Pharmacological Reports 71 (2019) 636–643

Contents lists available at ScienceDirect

Pharmacological Reports

journal homepage: www.elsevier.com/locate/pharep

Original article

Does kisspeptin participate in GABA-mediated modulation of GnRH and GnRH receptor biosynthesis in the hypothalamic-pituitary unit of follicular-phase ewes?

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ARTICLE INFO

Article history:
Received 26 October 2018
Received in revised form 25 February 2019
Accepted 28 February 2019
Available online 5 March 2019

Keywords:
Ewe
Hypothalamus
$GABA_AR$
GnRH/GnRHR
Kiss1

ABSTRACT

Background: The inverse relationship between GnRH transcript level and GABA neurons activity has suggested that GABA at the hypothalamic level may exert a suppressive effect on subsequent steps of the GnRH biosynthesis. In the present study, we analyzed the effects of GABA type A receptor agonist (muscimol) or antagonist (bicuculline) on molecular mechanisms governing GnRH/LH secretion in follicular-phase sheep.

Methods: ELISA technique was used to investigate the effects of muscimol and/or bicuculline on levels of post-translational products of genes encoding *GnRH* ligand and *GnRH* receptor (*GnRHR*) in the preoptic area (POA), anterior (AH) and ventromedial (VMH) hypothalamus, stalk/median eminence (SME), and *GnRHR* in the anterior pituitary (AP). Real-time PCR was chosen for determination of the effect of drugs on kisspeptin (Kiss 1) mRNA level in POA and VMH including arcuate nucleus (VMH/ARC), and on Kiss1 receptor (Kiss1r) mRNA abundance in POA-hypothalamic structures. These analyses were supplemented by RIA method for measurement of plasma LH concentration.

Results: The study demonstrated that muscimol and bicuculline significantly decreased or increased GnRH biosynthesis in all analyzed structures, respectively, and led to analogous changes in plasma LH concentration. Similar muscimol- and bicuculline-related alterations were observed in levels of GnRHR. However, the expression of Kiss 1 and Kiss1r mRNAs in selected POA-hypothalamic areas of either muscimol- and bicuculline-treated animals remained unaltered.

Conclusions: Our data suggest that GABAergic neurotransmission is involved in the regulatory pathways of GnRH/GnRHR biosynthesis and then GnRH/LH release in follicular-phase sheep conceivably *via* indirect mechanisms that exclude involvement of Kiss 1 neurons.

Introduction

Substantial body of evidence supports the notion that GABA, the dominant inhibitory neurotransmitter in the hypothalamus of mammals, affects GnRH release by two classes of membrane receptors: $GABA_A$ ($GABA_AR$) [1] and $GABA_B$ ($GABA_B$ R) [2]. Studies in rodents and sheep have documented that GnRH cells express these receptor molecules [3–6]. Furthermore, GABAergic synapses have also been identified on GnRH neurons [7,8]. GABA acting through $GABA_A$ and $GABA_B$ receptor mechanisms may stimulate [9–12] or inhibit [13–15] activity of GnRH cells and GnRH/LH release depending on the animal's physiological state, experimental conditions, and site of its action. *In vitro* experiments in mice and rats have indicated that the activation of $GABA_AR$ depolarizes as well as hyperpolarizes GnRH cells [6,16–18]. Some studies have also suggested that GABA could excite [12,19,20] or inhibit [21] the firing rate of GnRH neurons. The GABAergic system may serve as a primary integrating center in the hypothalamus for many inputs to GnRH cells. It has been proven that signals from gonadal hormones [22,23] and many neuronal systems [22,24] alter GABAergic drive to GnRH neurons and ultimately affect the GnRH output.

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http://dx.doi.org/10.1016/j.pharep.2019.02.019
1734-1140/

It is generally accepted that muscimol, a selective agonist of $GABA_AR$, decreases GnRH/LH secretion in sheep [13,14,25]. The effects of $GABA_AR$ blocker, bicuculline, on GnRH/LH release have been variable and largely dependent upon physiological state of the animal, its sex and experimental conditions. In ovariectomized ewes, bicuculline suppressed LH release [13], but in follicular-phase animals it had no evident effect on the extracellular concentration of GnRH in preoptic area (POA) and ventromedial hypothalamus (VMH) [14]. The dialysis of bicuculline into POA of the castrated rams resulted in reduced LH release [26]. On the other hand, its infusion into the third cerebral ventricle of follicular-phase sheep stimulated LH secretion [25]. The mechanisms and mediators by which GABA affects hypothalamic-pituitary GnRH/LH system in sheep are not well recognized. However, it seems highly probable that GABA may involve interneurons that provide afferents to GnRH system [11], because as yet, there is no information in the subject literature about direct GABA action on GnRH/LH secretion in ewes. Identification of kisspeptin (Kiss 1) and its G protein-coupled receptor 54 (Kiss1r) as an essential component of the hypothalamic-pituitary-gonadal (HPG) axis controlling GnRH/LH release [27] raises the possibility that Kiss 1-Kiss1r signaling may play an essential role in the transduction of GABA-induced changes in GnRH/LH secretion. The interactions between GABA and kisspeptin neurons in the control of GnRH/LH release are not elucidated in sheep. However, studies in other species imply the existence of GABA-kisspeptin relationship, at least in some aspects of the hypothalamo-pituitary unit essential for reproduction [28,29]. Thus, one important issue for clarification of the potential role of GABA-kisspeptin interconnections in the regulation of hypothalamic-pituitary reproductive axis of sheep is to explain whether kisspeptin neurons participate in $GABA_AR$-mediated control of GnRH/LH system.

Limited data in rats have also shown that changes in GABAergic neurotransmission affect the expression of *GnRH* [30–32] and *GnRH* receptor (*GnRHR*) [33,34] genes. Based on our previous study on follicular-phase sheep, GABA is believed to inhibit the transcriptional activity of *GnRH* and *GnRHR* genes in the hypothalamus and *GnRHR* in the anterior pituitary (AP). This effect of GABA is probably indirectly mediated *via* $GABA_A$ R in the hypothalamus [25], thus suggesting the significance of this receptor mechanism in the control of GnRH and GnRHR biosynthesis. To get more insight in the role of GABAergic system in molecular control of the hypothalamic-pituitary reproductive axis, we analyzed the effects of prolonged intermittent infusions of $GABA_AR$ agonist (muscimol) or antagonist (bicuculline) (1) on levels of post-translational products of genes encoding *GnRH* and *GnRHR* in the POA-hypothalamus and *GnRHR* in the AP, (2) on the expression of mRNAs encoding Kiss 1 and Kiss1r in the POA-hypothalamic tissue continuum. The level of LH in the blood was also determined as an indicator of pituitary gonadotrophs activity. These interferences were studied in follicular-phase sheep – the animal species that are considered to be a good model in experiments on the neuroendocrine mechanisms regulating the process of reproduction [5,10,11,25,27].

Material and methods

Animals

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

The estrous cycle in the ewes was tested by running them with a vasectomized ram twice daily; only ewes that showed two consecutive normal estrous cycles were chosen for subsequent experiments. The experimental schedule with periodic socio-sexual isolation does not allowed to habituation, according to suggestion of Hawken et al. [35]. First, females were brought from foreign herd as opposed to the ram which was derived from local source. Second, ram was kept in another building; however, he was introduced to ewes within the anticipated period of occurrence of oestrus. Furthermore, evaluation of the ovaries was performed *post-mortem* as per routine veterinary practice to finally confirm the occurrence of oestrus. Six ewes were used in each group. The day of the onset of the estrous cycle is referred to as day 0.

Surgical procedures

Guide cannulas were implanted into the third cerebral ventricle of ewes under general anaesthesia (xylazine: 40 μg/kg of body mass, intravenously; Xylapan and ketamine: 10–20 mg/kg of body mass, intravenously; Bioketan; Vetoquinol Biowet, Puławy, Poland). Using a stereotaxic procedure [36,37], stainless steel guide cannulas were directed towards the third ventricle and secured to the skull with screws and dental cement. Each cannula was fitted with an indwelling stylette to prevent back flow of cerebrospinal fluid. After surgery, the ewes were given antibiotics subcutaneously for 4 consecutive days.

Infusion procedures and collection of material

One hour before infusion, cannulas were introduced through the guide cannula and secured in position with tips placed approximately 2.0–2.5 mm above the base of brain; when the tips of the cannulas were in the third ventricle, cerebrospinal fluid was seen to flow into the infusion cannula. For precise analysis of the effects of muscimol (Sigma-Aldrich, St. Louis, MO, USA) or bicuculline (Sigma-Aldrich, St. Louis, MO, USA) on LH secretion in ewes, each animal received two infusions: first, a control infusion with Ringer's solution on days 13–16 of the estrous cycle; and second infusion of either muscimol (20 μg muscimol/ml Ringer's solution) or bicuculline (20 μg bicuculline/ml Ringer's solution) over the same period in the next estrous cycle. The doses of drugs were selected based on our previous study and many years of experience [25]. Thus, for LH measurement each ewe served as its own control. The infusion of both control Ringer's solution and drugs was performed at the rate of 2 μl/min for 20 min of every hour for 5 h daily (from 08.00 to 13.00). This infusion protocol resulted in daily doses of 4 μg muscimol and 4 μg bicuculline per animal. Infusions were applied using calibrated glass-tight syringes and a CMA/100 microdialysis microinjection pump (CMA Microdialysis AB, Stockholm, Sweden). To determine the LH concentration, a series of blood samples were collected *via* an indwelling jugular catheter at 10-min intervals on the last day of infusion (Ringer's solution or muscimol or bicuculline). Blood samples were taken into tubes containing 100 μl of heparine (100 U/ml) and centrifuged within 1 h from collection. Plasma was stored at −20 °C until assay. Immediately after the last infusion of muscimol and/or bicuculline, ewes were euthanized and brain tissue were taken for analyses of GnRH and GnRHR levels as well as for Kiss 1 and Kiss 1 receptor (Kiss1r) mRNAs. For these analyses, a separate group of ewes which received an infusion of Ringer's solution served as a control for muscimol and bicuculline-infused animals. Control for Ringer's solution group was non-treated (intact) animals.

Following the euthanasia, brain was removed rapidly from the skull and the stalk/median eminences (SME) were isolated. Using surgical instruments (scalpel, scissors, narrow forceps and

micrometer) fresh hypothalamic blocks were sectioned sagitally and dissected from both sides into three, 3 mm thick parts (*i.e.* preoptic area - POA, anterior hypothalamus - AH, ventromedial hypothalamus including arcuate nucleus – VMH/ARC) according to the stereotaxic atlas of the ovine brain [37] for measuring the levels of GnRH/GnRHR and Kiss1r mRNAs. The pars distalis of the anterior pituitary gland (AP) was also taken for GnRHR analysis. The measurement of Kiss 1 mRNA was limited to the POA and VMH/ARC. After fresh dissection samples were frozen in liquid nitrogen and stored at −80 °C until assay.

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

Determining the GnRH and GnRHR levels

The quantitative measurement of GnRH and GnRHR levels in the POA-hypothalamus tissue and GnRHR in the AP was performed using an enzyme-linked immunosorbent assay (ELISA). All steps of the assay were performed according to the manufacturer's instructions for GnRH (Cusabio Biotech Co., Wuhan, China) and GnRHR (Cusabio Biotech Co., Wuhan, China).

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

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

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

Quantitative gene expression assay

Isolation of RNA and cDNA synthesis

Total RNA from hypothalamic structures and from pituitary was extracted using GeneElute Mammalian Total RNA Miniprep Kit (Sigma Aldrich, St. Louis, MO, USA) according to manufacturer's protocol. In order to quantify the concentration and purity of total extracted RNA, the optical density was determined with a spectrophotometer (NanoDrop™ 2000, Thermo Fisher Scientific, Wilmington, DE, USA). The RNA integrity was checked in a 1.5% agarose gel stained with ethidium bromide (Sigma Aldrich, St. Louis, MO, USA). To eliminate the probe contamination by genomic DNA, the total RNA was treated with RNase free DNase 1 (Sigma Aldrich, St. Louis, MO, USA). 1 μg of RNA was treated with 1U of DNase 1 for 15 min at room temperature. The reaction was stopped by adding stop solution, and DNase was inactivated at 70 °C for 10 min. Reverse transcription was carried out using 1 μg of DNase-treated RNA and an Enhanced Avian HS RT-PCR Kit (Sigma Aldrich, St. Louis, MO, USA), in line with the manufacturer's instructions. Following incubation for 10 min at 70 °C, all remaining components were added and the reaction runs at 45 °C for 50 min. A negative control (no reverse transcriptase added) was included for all samples. The cDNA was used immediately in the quantitative real-time PCR (qPCR) or stored at −20 °C.

Primer design

Specific primer pairs for target amplification were designed using Primer3 software [38,39] from sheep sequences available in GenBank. The primers sequences are presented in Table 1. The oligonucleotides were synthesized by Oligo IBB PAN, Poland. Based on the work of Peletto and co-workers [40], a combination of two reference genes *SDHA/YWHAZ* (geometric mean) was selected for normalization of Kiss 1 and Kiss1r mRNAs expression.

Quantitative real-time PCR

Real-time PCRs were performed on the RotorGene 6000 system (QIAGEN Company, Switzerland). Reactions were carried out using LuminoCt SYBR Green qPCR Master Mix (Sigma Aldrich, St. Louis, MO, USA), while cycle threshold (Ct) estimates were obtained using the relative quantification module in the software package. PCR reactions were performed in a final volume of 20 μl containing 2 μl of template cDNA, 2 μl of forward primer, 2 μl of reverse primer (working concentration 0.25 μmol of each), 10 μl of the 2x qPCR Master Mix, and 4 μl of RNAase-free water. After 10 min at 95 °C, the cycling conditions were as follows: 40 cycles at 95 °C for 10 s, 56 or 60 °C for 10 s, and 72 °C for 10 s. The positive (1 μl cDNA each sample) and negative (no template and no RT) controls were run for each reaction. To validate the specificity of amplification PCR products were sequenced on the Genetic Analyzer Applied Biosystems 3500xL (Thermo Fisher Scientific, Wilmington, DE, USA). The amplificon sequence was consistent with the information provided in GeneBank.

Table 1
Primers for experimental and housekeeping genes amplification.

Gene	Sequence	Temperature (°C) of annealing	Amplicon size (bp)
Kiss1 Kisspeptin (NM_001306104.1)	Forward: ATCCTAGAACCACAGGCTCG Rewerse: AAGGAGTTCCAGTTGTAGGCG	56	102
Kiss1r Kisspeptin receptor (NM_001318077.1)	Forward: TACATCCAGCAGGTCTCGGTG Rewerse : ACGTACCAGCGGTCCACACT	56	71
YWHAZF Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (NM_00126J887,1)	Forward: AGACGGAAGGTGCTGAGAAA Rewerse: CGTTGGGGATCAAGAACTTT	60	123
SDHAF Succinate dehydrogenase complex, subunit A (AY970969)	Forward: CATCCACTACATGACGGAGCA Rewerse:ATCTTGCCATCTTCAGTTCTGCTA	56	90

Data analysis

The $2^{\Delta\Delta Ct}$ method [41] was used in calculating the relative ratio, but instead of value 2, the correct amplification efficiency was used. We used a noise-resistant iterative nonlinear regression algorithm (Real-time PCR miner; www.miner.ewindup.info) to determine the efficiency of the PCR reaction [42]. The results are presented as relative gene expression of target genes *vs.* geometric mean of references genes.

Determining the LH concentration

Plasma LH concentrations were analyzed by double-antibody radioimmunoassay using anti-ovine LH and anti-rabbit gamma globulin antisera and ovine LH standard (NIH-LH-SO18, Sigma-Aldrich), as described by Stupnicki and Madej [43]. The sensitivity of the assay was 0.06 ng/ml and the intra and inter assay coefficients of variation were 9% and 11%, respectively.

Statistical analysis

All data were expressed as the mean + standard error of the mean (SEM). The one-way ANOVA and *t*-test were used for statistical analysis of ELISA and RT-PCR data. The significance of differences in LH secretion between control and drugs-treated ewes was assessed by one-way ANOVA followed by the least significant differences (LSD) test (STATISTICA; Stat-Soft, Inc., Tulsa, OK, USA). Differences in LH pulse frequency and amplitude between groups were analyzed with unilateral Wilcoxon test. The frequency of LH pulses was defined as the number of pulses identified per collection period [44]. The amplitude of LH pulses was defined as the differences between peak and nadir values. *p*-Values < 0.05 were considered significant.

Results

Effects of muscimol and bicuculline on the GnRH concentration in the POA-hypothalamus region of follicular-phase ewes

In control ewes, GnRH was found in structures throughout the POA, AH, VMH and SME (Fig. 1). Following muscimol treatment, GnRH levels decreased significantly in POA-hypothalamic areas (POA: $p < 0.01$; AH: $p < 0.05$; VM: $p < 0.01$; SME: $p < 0.01$), whereas administration of bicuculline resulted in a significant increase in GnRH quantities in all of the analyzed tissue material (POA: $p < 0.01$; AH: $p < 0.01$; VM: $p < 0.01$; SME: $p < 0.001$) compared to control values.

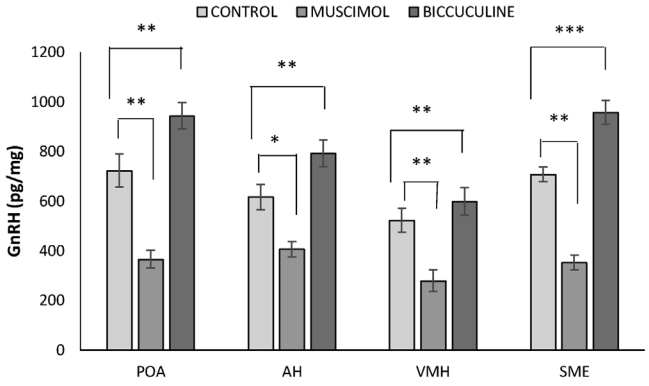

Fig. 1. Effect of muscimol and bicuculline on the level of GnRH in the preoptic area (POA), anterior hypothalamus (AH), ventromedial hypothalamus (VM) and the stalk median eminence (SME) of follicular-phase ewes, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Asterisks indicate values that differ significantly from the control group animals. Data are the mean ± SEM, n=6 animals per group (one-way ANOVA).

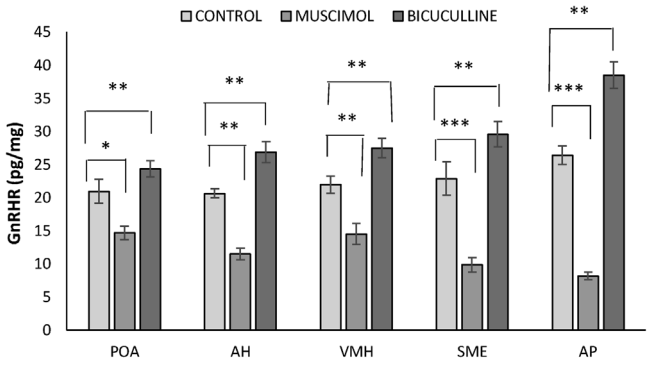

Fig. 2. Effect of muscimol and bicuculline on the level of GnRHR in the preoptic area (POA), anterior hypothalamus (AH), ventromedial hypothalamus (VM), stalk median eminence (SME) and the anterior pituitary gland (AP) of follicular-phase ewes, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Asterisks indicate values that differ significantly from the control group animals. Data are the mean ± SEM, n=6 animals per group (one-way ANOVA).

Effects of muscimol and bicuculline on levels of GnRHR in the POA-hypothalamus and in the AP of follicular-phase ewes

In control animals, GnRHR protein was found in the tissue continuum throughout the POA, AH, VMH, SME, and in the AP (Fig. 2). Administration of muscimol decreased significantly the level of GnRHR in the POA ($p < 0.05$), AH ($p < 0.01$), VMH ($p < 0.05$), SME ($p < 0.001$) and also in the AP ($p < 0.001$). The effect of bicuculline on GnRHR biosynthesis was opposite to those induced by muscimol. GnRHR levels increased significantly in the POA ($p < 0.01$), AH ($p < 0.01$), VMH ($p < 0.01$), SME ($p < 0.01$) as well as in the AP ($p < 0.01$) of bicuculline-treated animals.

Effects of muscimol and bicuculline on the expression of Kiss 1 mRNA in selected hypothalamic structures of follicular-phase ewes

In control ewes, Kiss 1 mRNA was detected in the POA and VMH/ARC (Fig. 3). Both activation and blockade of $GABA_A$ receptors had no significant effect on either Kiss 1 mRNA level in the POA and in the VMH/ARC.

Effects of muscimol and bicuculline on the expression of Kiss1r mRNA in the POA-hypothalamus of follicular-phase ewes

In control animals, Kiss1r mRNA was found at different concentrations in all POA/hypothalamus structures, *i.e.* POA, AH, VMH/ARC and in the SME (Fig. 4).

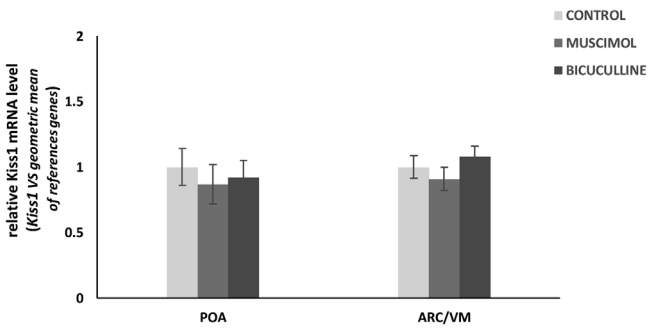

Fig. 3. Effect of muscimol and bicuculline on the level of Kiss1 mRNA in the preoptic area (POA) and the ventromedial hypothalamus including arcuate nucleus (VMH/ARC) of follicular-phase ewes. Data are the mean ± SEM., n = 6 animals per group (*t*-test).

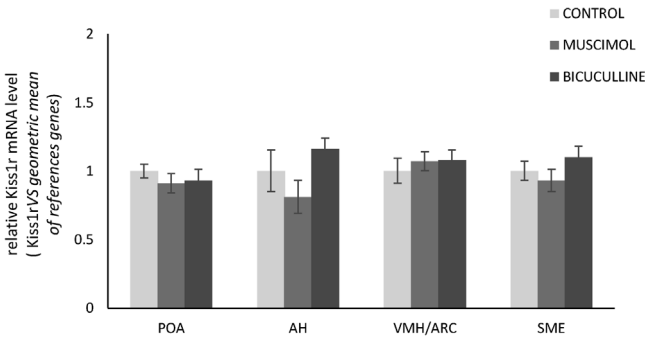

Fig. 4. Effect of muscimol and bicuculline on the level of Kiss1r mRNA in the preoptic area (POA), anterior hypothalamus (AH), ventromedial hypothalamus including arcuate nucleus (VMH/ARC) and the stalk median eminence (SME) of follicular-phase ewes. Data are the mean ± SEM, n = 6 animals per group (*t*-test).

The level of Kiss1r mRNAs in selected POA-hypothalamic areas of either muscimol- and bicuculline-treated ewes remained unaltered.

Effects of muscimol and bicuculline on plasma LH concentrations

Infusion of muscimol into the third cerebral ventricle significantly decreased LH concentrations in the blood plasma ($p < 0.05$), as compared to control values (Fig. 5A). This down-regulation of LH release induced by muscimol administration is probably due to a decrease in LH pulse frequency ($p < 0.05$, Fig. 5B) because the amplitude of LH pulses, despite its upward tendency, did not differ significantly from control (data not shown). In ewes treated with bicuculline, LH secretion and LH pulse frequency increased significantly as compared to control animals ($p < 0.05$, Fig. 5AB). The LH pulse amplitude did not differ significantly between control and bicuculline-treated groups (data not shown).

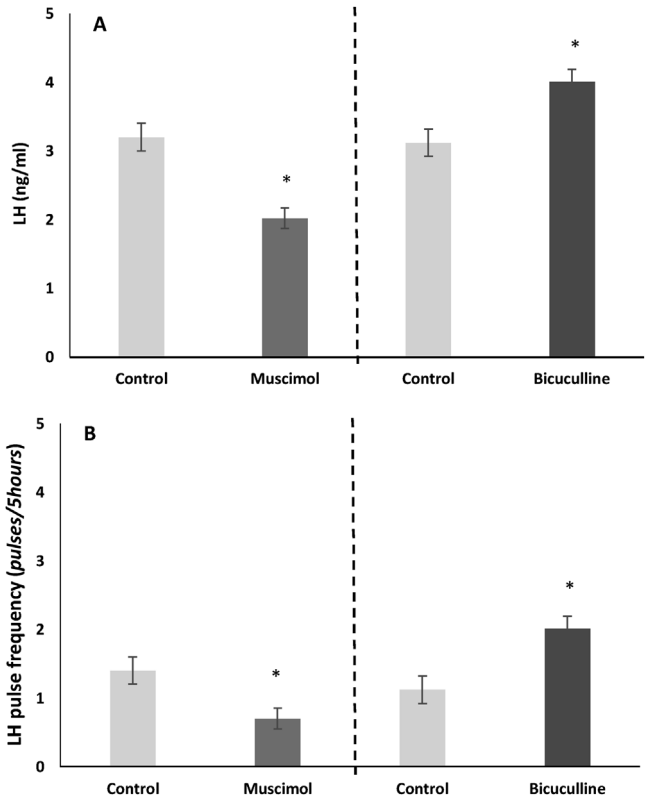

Fig. 5. Effect of muscimol and bicuculline on the level of LH (A) and LH pulse frequency (B) in blood plasma of follicular-phase ewes, $^{*}p < 0.05$. Asterisks indicate values that differ significantly from the control group animals. Data are the mean ± SEM, n=6 animals per group (one-way ANOVA, Wilcoxon test).

Discussion

The inhibitory action of a GABA agonist, muscimol, on the level of mRNA encoding GnRH in the POA-hypothalamus [25] of follicular-phase sheep has suggested that at the hypothalamic level GABA may exert a suppressive effect on subsequent steps of the GnRH biosynthetic pathway. Our current study provides direct support for this suggestion, when similar muscimol-related changes in the level of post-translational product of the *GnRH* gene were determined in sheep. Specifying, our results demonstrated for the first time that muscimol infused into the third cerebral ventricle of follicular-phase sheep decreased levels of GnRH in the POA, AH and VMH, and SME. Thus, the possibility that decapeptide level is exclusively coupled with cytoplasmic GnRH mRNA cannot be excluded, even if studies on mice and rats suggested that GnRH biosynthesis regulated *via* post-transcriptional mechanism(s) into the mature GnRH form is not strictly related with GnRH mRNA levels [45,46]. However, it should be noted that GnRH mRNA amount in various parts of the hypothalamus results not only from the transcriptional activity of *GnRH* gene, but also from the stability of GnRH transcript and its utilization in GnRH biosynthesis. Hence, in some circumstances transcription and translation may be far from having a linear and simple relationship [47]. Since GnRH mRNA was not detected in the SME of sheep in our previous study [25], it is certain that the post-translational product of *GnRH* gene in this structure results from the axonal transport of the hormone from other hypothalamic areas. It is now indisputable that the most important source of hormone conveyed to SME are GnRH neurons located in POA, *i.e.*, the hypothalamic structure where most of GnRH perikarya are located [48] and most of GnRH transcript is synthesized [49,50].

Therefore, it could be suspected that the content of GnRH protein should be definitely higher in the POA in comparison to other hypothalamic structures. Surprisingly, such a dependency was not observed in the present study. One probable reason for these apparently conflicting results may be due to the method (relative measurement *vs* total protein concentration) that we used in the experiment. Furthermore, our studies were conducted on sheep in the late follicular phase of the oestrous cycle, just before the preovulatory GnRH/LH surge, and it is thus probable that in this period the differences in the GnRH content in particular hypothalamic areas of this breed of sheep could be less pronounced. This last opinion seems to be more justified, especially that the level of GnRH in the hypothalamus of follicular-phase sheep is significantly higher as compared to anestrous animals [51].

In the present study, the blockade of $GABA_AR$ induced by bicuculline increased the level of GnRH in the POA-hypothalamus. We previously reported the existence of a similar relationship between the administration of bicuculline and GnRH transcript level in the POA, AH and VMH of follicular-phase sheep [25]. Importantly, the decrease or increase in GnRH amount in the POA-hypothalamus of muscimol- and/or bicuculline-treated ewes, respectively, was associated with similar changes in LH secretion. Although we did not measure the level of GnRH in the hypophyseal portal blood, the diminished LH secretion following muscimol treatment indicates that GABA also suppresses the release of GnRH into the portal circulation. Therefore, it seems that GABA could reduce GnRH/LH release *via* the inhibition of GnRH biosynthesis. Thus, the possibility that GABA-mediated mechanisms controlling *GnRH* gene expression are similar to that governing decapeptide

release cannot be excluded, even if such a point of view is in contrast with the earlier studies in sheep, suggesting that neural mechanism(s) controlling GnRH release is/are distinct from those regulating *GnRH* gene expression [52,53].

Overall, our results indicated the involvement of $GABA_AR$ in controlling GnRH/LH release through mechanism which included GABA action on molecular processes leading to GnRH biosynthesis. This is consistent with previous *in vivo* [14,54] and *in vitro* [55] studies in ewes suggesting that GABA inhibits GnRH release with participation of $GABA_A$, rather than $GABA_B$, receptor mechanism. In support, the present study was carried out on ewes in the breeding season and the inhibition of LH secretion in sheep during this period is strongly controlled by $GABA_AR$ *in vivo* [13]. A study in rats, in which down-regulation of pulsatile LH release induced by stress was inhibited by bicuculline *in vivo* [15] further strengthens the importance of $GABA_AR$ mechanism in GABA-induced suppression of GnRH/LH secretion.

The mode of GABA action *via* the mechanism including $GABA_AR$-mediated effect on molecular processes leading to GnRH biosynthesis is poorly recognized. However, immunohistochemical studies in sheep showing that GABA terminals are in close contact with GnRH neurons in the POA [8] provide an opportunity for a direct action of GABA on the expression of gene encoding *GnRH*. Subsequently, from studies in mice it appears that muscimol and/or bicuculline could inhibit or activate *GnRH* gene expression directly through $GABA_AR$ located on GnRH cells [56,57]. The question remains whether GnRH cells in ewes, just as it is in mice, contain $GABA_AR$. Nonetheless, we cannot ignore the possibility of indirect GABA action *via* this class of receptor molecules which are expressed in other neuronal systems in the hypothalamus having a synaptic contact with the GnRH neurons. From studies in rats and ewes, it appears that such intermediary function may be performed by noradrenergic, dopaminergic and β-endorphinergic neurons [11,22,58]. However, further investigations are needed to clarify this point.

In the present study, both activation and blockade of $GABA_AR$ had no evident effect on the levels of mRNAs encoding *Kiss 1* and *Kiss1r* in hypothalamus. However, functional interconnections between inhibitory GABAergic and stimulatory kisspeptidergic inputs appears to govern pronounced changes in the pattern of GnRH/LH secretion in other species. Indeed, in the female rhesus monkey, administration of bicuculline during the prepubertal period, but not during the pubertal period, stimulated kisspeptin release, and the bicuculline-induced GnRH secretion was blocked by the kisspeptin antagonist, peptide 234 [29]. Furthermore, an *in vitro* study in rats indicated that the activation of $GABA_A$ R by muscimol can increase *Kiss 1* gene expression in kisspeptin-producing cell model [59]. There was also an inverse relationship in mice suggesting that GABAergic cells mediate the effect of kisspeptin on inhibition of GnRH secretion [28]. Although it is premature to exclude GABA action *via* kisspeptin in sheep, our mRNAs expression data did not confirm the dogma that kisspeptin is a mandatory intermediate for GABA-induced changes in GnRH release. Given that there are many processes between Kiss 1 mRNA and mature peptide, and also Kiss 1 stability is an important factor, we were taking into account that transcription and translation might be far from having a simple relationship. It must be emphasized that due to lack of information on transcript degradation or utilization in biosynthetic processes, it is now impossible to define the relationship between the post-transcriptional and post-translational products of *Kiss 1* and *Kiss1r* genes.

Limited data on rats suggested that changes in GABA-ergic neurotransmission affect *GnRH* gene expression in a different manner, depending on the mode of agonist administration. For example, chronic activation of $GABA_AR$ by muscimol decreased GnRH transcript level [32], whereas acute treatment with low, but not high, doses of this drug increased GnRH mRNA [31]. From these conflicting results, it is impossible to clarify the mode of GABA action on *GnRH* gene expression. A study in mice suggested that the inconsistency between the results might be attributable to the heterogeneity of GnRH neurons in their response to GABA depending on GnRH cells location in hypothalamus [60,61]. Another possible explanation for the contradictory data may be the assumption that $GABA_AR$ exist in numerous stimulatory and inhibitory hypothalamic neuronal systems with respect to the control of GnRH expression. Furthermore, the discordance between transcript and protein expression levels should also be considered.

In the present study, administration of both muscimol and bicuculline also affected the biosynthesis of GnRHR. A significant decrease in GnRHR level in the POA, AH, VMH and SME following muscimol treatment as well as the opposite effect induced by bicuculline indicates that *GnRHR* gene expression is, as in the case of the ligand, under GABA inhibitory control. The neuroendocrine processes that lead to decreased GnRHR levels in the POA-hypothalamic structures of sheep in response to muscimol with concomitant decrease in GnRHR mRNA [25] in the POA-hypothalamus are unknown. However, it cannot be excluded that the subpopulations of GnRHR-expressing neurons in various parts of the hypothalamus belong to different neuronal systems and are innervated by different inhibitory and/or disinhibitory afferents which specifically act on *GnRHR* gene transcription and/or post-transcriptional steps of GnRHR biosynthetic pathway. Hence, the final effect of all these events on GnRH biosynthesis may be determined by the net results of the inhibition and/or disinhibition of all these afferents on GnRHR-expressing neurons. The precise role of hypothalamic GnRHR in the control of gonadotropin secretion is yet to be determined. The question remains as to what the regulatory function is for muscimol-induced changes in GnRHR levels in particular in POA-hypothalamic areas. Studies in rats [33,62,63] and sheep [64] provided some evidence to believe that GnRHR in the VMH may participate in the regulation of GnRH secretion from the axon terminals of GnRH neurons in the SME. According to this, the activation of GnRHR in VMH exerts an inhibitory effect on GnRH release. Our current data potentially eliminated a critical role for the VMH population of GnRHR in GABA-induced suppression of GnRH release. This possibility has also been eliminated when highest levels of hypothalamic GnRHR during the estrous cycle *vs.* anestrous period were determined in sheep [51]. Since biosynthesis of GnRHR has not been switched-down in the hypothalamus of follicular-phase ewes, it seems that GnRHRs in this structure are not critical for season-mediated control of GnRH release. However, the mechanisms of stress-induced suppression of GnRH release may involve an active GnRHR role in the VMH [64].

The physiological significance of GnRHR has been well recognized in the AP and muscimol-induced down-regulation of GnRHR amount in this gland is not surprising. Indeed, it is well documented in rats and sheep that small doses of GnRH applied in a physiological pulsatile manner into the pituitary gland have a stimulatory effect on *GnRHR* gene expression [65,66], GnRHR activity [67,68], biosynthesis of LHβ subunit [69,70] and LH secretion [45]. Hence, the decrease and increase in GnRHR level in the AP of muscimol- or bicuclline-treated ewes, respectively, results mainly from changes in the hypothalamic GnRH release. On the other hand, a decrease in the level of LH in peripheral blood after muscimol administration may be due to diminished GnRHR activity in the AP, as a consequence of low secretory activity of GnRH cells.

In conclusion, the present study emphasizes the significance of $GABA_AR$ in controlling GnRH/LH secretion. It provides strong support for the assumption that GABA-induced suppression of

GnRH/LH release in the hypothalamic-pituitary unit of follicular-phase sheep occurs by inhibition of molecular processes governing the biosynthesis of GnRH and GnRHR in hypothalamus as well as GnRHR in the AP. Lack of changes in the expression of mRNAs encoding *Kiss 1* and *Kiss1r* in the POA-hypothalamus of muscimol- and bicuculline- treated ewes potentially excludes the participation of Kiss1/Kiss1r signaling in $GABA_AR$-mediated control of GnRH and GnRHR biosynthesis. However, further work is required to clarify this point and to precisely determine the mechanisms of GABA action on GnRH system activity. It should be also emphasized that our data are still preliminary and need to be verified by further experiments in which the relationship between primary transcript and post-translational products of *Kiss 1* and *Kiss1r* genes should first be determined.

Conflict of interest

All authors declare no conflict of interest.

Funding

This work was supported by grant National Science Centre No UMO-2012/05/B/NZ4/02443.

Author contributions

M.O. Ciechanowska: Conceptualization, Data curation, Investigation, Methodology, Writing - original draft. M. Łapot: Formal analysis, Investigation, Methodology. M. Kowalczyk: Writing - review and editing. T. Malewski: Methodology. M. Brytan: Visualization. B. Antkowiak: Visualization. F. Przekop: Investigation, Methodology.

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