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Effect of central administration of indomethacin on anandamide-induced GnRH/LH secretion in the hypothalamus of anoestrous ewes

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Abstract

Introduction: It is suggested that cannabinoids (CBs) may disturb reproduction through action on hypothalamic gonadotropin-releasing hormone (GnRH) neurons directly or indirectly through intermediates such as prostaglandins. The study aimed to determine the influence of intracerebroventricular (i.c.v.) injection of the endogenous cannabinoid anandamide (N-arachidonoylethanolamine – AEA), alone or with the prostaglandin synthesis inhibitor indomethacin (IND), on GnRH/luteinising hormone (LH) secretion. The purpose of the research was to clarify the role of endocannabinoids and their interaction with prostaglandins in the regulation of reproduction at the level of the hypothalamus and pituitary in anoestrous sheep. **Material and Methods**: The study was performed on 24 anoestrous ewes divided into four experimental groups: a control group receiving i.c.v. injection of Ringer–Locke solution, an AEA group that received i.c.v. injection of 30 μM of AEA, an IND group receiving i.c.v. injection of 5 μM of IND and an AEA + IND group that received i.c.v. injections of 30 μM of AEA and 5 μM of IND. **Results:** Anandamide stimulated GnRH protein and gene expression in the median eminence and protein expression in the preoptic area without influencing GnRH messenger RNA (mRNA) in this structure. Indomethacin reversed the changes in GnRH secretion after AEA administration. It was also found that AEA stimulated LH mRNA in the pituitary without influencing LH release. **Conclusion:** Our results support the role of endogenous cannabinoids in the regulation of reproductive processes at the central nervous system level. They may act directly on the hypothalamic GnRH neurons or indirectly through intermediates such as prostaglandins.

Keywords: anandamide, indomethacin, gonadotropin-releasing hormone, luteinising hormone.

Introduction

It is well recognised that tetrahydrocannabinol (THC) influences many reproductive functions in both females and males (37). The presence of endogenous receptors for THC in vertebrates and the availability of highly selective and potent agonists of these receptors led to the identification of a naturally occurring lipid-signalling system termed the endocannabinoid system. Endocannabinoids (ECBs) are lipid mediators isolated from brain and peripheral tissues that include amides, esters and ethers of long-chain polyunsaturated fatty acids (2). The best-known ECBs are anandamide (N-arachidonoylethanolamide – AEA) and 2-arachidonoylglycerol (2-AG). Endocannabinoids can activate a wide range of cannabinoid receptors (CBRs), among them cannabinoid receptor type 1 (CB1R) cloned by Matsuda in 1990 and cannabinoid receptor type 2 (CB2R) identified by Munro in 1993 (20). Cannabinoid receptor type 1 was mainly expressed in the central nervous system (CNS) (41), while expression of CB2R was detected mainly in immune cells. However, CB2R expression was also found in neuronal microglia cells, brain stem cells, the cerebellum, striatum, midbrain and hippocampus (41). There is evidence for the presence of other CBRs that work differently to CB1R and CB2R, such as G protein-coupled receptors 55 and 119 and type 1 vanilloid receptor (28). Anandamide binds to CB1R in the brain with high affinity and mimics the behavioural actions of the exogenous cannabinoid THC when injected into rodents, while 2-AG has similar affinities for CB1R and CB2R receptors, which are comparable to but productive of more efficient bonds than those of anandamide (41). Endocannabinoids and their receptors have been identified in the central and peripheral

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nervous system as well as in gonads and gametes, suggesting the involvement of this system in the control of reproductive function, at both the central and local levels (2). It was shown in human marijuana smokers as well as in animal models that cannabinoids (CBs) and ECBs impair gonadotropin-releasing hormone (GnRH) and luteinising hormone (LH) secretion. In females, chronic CB exposure delays sexual maturation, disrupts the menstrual or oestrous cycle, depresses follicular maturation in the ovary, alters uterine and vaginal cytology, and may reduce the contents of LH and sex steroids in blood (36). It was shown in a study on female rats that acute THC exposure inhibited pulsatile LH secretion (35) and also blocked oestrogen or oestrogen/progesterone-mediated positive feedback on the LH surge in ovariectomised rats (29). It was also shown that in rats that AEA inhibited GnRH release (26) and GnRH transcription (6). It was suggested that the principal site of action of CBs and ECBs to inhibit reproductive processes is the hypothalamus, but that direct action at the level of the pituitary cannot be excluded (34). It is worth mentioning that ECB binding sites and the expression of enzymes which biosynthesise and hydrolyse CBs were reported in the hypothalamus and pituitary (12). In the present authors' earlier study on ewes (34), expression of the *CB1R* gene was determined both in the hypothalamus and pituitary gland. In that study, in an *ex vivo* experiment conducted on pituitary explants from anoestrous ewes, AEA inhibited LH and follicle-stimulating hormone (FSH) secretion (34).

Cannabinoids may influence reproduction through action on hypothalamic GnRH neurons directly or indirectly through intermediates. In ewes, regulation of GnRH/LH secretion is complex and involves many factors such as steroid hormones, γ-aminobutyric acid and biogenic amines, kisspeptin, neuropeptide Y and melatonin. Additionally the mechanism regulating GnRH/LH secretion regulation is a different one during the oestrous cycle to the mechanism in the anoestrous season. Endocannabinoids could be considered some of the factors influencing reproductive functions. The mechanism through which ECBs exert effect is not well recognised; however, it is suggested that prostaglandins may play an important role in this interaction. It is known that AEA metabolism produces arachidonic acid (AA) as a main metabolite. Arachidonic acid is a polyunsaturated fatty acid that is present in the phospholipids of cell membranes and is abundant in the brain (11). Prostaglandins, thromboxane and leukotrienes are mediators synthesised *de novo* through enzyme actions on AA. Previous studies showed that exogenous AA exerted several physiological and pharmacological effects by increasing synthesis of prostaglandins (4). The most abundant prostaglandin, E2 (PGE2), is formed as a result of the conversion of AA (19) and was observed to be an important upregulator of GnRH secretion (24).

In the present study, the hypothesis that AEA can influence GnRH/LH secretion involving prostaglandins at the hypothalamic level was tested. The research aimed to determine the influence of intracerebroventricular (i.c.v.) injection of AEA alone or with the prostaglandin synthesis inhibitor indomethacin (IND) on GnRH/LH secretion. The influence of the studied substances on the *GnRH* gene and GnRH protein expression was determined as well as their influence on the *LH* gene expression and LH release. The purpose of the research was to clarify the role of endocannabinoids and their interaction with prostaglandins in the regulation of reproduction at the level of the hypothalamus and pituitary in anoestrous sheep. Taking into account that the expression pattern of cannabinoid receptors is characterised by diurnal changes and that pharmacological alteration of CBR signalling affects processes regulated in a cyclical, circadian or diurnal manner, it would seem reasonable to undertake studies on animals active in the daytime. Most studies concerning the influence of cannabinoids were undertaken on rodents. These animals being active at night, they would not appear to be a good model for the investigation of CB influence on primate, and especially human, organisms. The results obtained in rats *in vivo* or from their explants *in vitro* would not be translatable directly to other species. In anoestrous ewes, the circulating level of oestrogen is low. This obviates the need for ovariectomisation and makes possible observation of the changes in the activity of neuroendocrine system without the hindering influence of diurnal and cyclical changes in the oestrogen secretion.

Material and Methods

Animals. The study was performed on 24 three-year-old adult Blackhead ewes in the anoestrous season (April–May). Anoestrus was confirmed by very low LH concentrations in plasma during the experiment as well by heat testing by a vasectomised ram, and only animals which had not exhibited regular cycles for at least three weeks before the experiment were used. The animals were kept indoors in individual pens, yet exposed to natural daylight. The ewes' body condition scores were kept at an estimated 3 points or higher on a 5-point scale. The ewes were well adapted to the experimental conditions and always had visual contact with their neighbours during the experimental period to prevent the stress of social isolation. The animals were fed a constant diet of commercial concentrates with hay and water available *ad libitum*. One month before the start of the experiment, stainless steel guide cannulae of 1.2 mm outer diameter were placed into the third ventricle of the brain under stereotaxic control according to a method described elsewhere (15). All procedures were performed with the consent of the Local Ethics Committee of Warsaw University of Life Sciences – SGGW, given in resolution no. 18/2014.

Experimental procedure. The animals (n = 24) were randomly assigned to four experimental groups: a control group (n = 6) that received an i.c.v. injection of

Ringer–Locke solution (RLs), an AEA group (n = 6) that received an i.c.v. injection of 30 μM/animal of AEA solution, an IND group (n = 6) that received an i.c.v. injection of 5 μM/animal of IND solution, and an AEA + IND group (n = 6) that received i.c.v. injections of 30 μM/animal of AEA and 5 μM/animal of IND solutions. The dose was chosen on the basis of a preliminary study with doses of AEA of 1 μM, 10 μM, 30 μM and 100 μM, in which the dose of 30 μM was found optimal taking into account LH release and the behaviour of the animals. Jugular blood samples were taken from each ewe for LH measurement at 15-min intervals, beginning 2 h before and continuing until 3 h after i.c.v. administration of the cannabinoids or the control solution. The experiment started at 9 am and finished at 2 pm. The animals were euthanised immediately after the experiment (3 h after i.c.v. administration of cannabinoids or only their vehicle, the Ringer–Locke solution), the brains were rapidly removed from the skulls and then the chosen hypothalamic structures, namely the preoptic area (POA), median eminence (ME) and anterior pituitary (AP), were dissected. All tissues were frozen immediately after collection in liquid nitrogen and stored at −80°C until assay.

Radioimmunoassay to determine plasma LH concentration. The LH concentration in the plasma was estimated with a double-antibody radioimmunoassay using anti-ovine-LH antibodies and standards (US National Hormone and Pituitary Program and Dr. A.F. Parlow of Harbor-UCLA Research and Education Institute c/o Los Angeles Biomedical Research Institute, Los Angeles, CA, USA) and anti-rabbit-ɤ-globulin antisera (Sigma-Aldrich, St. Louis, MO, USA) according to the method of Stupnicki and Madej (30). The assay sensitivity was 0.3 ng/mL and the intra- and inter-assay coefficients of variation were 8% and 11%, respectively.

ELISA assay for the GnRH concentration in the POA and ME. The hypothalamic tissues were homogenised in 400 μL of phosphate-buffered saline (0.02 mol), and the concentrations of GnRH in the POA and ME were determined with a commercial ELISA kit purpose-made for the measurement of sheep GnRH (CUSABIO BIOTECH Co., Wuhan, China). All steps in the assays were performed according to the manufacturer's instructions and as described by Herman *et al.* (17). The assay sensitivity was 1.0 pg/mL. Gonadotropin-releasing hormone concentrations were normalised against the total protein content in each sample and assayed using the Bradford method (5).

Relative gene expression assays. The POA and ME tissues were homogenised in liquid nitrogen, and the total RNA was extracted using a NucleoSpin RNA kit (MACHEREY-NAGEL, Düren, Germany) according to the manufacturer's instructions. Complimentary DNA (cDNA) was synthesised and real-time PCRs were carried out according to the method earlier described by Wojtulewicz *et al.* (38). A 2-μg mass of total RNA was used as the starting material for the synthesis of cDNA by reverse transcription. The real-time PCR reactions were carried out using a Rotor-Gene Q instrument (Qiagen, Hilden, Germany), HOT FIREPol EvaGreen quantitative PCR Mix Plus (Solis BioDyne, Tartu, Estonia) and the primers (Genomed, Warszawa, Poland) listed in Table 1.

Table 1. Specific primers used in real-time PCR for determining the expression of housekeeping genes and genes of interest

	GenBank accession No.	Gene	Amplicon size (base pairs)	Forward/reverse	Sequence 5′→3′	Reference
Housekeeping genes	NM_001034034	*GAPDH* glyceraldehyde-3-phosphate dehydrogenase	134	forward	AGAAGGCTGGGGCTCACT	(31)
				reverse	GGCATTGCTGACAATCTTGA	
	U39357	*ACTB* β-actin	168	forward	CTTCCTTCCTGGGCATGG	
				reverse	GGGCAGTGATCTCTTTCTGC	
	BC108088.1	*HDAC1* histone deacetylase1	115	forward	CTGGGGACCTACGGGATATT	
				reverse	GACATGACCGGCTTGAAAAT	
Genes of interest	NM_001009397	*GnRHR* gonadotropin-releasing hormone receptor	150	forward	TCTTTGCTGGACCACAGTTAT	(14)
				reverse	GGCAGCTGAAGGTGAAAAAG	
	U02517	*GnRH* gonadotropin-releasing hormone	123	forward	GCCCTGGAGGAAAGAGAAAT	
				reverse	GAGGAGAATGGGACTGGTGA	
	X52488	*LHB* luteinising hormone β-subunit	184	forward	AGATGCTCCAGGGACTGCT	
				reverse	TGCTTCATGCTGAGGCAGTA	

The relative gene expression was calculated using the comparative quantification option (23) in Rotor-Gene Q software (Qiagen) measuring against the mean expression of three housekeeping genes: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), β-actin (*ACTB*) and histone deacetylase 1 (*HDAC1*). Data for a specific gene's expression were normalised to the average relative level of that gene's mRNA expression in the control group, which was set to 1.0, in the POA and ME separately.

Statistical data analysis. The statistical analyses were performed using Statistica software (StatSoft, Tulsa, OK, USA). The blood hormone concentrations after the treatment period (from 1 to 3 h after i.c.v. administration), GnRH content in the ME and POA and expression levels of all examined genes were subjected to two-way analysis of variance and a post-hoc Fisher's least significance test comparing groups. The results are presented as the mean ± standard error of the mean, and statistical significance was set at P-value ≤ 0.05.

Results

Effect of central injection of AEA, IND and AEA + IND on the expression of the *GnRH* and *GnRHR* genes in the POA and ME. Central injection of AEA increased *GnRH* gene expression in the ME significantly (P-value ≤ 0.05) (Fig. 1B), but no effect was observed in the POA (Fig. 1A). Injection of IND alone did not influence *GnRH* gene expression in the studied hypothalamic structures. In the ME, the administration of IND together with AEA reduced the AEA-mediated stimulation of *GnRH* gene expression from that in the AEA group significantly (P-value ≤ 0.05) (Fig. 1B). Expression of the *GnRHR* gene in the ME was significantly increased (P-value ≤ 0.05) in the AEA and AEA + IND groups compared with the control group. Indomethacin alone did not influence *GnRHR* gene expression in this structure (Fig. 2A). In the AP no effect of administration of AEA or IND was observed on the level of GnRHR mRNA (Fig. 2B). Only simultaneous administration of AEA and IND increased the GnRHR mRNA level, doing so significantly (P-value ≤ 0.05) compared with the control group level.

Effect of central injection of AEA, IND, and AEA + IND on the GnRH peptide concentration in the POA and ME. Intracerebroventricular administration of AEA significantly increased (P-value ≤ 0.01) the GnRH peptide concentration in both the POA and ME (Figs 3A and 3B). The injection of IND, when with AEA, significantly reduced (P-value ≤ 0.01) AEA-mediated stimulation of the GnRH peptide concentration (Figs 3A and 3B).

Effect of central injection of IND and AEA on LH secretion in the AP. Central injection of AEA and AEA + IND did not reduce LH release in the AP compared with the control animals' LH level, while administration of IND alone decreased it (P-value ≤ 0.05) in this tissue of the experimental anoestrous ewes (Fig. 4A). However, i.c.v. injection of AEA alone as well with IND stimulated (P-value ≤ 0.05) *LH*β gene expression (Fig. 4B). Indomethacin administered alone did not influence LH mRNA.

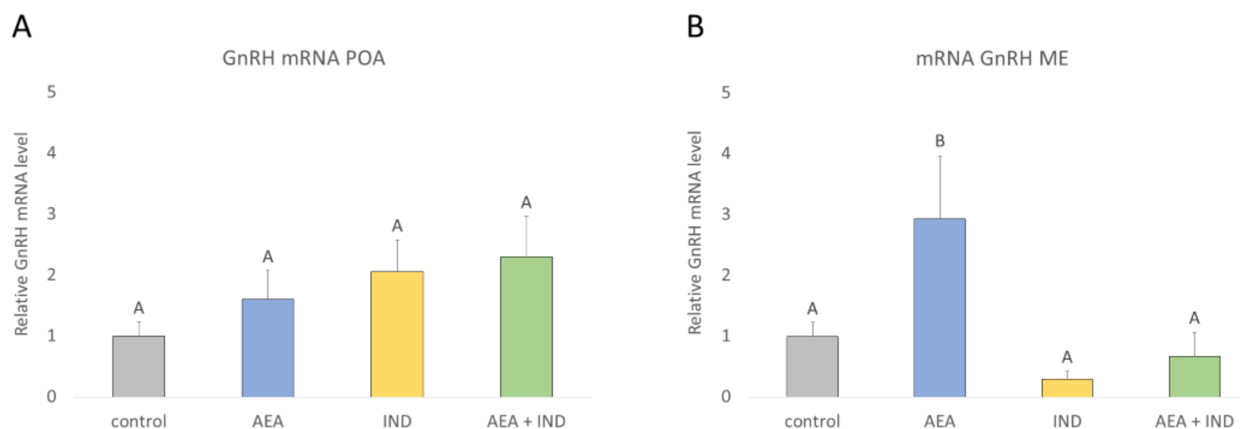

Fig. 1. Effect of intracerebroventricular injection of anandamide (AEA) indomethacin (IND) and AEA + IND on the expression of the gonadotropin-releasing hormone (*GnRH*) gene in the preoptic area (POA – A) and median eminence (ME – B) of anoestrous ewes. Data are presented as the mean ± standard error of the mean. The results were analysed using two-way analysis of variance with a post-hoc Fisher's least significance test (P-value ≤ 0.05)

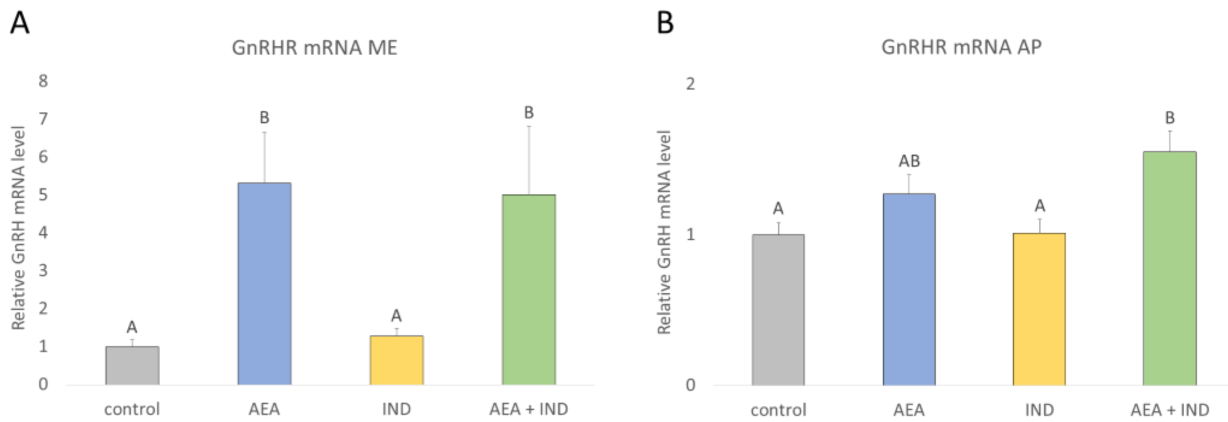

Fig. 2. Effect of intracerebroventricular injection of anandamide (AEA) indomethacin (IND) and AEA + IND on the gene expression of gonadotropin-releasing hormone receptor (GnRHR) in the median eminence (ME – A) and in the anterior pituitary (AP – B) of anoestrous ewes. The data are presented as the mean ± standard error of the mean. The results were analysed using two-way analysis of variance with a post-hoc Fisher's least significance test (P-value ≤ 0.05)

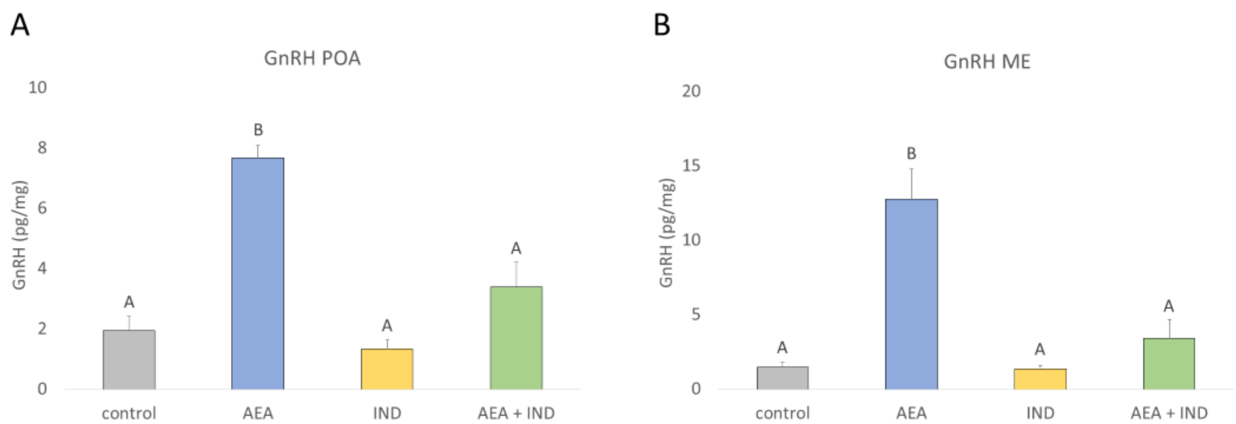

Fig. 3. Effect of intracerebroventricular injection of anandamide (AEA) indomethacin (IND) and AEA + IND on the content of gonadotropin-releasing hormone (GnRH) in the preoptic area (POA – A) and median eminence (ME – B) of anoestrous ewes. The data are presented as the mean ± standard error of the mean. The results were analysed using two-way analysis of variance with a post-hoc Fisher's least significance test (P-value ≤ 0.01)

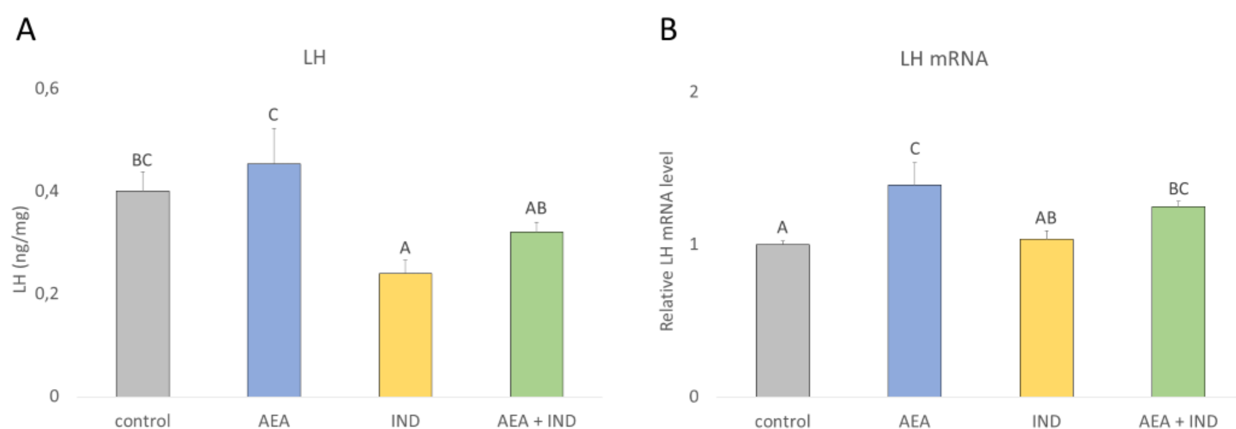

Fig. 4. Effect of intracerebroventricular injection of anandamide (AEA), indomethacin (IND) and AEA + IND on the plasma concentration of luteinising hormone (LH –A) and on the expression of the *LH*β gene (B) in the anterior pituitary (AP) of anoestrous ewes. The data are presented as the mean ± standard error of the mean. The results were analysed using two-way analysis of variance with a post-hoc Fisher's least significance test (P-value ≤ 0.05)

Discussion

The present study demonstrates that the prostaglandin synthesis inhibitor indomethacin can effectively reverse the effect of anandamide on GnRH secretion in anoestrous ewes. Anandamide alone injected i.c.v. at a dose of 30 μM increased *GnRH* gene expression in the ME but not in the POA. Interestingly, the stimulatory effect of AEA on the GnRH peptide concentration was observed in both the ME and the POA. In studies mainly conducted on rats, CBs and ECBs usually exerted inhibitory influence on the reproductive axis. Scorticati *et al.* (26) showed that AEA inhibited GnRH release from the mediobasal hypothalamus (MBH) in male rats but had no effect on the hypothalamic GnRH release in ovariectomised (OVX) rats. However, *in vitro* experiments on the MBH of ovariectomised and oestrogen-supplemented (OVX-E) rats found that AEA stimulated GnRH release, while in this tissue in OVX rats AEA did not. The finding indicated that oestrogen reverses the inhibitory action of AEA on the secretion of GnRH in female rats. It was demonstrated that AEA influenced the release of GABA – a neurotransmitter important in the regulation of GnRH secretion. In *in vitro* experiments on the MBH of male rats, Fernandes-Solari *et al.* (10) demonstrated that incubation of this structure with AEA at a concentration of 10^{-9} M significantly increased GABA secretion. It was also reported that CB1R was expressed in a subpopulation of GABAergic interneurons that also express cholecystokinin in the hippocampus and neocortex (22). Generally, GABA is involved in the inhibition of GnRH secretion, thus stimulation of GABA release could lead to a decrease in the secretion of GnRH. In ewes, GABA(A) receptors play an important role in the control of GnRH secretion, but different neural mechanisms could be involved in the suppression of GnRH release by GABA. This process could operate through β-endorphinergic and catecholaminergic systems. The influence of GABA on these neural systems' activity depends on the physiological state of animals which release or receive it (32, 33). It was noted that cannabinoids influenced the activity of catecholaminergic and serotoninergic systems, which are involved in the regulation of GnRH secretion at the hypothalamic level. Experiments on mice established that i.c.v-injected AEA increased the content of hypothalamic norepinephrine (13), this hormone being one well known to increase GnRH secretion. It was also demonstrated that the dopaminergic system in the hypothalamus plays an important role in the regulation of GnRH secretion. In ewes, this system has a predominantly inhibitory effect on GnRH release in the non-breeding season, but does not in the breeding season. The most likely location of dopaminergic cells that inhibit GnRH secretion during anoestrus in ewes is the A15 cell group (7). Dopaminergic neurons of this group are involved in oestradiol-mediated feedback action on GnRH secretion during anoestrus. Scorticati *et al.* (27) demonstrated that cannabinoids can affect brain dopamine (DA) concentration and DA receptor expression, and that sex steroids may modulate the density and affinity of CB1R in certain brain areas. This study demonstrated that AEA increased the synthesis and release of DA from the MBH explants. The 3,4-dihydroxyphenylacetic acid : DA ratio in the MBH was also increased, which indicates the increased turnover of DA. The influence of AEA on dopaminergic neuron activity was different in male rats to its influence in female OVX and OVX-E rats (27). These findings suggest that oestrogen induces the release of AEA from AEA neurons in the MBH, which acts on AEA receptors on tuberoinfundibular dopamine neurons to inhibit the release of DA. This hypothesis is confirmed by the finding that CBR1 receptors are colocalised on the cell bodies of periventricular tyrosine hydroxylase–containing neurons, DA neurons that are known to project to the ME, where DA can influence GnRH release (27). Summarising, the observed effect of AEA on GnRH secretion in our experiment is the result of complex interactions between the endocannabinoid system and various neurotransmitters such as catecholamines and GABA. It is also undoubtedly important that the sheep were in the anoestrous season because oestrogen levels influence the effect of AEA on GnRH secretion.

In our study, we observed that i.c.v. administration of IND together with AEA reduced the stimulation of GnRH peptide expression by AEA in both the POA and ME, while it upregulated *GnRH* gene expression only in the ME. Prostaglandins including PGE2 are produced from AA, which is the main metabolite of AEA degradation, and so the inhibition of prostaglandin synthesis may lead to an increase in AA concentration. In the pituitary and hypothalamus, AA and its metabolites regulate the secretion of many peptide hormones. In some studies, it was shown that centrally injected phospholipase A2 activator melittin, AA and thromboxane A2 can activate the catecholaminergic, vasopressinergic and renin-angiotensin system (1, 39). These neuropeptides can modulate GnRH/LH secretion. It was also shown that i.c.v-administered AA increased plasma FSH, LH and testosterone levels in conscious male rats (9). Thus inhibition of prostaglandin synthesis leads to an increased concentration of these eicosanoids in studied structures. On the other hand, experiments also indicated that PGE2 was an important upregulator of GnRH secretion (24). It is known that prostaglandins could influence GnRH secretion directly at the GnRH nerve terminal or could be involved in the regulation of activity of the other neurotransmitter systems influencing GnRH secretion. Moreover, it was shown that GnRH neurons express PGE2 receptor mRNA and protein (8). Ojeda *et al.* (21) showed that prostaglandins are involved in the regulation of GnRH secretion in the hypothalamus. In a study on OVX rats, they showed that PGE2 injected into the POA or MBH induced the secretion of LH. Also, through *in vitro* studies using fragments of the MBH, Bigdeli and Snyder (3) demonstrated that PGE2 stimulated GnRH secretion, while Ojeda *et al.* (21) noted that inhibition of PGE2 synthesis suppressed catecholamine-

induced GnRH secretion. To sum up, the negation of AEA-dependent stimulation of GnRH expression by administration of IND may result from the increase of the AA concentration or the inhibition of PGE2 synthesis.

Unexpectedly, in our study, we did not find any effect of AEA injection on the LH concentration. We only observed that i.c.v. administration of AEA stimulated LH mRNA in the AP without influencing the release of this hormone. Our results differ from those of most studies, which were performed on rats. It was shown that exogenous cannabinoids such as THC as well as endogenous ones such as AEA decreased gonadotropin release. Chronic administration of CBs to prepubertal female rats was observed to reduce the contents of LH and sex steroids in serum (36). Research also found that acute THC administration inhibited pulsatile LH secretion and also blocked oestrogen- or oestrogen/progesterone-mediated positive feedback on the LH surge in ovariectomised rats (29). In our experiment on AP explants from anoestrous ewes, we also demonstrated the inhibitory influence of AEA on the secretion of LH (34). However, in that experiment, which was *in vitro*, we observed the direct influence of AEA on the explanted AP cells. In the present experiment, which was *in vivo*, i.c.v. injection of AEA stimulated GnRH secretion, so the effect of our manipulation on LH is probably the effect of GnRH stimulation of LH synthesis, which has been expressed as stimulation of *LH* gene expression. The lack of stimulation of LH release could be caused by too short a blood collection time. Administration of IND did not reverse the stimulatory effect of AEA on *LH* gene expression. We only observed that IND given alone inhibited the release of LH, which is not surprising taking into account that experimental evidence indicated that the administration of prostaglandins can evoke the release of gonadotropic hormones (LH and FSH) in the AP (21).

We found that AEA alone as well as with IND increased *GnRHR* gene expression in the ME, but we did not observe such effects in the POA or AP. In our earlier study on the AP explants, we demonstrated that AEA could inhibit LH secretion by *GnRHR* downregulation (34). However, in the current experiment, we did not determine any AEA effect on the release of LH. We only observed an increase in the expression of LH mRNA in the AP, which is not surprising taking into account that AEA stimulated GnRH synthesis in the POA and ME, and GnRH release in the ME. The obtained results suggest that AEA affects GnRH synthesis rather by influencing it in the post-transcriptional stages. It is worth mentioning that previous experiments estimated the ratio of GnRH nuclear mRNA to GnRH cytoplasmic mRNA at 1:2.5 and 1:1.5 (18, 40). A greater amount of nuclear transcript than cytoplasmic mRNA provides a steady flow of GnRH mRNA to the cytoplasm; therefore, the reduction in the amount of GnRH mRNA in the perikaryons is considered rather to be dependent on this mRNA turnover, as both rapid accumulation and as fast degradation (16). In contrast, increased stability of cytoplasmic GnRH mRNA could result in increased synthesis of this neurohormone in the hypothalamus. This assumption may also be supported by the GnRH mRNA level in the ME, the part of the hypothalamus where only GnRH nerve terminals are located. Axonal transport of mRNA from cell bodies to nerve terminals has been described in the number of neurons, and recent evidence suggests that many neuronal proteins are synthesised in axons (25). This mRNA is transported and stored in the nerve terminals as a part of nucleoprotein structure in the form of a granule. The granule represents a complete translational unit that allows protein biosynthesis in the distal part of the neuron (19). Our finding that AEA administration caused a great increase in the GnRH mRNA content in the ME suggests that AEA induced mechanisms increasing the stability of this mRNA. Our study also showed that i.c.v. AEA injection increased the level of mRNA encoding GnRHR in the ME, the part of the hypothalamus where GnRH nerve terminals are located. This increase in *GnRHR* gene expression in the ME could simply result from the increased release of GnRH into the portal blood system and an increase in the feedback stimulation of this structure, because GnRH is known as one of the most potent stimulators of its receptor expression.

Conclusion

Our *in vivo* experiment on anoestrous ewes showed that the endogenous cannabinoid AEA stimulated GnRH protein and gene expression in the ME and protein expression in the POA without influencing mRNA GnRH in this structure. It was also found that i.c.v-injected AEA stimulated LH mRNA without influencing LH release. The influence of i.c.v-injected AEA on LH secretion is probably caused by changes in GnRH neuron activity. Indomethacin, the inhibitor of prostaglandin synthesis, reversed the changes in GnRH secretion after AEA administration. Our results support the role of endogenous cannabinoids in the regulation of reproductive processes at the CNS level. They could act directly on the hypothalamic GnRH neurons or indirectly through prostaglandins, which may play an important role in this interaction. However, the exact role of prostaglandins in these processes is not clear and requires further research. The participation of other neural systems such as those in which catecholamines or GABA are implicated cannot be excluded. Taking into account that the inhibition of prostaglandin synthesis from AA, the main metabolite of AEA, could increase the AA concentration in the studied structures, the mediation of the effect of AEA on GnRH/LH secretion by AA must be regarded as a possibility.

In recent years the need to perform research on non-rodent animal models has been increasingly emphasised. Sheep are a long-lived and widely adopted animal model lending itself to investigation of the regulation of

neuroendocrine processes. The increasing interest of the scientific community in research performed on animals other than laboratory rodents should be also underscored. Rodents may be possibilities among the alternative research models for testing which are to be used before clinical trials are undertaken, which is a recommendation of the Food and Drug Administration of US Department of Health and Human Services for all new drugs which are planned for therapeutic application in human medicine. A contribution to the exploration of cannabinoids for medical use may be constituted by the presented results of this alternative-research-model study, which enable the role of the endocannabinoid system in the regulation of reproductive functions to be better understood.

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Animal Rights Statement: All procedures were performed with the consent of the Local Ethics Committee of Warsaw University of Life Sciences – SGGW, resolution no. 18/2014 in adherence to all the methods and guidelines of the Polish parliamentary Act of 15 January, 2015 on the protection of animals used for scientific or educational purposes, and to the international standards on use and handling of animals.

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