guide cannulae were directed towards the ventromedial-infundibular region by a stereotaxic procedure and were secured to the skull with screws and dental cement. The animals were maintained indoors at temperature 12—15°C in individual pens throughout the study and exposed to natural daylight. They always had visual contact with their neighbors, event during the sampling periods, to prevent the stress of social isolation.

Food and water available ad libitum. On the day of experiment two hours prior to a perfusion, the pull-push cannulae were introduced through the guide cannulae and placed in the proper position according to coordinates for the ovine hypothalamus. The design and details of construction of the push-pull cannulae were described previously (22). For this study the outer cannulae was constructed from 1,0 × 100 mm hypodermic needles. The inner cannula was constructed from stainless steel tubing o.d. 0,5 mm. The inner cannula extended 1,5 mm beyond the end of the external cannula. Each animal received two perfusions: first, a control perfusion with Ringer solution and a subsequent perfusion conducted at 1 week interval with muscimol at a concentration 10 μg muscimol/ml Ringer solution or bicuculline at concentration 30 μg bicuculline/ml Ringer solution, respectively. Each ewe served as a control. After the experiments the animals were sacrificed with barbiturate overdose. These procedures were done with the consent of the Institutional Animals Care Committee. All experiments were performed during mid period of seasonal anestrous (April-May).

Perfusate collection

The perfusion were performed at a flow rate 5 μl/min and perfusates were coolected continuously during six hours (9^{00}—15^{00}), in 30 min fractions into the tubes containing 50 μl 0.1 mM ascorbid 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, next frozen in liquid nitrogen 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 of Prussian blue for 10 min. Then the brains were removed and sectioned sagitally under stereoscopic binocular. Stained tissue was estimated in a spherical fraction about 2.0—2.5 mm around the tip of the cannula. The perfusates of animals with misplaced location in the proper structure were excluded from analysis.

Analysis of catecholamines and their metabolites

Catecholaminergic system activity in the chosen areas was evaluated according to the extracellular concentration of dopamine (DA), noradrenaline (NE) and their main metabolites: 4-hydroxy-3-methoxy-phenylglycol (MHPG), 3,4,-dihydroxy-phenylacetic acid (DOPAC). The concentration of DA, NE and their metabolites were analyzed using high performance liquid chromatography with electrochemical detection as described previously (25). The limit of detection was 4 pg/50 μl for NE,
5 pg/50 μl for DA, 3 pg/50 μl for MHPG, 3 pg/50 μl for DOPAC.

Analysis of β-endorphin in perfusates

Extraction of β-endorphin-like immunoreactive compound(s) was performed by a method similar to the procedure described by Leshin and Malven (26), Briefly, 50—60 μl perfusate were added to plastic tubes containing 100 mg of silica acid powder (70—325 Mesh, Merck), incubated

for 20 min. at 4°C with wortexing every 5 min. The suspension was centrifuged at 1000 g for 5 min. and supernatant decanted. The pellets were washed three times with 1 ml pre-cooled distilled water, b-endorphin-like immunoreactive compound(s) was eluted from silica 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 RIA analysis. B-endorphin-like immunoreactivity was measured by specific RIA according to previously described method (22). The detection limit was 6 pg/100 µl. The recovery of β-endorphin-like immunoreactivity calculated on the base of extraction using ^{125}I-β-endorphin added to plasma samples was 85—87%. Intra- and interassay coefficients of variation were 11 and 16 respectively.

Radioimmunassay of GnRH

The RIA procedure for GnRH was similar as that previously reported (27). The reference GnRH was obtained from Sigma. GnRH was radioodinated using the chloramine-T method. GnRH antibody (kindly provided by J. Kosowicz, Medical School, Poznań) at a final dilution 1:16000 was used for wach determination. The detection limit of the assay was 1.2 pg/100 µl. The intra- and inter assay coefficients were 7% and 10% respectively.

Data analysis

Control levels of NE, DA, MHPG, DOPAC in the VEN/NI were evaluated in perfusate fractions collected from Ringer's solution perfused animals. The effect of perfusion with muscimol or bicuculline in this structure on the concentration of these compounds was expressed as a percent change in the respective fraction pairs of control to muscimol- or bicuculline-trated animals, respectively.

All results are presented as a mean ± SEM. One way ANOVA was use followed by Tukey's test (28) to evaluate differences between concentration means of their concentration in respective pair fractions in perfusates of control and muscimol- or bicuculline-treated animals.

GnRH, β-END-LI are expressed as mean ± SEM. GnRH and β-END-LI levels were assessed by one way ANOVA. Differences between mean value in control and during muscimol or bicuculline treatment in particular fraction were evaluated by Tukey's test.

RESULTS

Effect of muscimol perfusion into the ventromedial-infundibular (VEN/IN) on the concentration of GnRH in perfusates

Schematic diagram showing location of the perfusion sites of muscimol in 8 ewes in the VEN/NI region of hypothalamus is illustrated in *Fig. 1*. In the control treatment of anestrous ewes, the GnRH concentration was stable during entire period of perfusate collection. The onset of muscimol perfusion caused a significant decrease in GnRH concentration in 6 out of 8 ewes (*Fig. 2A*); in the remaining 2 animals the decreased concentration of this hormone did not attain statistical significance.

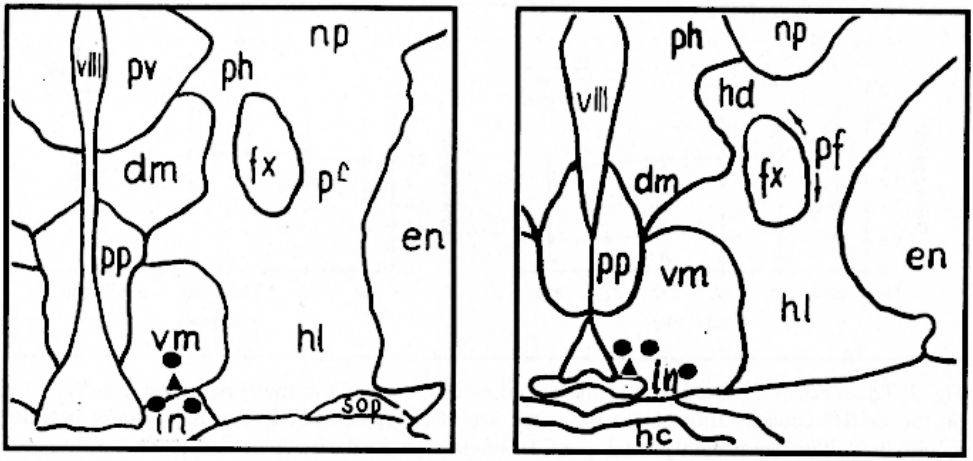

Fig. 1. Schematic diagram showing location of the perfusion sites of muscimol in 8 ewes in the VEN/NI region of hypothalamus; circle — responsive animal in GnRH release, triangle — non-responsive animal in GnRH release dm — nucleus dorsomedialis, en — nucleus entopeduncularis, fx — formix, hd — area dorsalis hypothalami, hl — area hypothalamica lateralis, in — nucleus infundibularis, np — nucleus hupothalamicus parvocellularis, pf — nucleus perifornicalis, ph — nucleus paraventricularis, pp — nucleus periventricularis, pv — pars verticalis nuclei paraventricularis, sop — nucleus supraopticus, vm — nucleus ventromedialis, VIII—III ventricle.

Effects of bicuculline perfusion in the VEN/IN on the concentration of GnRH in perfusates

Perfusion with bicuculline into the VEN/IN region did not cause evident changes in the extracellular GnRH concentration in 6 out of 8 animals (*Fig. 2B*). In the remainder 2 ewes the GnRH level increased significantly as compared to controls.

Effects of muscimol perfusion into the VEN/IN on the concentration of β-endorphin-like immunoreactivity (β-END-LI) in the perfusates

In the anestrous ewes the extracellular concentration of β-END-LI were at a rather stable level during control perfusion. Muscimol perfusion elicited a significant increase in the extracellular concentration of β-END-LI in 5 out of 8 animals (*Fig. 3A*). In the remaining 3 ewes the levels of β-END-LI did not differ significantly from the control values.

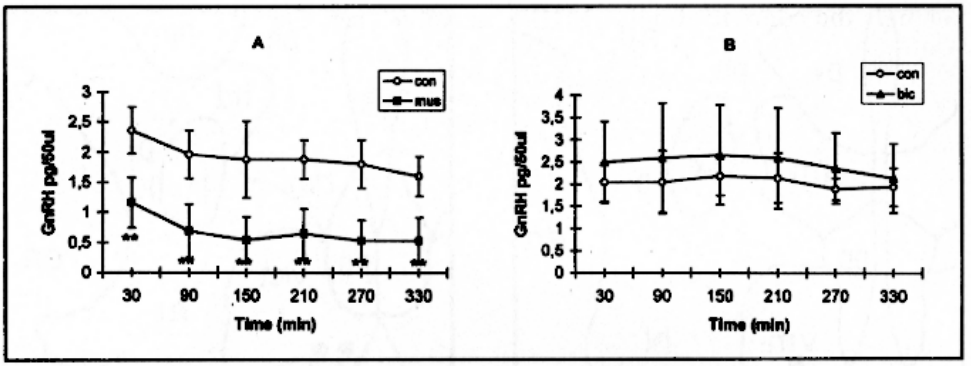

Fig. 2. The effect of muscinol (solid square) and bicuculline (solid triangle) perfusion into VEN/IN on the GnRH concentration. Data presented are mean value ± SEM (n = 6). Asterisks indicate values that differ significantly from control animals (opened circle) according to Tukey's test (*$P \leqslant 0.05$, **$P \leqslant 0.01$).

Effects of bicuculline perfusion on the concentration of β-END-LI in the VEN/IN of anestrous ewes

During bicuculline perfusion a significant decrease in the β-END-LI concentrations was found in 6 out of 8 animals (*Fig. 3B*); in two others bicuculline failed to affect β-END-LI concentration in perfusates.

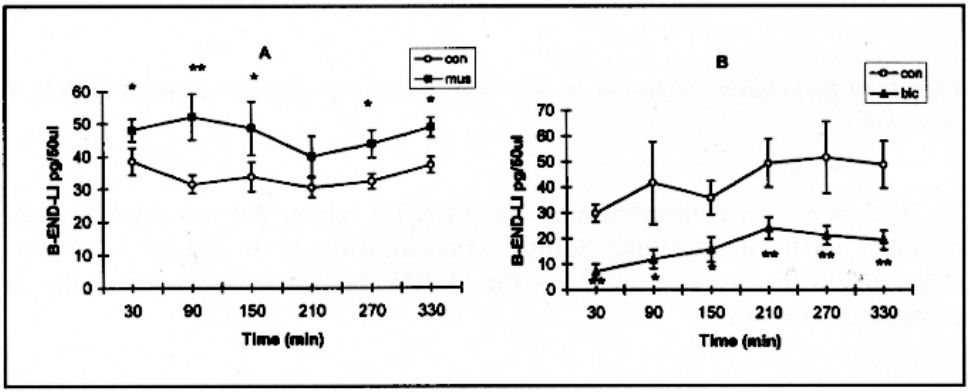

Fig. 3. The effect of muscinol (solid square) and bicuculline (solid triangle) perfusion into VEN/IN on the B-END-LI concentration. Data presented are mean value ± SEM (n = 5 or 6). Asterisks indicate values that differ significantly from control animals (opened circle) according to Tukey's test (*$P \leqslant 0.05$, **$P \leqslant 0.01$).

Effects of muscimol perfusion into the VEN/IN on the concentration of NE, DA, MHPG, DOPAC

Perfusion of the $GABA_A$ agonist, muscimol had no effect on NE concentration in the perfusates as compared with control levels. However in all of these

animals the concentration of MHPG increased significantly during muscimol perfusion (*Fig. 4*).

There were two distinct responses with respect to DA and DOPAC values. In 6 of 8 ewes muscimol increased DA and DOPAC (*Fig. 4*), whereas in the remaining 2 animals it did not cause a significant changes in the concentration of these neurochemicals. It is noteworthy, however, that levels of DA and DOPAC in these animals remained detectable throughout the entire period of perfusion.

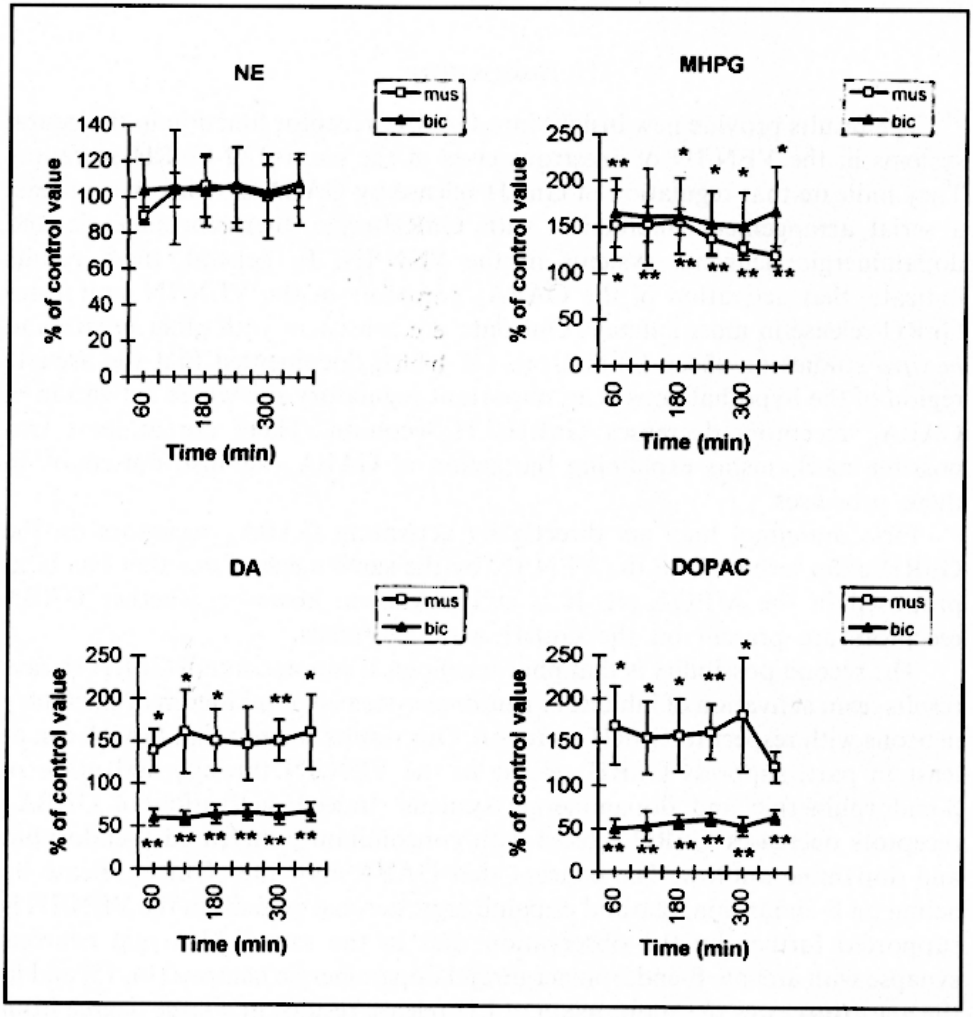

Fig. 4. Extracellular concentrations of NE, MHPG, DA, DOPAC in VEN-IN of ewes during perfusion of muscinol (opened square) and bicuculline (solid triangle). Data are expressed as a percent of the control value in the respective pair of animals, ($n = 6$, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$).

Effects of bicuculline perfusion into the VEN/IN on the concentration of NE, DA, MHPG, DOPAC

Perfusion of $GABA_A$ receptor antagonist, bicuculline, into the VEN/IN had variable effects on extracellular concentration of NE and MHPG. There was a marked increase in MHPG concentration in 6 out of 8 animals but no evident changes in NE levels were observed. Bicuculline decreased DA and DOPAC in 6 animals, in the remaining 2 ewes, their levels did not differ from the control value (*Fig. 4*).

DISCUSSION

The results provide new insight into $GABA_A$ receptor function in the neural systems in the VEN/IN of anestrous ewes in the control of GnRH secretion. They indicate that regulation of GnRH release by GABA may involve, at least a serial arrangement interaction with GnRH-ergic, β-endoorphinergic and dopaminergic neuronal systems in the VEN/IN. In general, these results indicate, that activation of the $GABA_A$ receptors in the VEN/IN suppresses GnRH release in most animals. Our data are consistent with other *in vivo* and *in vitro* studies on sheep (2) and rats (3), which documented that the arcuate region of the hypothalamus is an important regulatory site where activation of $GABA_A$ receptors decreases GnRH/LH secretion. There are at least two possible mechanisms explaining the action of $GABA_A$ agonist, muscimol, in these processes.

First muscimol may act directly by activating $GABA_A$ receptors on the GnRH axon terminals in the VEN/IN by the same mechanisms that has been proposed in the MPOA (1). It is still unknown, however, whether GABA receptors are present on the GnRH axon terminals.

The second possibility is that pharmacological suppression of GnRH release results from activation of inhibitory neuronal system(s) or inhibition of excitatory neurons with respect to GnRH secretion. Our results suggest that GABA can at least in part, suppress GnRH release in the VEN/IN through activation of β-endorphinergic and dopaminergic systems. Indeed, activation of $GABA_A$ receptors decreases GnRH release with concomitantly increased β-endorphin and dopamine outflow. The concept that GABA may alter GnRH release by acting on β-endorphinergic and dopaminergic neronal systems in the VEN/IN is supported further by the observation that in the rats GABAergic neurons synapse with arcuate β-endorphinergic and dopaminergic neurons (14, 15) and in the anestrous ewes the suppression of LH release results, in a large degree from inhibitory action of opioids and dopamine on hypothalamic GnRH neurons (21).

Lack of change in extracellular concentration of NE during muscimol or bicuculline treatment suggest, that GABA does not play an important role in

the GABA-ergic inhibitory action on GnRH release in the anestrous ewes. In light of numerous results implicating NE in the control of GnRH release the importance of the noradrenergic system in the inhibition of this hormone secretion from the hypothalamus of anestrous ewes is limited to the MPOA (21, 29).

Although our observations on the effect of $GABA_A$ receptor stimulation on GnRH/LH release are generally consistent with the results obtained in rats (30) and sheep (2), a note of caution in the interpretion of these data must be rise.

Blocking $GABA_A$ receptors in the VEN/IN leads to a decrease of β-endorphin and dopamine release but has no evident effect on GnRH secretion in most animals. Similar paradoxical effects of $GABA_A$ receptor agonist and antagonist was found in sheep and rats. Both muscinol and bicuculline elevated LH release when injected in the MPOA of some ovariectiomized, estrogen treated ewes, but both suppressed it when introduced into ovariectomized animals (1). Similar injection of either GABA or bicuculline into MPOA of ovariactomized rats reduced circulating concentration of LH (4). Thus pharmacological manipulation of $GABA_A$ receptors indicates, that these receptors are able to mediate both stimulatory and inhibitory effect of GABA on GnRH/LH release.

The significance of the dual GABA-ergic inhibitory-stimulatory action on GnRH release is not clear. Perhaps the most reasonable explanation for these contradictory data set may be assumption that GABA receptors are located on numerous stimulatory and inhibitory neurons with respect to GnRH release; the final effect of GABA is determined by net inhibition and disinhibition of neurons involved in the control of GnRH release. It could not be excluded that such effect results from neurotoxic influence of muscimol and/or bicuculline on the affecting neurons.

Presented results show that functional interaction between GABA-ergic system with other neuronal pathways throught which GABA may affect GnRH release from hypothalamus needs further studies. For example, it is suggested that in the rat GABA inhibits β-endorphinergic system activity in the arcuate nucleus — median eminence (15). Our results indicate that GABA may potentially stimulate β-endorphin release via $GABA_A$ receptor mechanism in the VEN/IN of anestrous ewes. It remains to be established if such differences of β-endorphinergic neurons response to GABA are species-specific or stimulatory effect of muscinol on β-endorphin release in the VEN/IN is characteristic only for anestrous state of ewes.

Analysis of the neurochemical relationship between stimulation-inhibition of $GABA_A$ receptors and dopaminergic system activity in the VEN/IN indicates that activation of these receptors in anestrous ewes increases dopaminergic activity, whereas blockade of $GABA_A$ receptors suppresses DA outflow. It needs further studies to define if such responses are characteristic for the sheep in all phases of reproduction or only for anestrous state.

Studies on rats documented that tuberinfundibular dopaminergic neurons in the male rats are tonically inhibited by GABA acting at $GABA_A$ receptors (30). Such point of view is supported by the finding that GABA activation inhibits potassium-induced ^3H-DA release from synaptosomes of the median eminence (31). Analysis of the influence of GABA on the dopaminergic system activity in the other structure of the CNS, including striatum (32), nucleus accumbens (33) and nigrostriatal dopaminergic neurons (34) provided controversial results. Perhaps the most reasonable explanation for this paradoxical effect is the hypothesis that $GABA_A$ receptors may simultaneously act on both DA neurons and inhibitory interneurons in different areas of the CNS, with the net effect of GABA on DA cell response depending on the functional balance of both direct inhibition and indirect disinhibition.

The increase of MHPG during muscinol or bicuculine treatment in the VEN/NI shows that stimulation or inhibition of $GABA_A$ receptors activates metabolic activity in the noradrenergic terminals. Further investigation is necessary to determine what neurochemical processes evoked by this drugs lead to this phenomenon. Pharmacological manipulation with $GABA_A$ and $GABA_B$ receptors on the NE outflow from the hypothalamus of rats has shown, that GABA might inhibit as well as facilitate noradrenaline release. It has generally been reported that the inhibition of basal and electrically evoked NE output is mediated primarily through $GABA_B$ receptors (35, 36); the facilitatory action of GABA on NE outflow occurs mainly through inhibitory $GABA_A$ chanel inerneurons (37, 38). However, it is still unknown what inhibitory neurotransmitters or neuromodulators might be acting within these GABA receptive interneurons.

In conclusion, these results indicate that stimulation of $GABA_A$ receptors significantly attenuates GnRH release with concomitant increase of β-endorphin, DA and MHPG levels, but blockade of these receptors does not affect GnRH outflow, but reduces β-endorphin and DA output. These results indicate that activation of $GABA_A$ receptors suppress GnRH release and suggest that GABA may operate in this process directly by $GABA_A$ receptors located on GnRH axon terminals in the VEN/IN, or indirectly through $GABA_A$ receptor mechanism activating β-endorphinergic and dopaminergic neurons in this structure. On the basis of these results it could not be distinguished between these two processes.

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