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Time-dependent effect of inflammation on the gene expression of pro-inflammatory cytokines and their receptors at the different levels of the somatotropic axis in ewe

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ABSTRACT. Pituitary-hypothalamic-somatotropic (HPS) axis plays a key regulatory role in the metabolic and physiological processes in mammals. It is considered that inflammatory mediators such as proinflammatory cytokines, whose receptors are widespread at the different levels of the HPS axis, may be responsible for disturbing this axis activity. Our study was carried out to determine the effect of lipopolysaccharide (LPS)-induced inflammation on the expression of genes encoding pro-inflammatory cytokines and their receptors in the organs belonging to the HPS axis. *In vivo* studies were carried out on ewes (n = 36) divided randomly into 3 time-dependent groups (n = 12; each). Each time group was divided into two subgroups: control (saline-treated; n = 6) and intravenously treated with LPS (400 ng/kg; n = 6). Animals were euthanized 1.5 h, 3 h, or 9 h after LPS injection; and the fragments of the dorsomedial hypothalamus and mediobasal hypothalamus, anterior pituitary, and liver were collected. The relative gene expression encoding IL1B, IL1R1, IL1R2, IL6, IL6R, IL6ST, TNF, TNFRSF1A, and TNFRSF1B was determined with real-time PCR. Our experiment showed that LPS-induced inflammation modulates the gene expression of pro-inflammatory cytokines such as *IL1B*, *IL6*, and *TNF* and their corresponding receptor at the different levels of the HPS axis in sheep at the time-dependent manner. Because these cytokines are known as potent modulators of the secretion of hormones and neurohormones, the paracrine action of these locally synthesized cytokines may disturb the activity of the HPS axis; however, it requires further deep-in research.

Introduction

Pituitary-hypothalamic-somatotropic (HPS) axis plays a key regulatory role in the metabolic and physiological processes in mammalian organisms. In the HPS axis, the hypothalamus is the most important regulator. It is responsible for the regulatory signal from both: other hormones, and feedback loops of the HPS axis hormones (Giustina and Veldhuis, 1998; Kojima et al., 1999; Le Roith et al., 2001). The hypothalamic regulation is made by the action of two neurohormones: stimulation by growth hormone-releasing hormone (GHRH) and inhibition by somatostatin (SST) (Müller et al., 1999; Patel, 1999). In the context of the HPS axis, both the dorsomedial hypothalamus (DMH) and mediobasal hypothalamus

(MBH) are involved in regulating the release of SST and GHRH. The DMH plays a role in influencing the release of SST, which acts as an inhibitor of growth hormone (GH) secretion by the pituitary gland. It helps control GH levels in the body. Conversely, the MBH, which includes the arcuate nucleus (ARC) and median eminence (ME), is a part of the neural control of the somatotropic axis. Within the MBH, the ARC produces GHRH, which is a stimulant for the pituitary gland to release GH. SST, produced in various brain regions, including the periventricular nucleus, acts as an inhibitory factor for GH secretion, while GHRH stimulates GH production. Both the DMH and MBH, in their respective roles, influence the release of SST and GHRH, contributing to the precise control of GH levels in the body for proper growth and metabolic regulation. Two hormones play the most significant role in its signal transduction, GH and insulin-like growth factor (IGF1). Inflammation may affect the activity of the HPS axis, modulating or disrupting its signal transduction (Soto et al., 1998; Renaville et al., 2002). The influence of inflammation on the HPS axis activity in mammals seems to be species-dependent (Soto et al., 1998; Wang, 2002). Noteworthy, the increase in growth hormone secretion is a common element in the pathophysiology of sepsis in sheep and primates, including humans (Briard et al., 2000; Daniel et al., 2005), whereas in rodents a decrease or no influence were only observed (Soto et al., 1998; Wang, 2002).

Inflammation, mediated by the three most crucial pro-inflammatory cytokines – interleukin (IL)1B, IL6, and tumour necrosis factor (TNF), along with malnutrition, chronic stress, and glucocorticoids, is identified as one of the most significant factors disrupting the HPS axis signal transduction (Witkowska-Sędek and Pyrżak, 2020). Although the main source of pro-inflammatory mediators is activated by immune cells, some amount of these cytokines could be synthesized locally at all levels of the HPS axis (Wojtulewicz et al., 2020; Szczepkowska et al., 2021; 2022; Gramignoli et al., 2022). These locally synthesized cytokines may significantly influence these tissues' function in a paracrine way. In experimental conditions, to induce systemic inflammation without introducing an active pathogen, the animals are injected with bacterial endotoxin – lipopolysaccharide (LPS), an ingredient of the outer cell membrane of a Gram(−) bacteria (Ding et al., 2004). During infection, LPS is released into the bloodstream during lysis and bacterial cell replication (Brade, 1999). Endotoxin induces its biological effects mainly by activation of Toll-like receptor 4 (TLR4) (Poltorak et al., 1998) which leads to the synthesis of inflammation mediators, IL1B, IL6, and TNF (Raetz and Whitfield, 2002; Copeland et al., 2005). The expression of TLR4 has been previously reported at the HPS axis (Guo and Friedman, 2010; Herman et al., 2013a; Sabatino et al., 2013) which suggests that LPS may directly influence the HPS axis cells among other promoting the local synthesis of inflammatory cytokines. It is worth mentioning that the expression of blood-borne cytokines after endotoxin exposure is time-dependent. IL1B and TNF are two early expressed cytokines after exposure to the LPS, and the most potent inducers of the following *IL6* gene expression by the stimulation of nuclear factor-kappa B (NF-κB) (Merola et al., 1996; Blanchard et al., 2009; Fonseca et al., 2009). Since different inflammatory cytokines may affect target cell functions in a partially different way, these time-dependent changes in their local synthesis may be important for the secretory activity of the HPS axis.

Therefore, the study aimed to determine time-dependent changes in the local expression of pro-inflammatory cytokines such as *IL1B*, *IL6*, and *TNF* and their receptors at all levels of the HPS axis during inflammation induced by single injection of LPS.

Material and methods

Animals

Experimental procedures were approved (authorization no. WAW2/052/2018 on 23 March 2018) by the 2nd Local Ethics Committee of the Warsaw University of Life Sciences – SGGW (Warsaw, Poland). The *in vivo* experiment was conducted on 36 adult blackface ewes in November. The animals were maintained indoors in individual pens and exposed to natural daylight. The stress of social isolation was limited by visual contact between animals. The ewes were fed a constant diet of commercial concentrates with hay and water available *ad libitum*. Before the beginning of the experiment (12 h), animals were deprived of food. Before the experiment, the stage of the oestrous cycle of ewes was synchronized using an intra-vaginal sponge impregnated with 20 mg of a synthetic progesterone-like hormone, the Chronogest® CR (Merck Animal Health, Boxmeer, The Netherlands) following the methodology outlined in Przybył et al. (2021). After sponge removal, the ewes received an intramuscular injection of 500 IU pregnant mare's serum

gonadotropin (PMSG) (Merck Animal Health, Boxmeer, The Netherlands). The experimental procedure was carried out on the 10th day of the luteal phase, during estradiol and progesterone plateau. A venous catheter was implemented into a jugular vein on the day before the experiment. Ewes (n = 36) were divided randomly into 3 time-dependent groups: 1.5 h (n = 12), 3 h (n = 12), and 9 h (n = 12). While each time group was divided into two subgroups: control (saline-treated; n = 6) and LPS treated (LPS from *Escherichia coli* (Sigma-Aldrich, St. Louis, MO, USA); intravenous (i.v.); dose of 400 ng/kg body weight; n = 6). The body temperature of the animals was measured throughout the experiment. Animals were euthanized 1.5 h, 3 h, or 9 h after i.v. injection of LPS. Tissues such as mediobasal hypothalamus (MBH), dorsomedial hypothalamus (DMH), anterior pituitary (AP), and liver were collected and immediately frozen in liquid nitrogen and stored at −80 °C until further analysis.

Table 1. Primers used for gene expression assay

Gene symbol	Primers (sequence 5'→3')		Gene Bank accession number	References
	Forward	Reverse		
HDAC1	CTGGGGACCTACGGGATATT	GACATGACCGGCTTGAAAAT	XM_004005023.3	Herman et al., 2014
GAPDH	TGACCCCTTCATTGACCTTC	GATCTCGCTCCTGGAAGATG	NM_001190390.1	Haziak et al., 2014
PPIC	TGGCACTGGTGGTATAAGCA	GGGCTTGGTCAAGGTGATAA	XM_004008676.5	Haziak et al., 2014
B2M	CTTCTGTCCCACGCTGAGTT	GGTGCTTAGAGGTCTCG	XM_012180604.3	Szczepkowska et al., 2022
IL1B	CAGCCGTGCAGTCAGTAAAA	GAAGCTCATGCAGAACACCA	NM_001009465.2	Herman et al., 2010
IL1R1	GGGAAGGGTCCACCTGTAAC	ACAATGCTTTCCCCAACGTA	NM_001206735.1	Herman et al., 2014
IL1R2	CGCCAGGCATACTCAGAAA	GAGAACGTGGCAGCTTCTTT	NM_001046210.2	Krawczyńska et al., 2019
IL6	GTTCAATCAGGCGATTTGCT	CCTGCGATCTTTTCCTTCAG	NM_001009392.1	Herman et al., 2014
IL6R	TCAGCGACTCCGGAAACTAT	CCGAGGACTCCACTCACAAT	NM_001110785.3	Herman et al., 2014
IL6ST	GGCTTGCCTCCTGAAAAACC	ACTTCTCTGTTGCCCACTCAG	XM_012096909.2	Król et al., 2016
TNF	CAAATAACAAGCCGGTAGCC	AGATGAGGTAAAGCCCGTCA	NM_001024860.1	Herman et al., 2014
TNFRSF1A	AGGTGCCGGGATGAAATGTT	CAGAGGCTGCAGTTCAGACA	NM_001166185.1	Herman et al., 2014
TNFRSF1B	ACCTTCTTCCTCCTCCCAAA	AGAAGCAGACCCAATGCTGT	NM_001040490.2	Herman et al., 2014

HDAC1 – histone deacetylase 1, *GAPDH* – glyceraldehyde-3-phosphate dehydrogenase, *PPIC* – peptidylprolyl isomerase C, *B2M* – beta-2-microglobulin, *IL1B* – interleukin 1 beta, *IL1R1* – interleukin 1 receptor type 1, *IL1R2* – interleukin 1 receptor type 2, *IL6* – interleukin 6, *IL6R* – interleukin 6 receptor, *IL6ST* – interleukin 6 family signal transducer, *TNF* – tumour necrosis factor, *TNFRSF1A* – TNF receptor superfamily member 1A, *TNFRSF1B* – TNF receptor superfamily member 1B

Real-time RT-PCR assay

Total RNA from collected tissues was isolated using the NucleoSpin RNA/Protein kit (Macherey-Nagel, Dueren, Germany). The quality and quantity of the RNA were verified using the spectrophotometer NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA) at 260, 280, and 230 nm wavelengths, and 1% agarose gel electrophoresis. Subsequently, RNA was transcribed using a reverse transcription kit Maxima™ First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific, Waltham, MA, USA). The obtained matrix has been used in the real-time PCR reaction, using FIREPol® HOT EvaGreen qPCR Mix® Plus kit (Solis Biodyne, Tartu, Estonia). The temperature profile for each gene was chosen based on optimization by standard protocol: 95 °C for 15 min for HOT FIREPol® DNA polymerase activation and 40 cycles at 95 °C for 5–10 s for denaturation, 60 °C for 10–30 s for annealing, and 72 °C for 15–30 s for the extension. The primers used for each examined gene are presented in Table 1. The PCR reactions were carried out using a thermocycler RotorGene Q (Qiagen, Germantown, MD, USA) with RotorGene Q software. The results obtained for the examined genes were normalized to the reference gene or combination of the reference genes chosen from among genes glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), histone deacetylase 1 (*HDAC1*), beta-2-microglobulin (*B2M*) or peptidylprolyl isomerase C (*PPIC*) using NormFinder (Molecular Diagnostic Laboratory, Aarhus University Hospital, Aarhus, Denmark) for identification of the optimal normalization gene. The results are presented in arbitrary units, as the ratio of the target gene expression to the expression of the reference gene with an appropriate control group is calculated as 1.

Statistical analysis

The statistical analysis was performed using TIBCO Statistica 13.3 (TIBCO Statistica Ltd., Palo Alto, CA, USA). The significant differences in gene

expression between the experimental groups were determined using the Student's t-test to compare expression between control and LPS-treated groups. The results are presented as mean ± standard error of the mean (SEM) and results $P \leq 0.05$ were deemed statistically significant.

Results

Hypothalamic structures

It was found that in the MBH, the expression of the gene encoding IL1B was increased in groups 1.5 and 3 h after LPS treatment but in animals sacrificed 9 h after the endotoxin injection *IL1B* expression was at the control level. The administration of LPS influenced *IL1R1* mRNA level in the MBH in a more complex way, it decreased *IL1R1* gene expression only in the group 1.5 h, whereas it increased this gene expression only in the 9 h group. The *IL1R2* mRNA level was increased after endotoxin injection in 3 h and 9 h groups. The expression of the gene encoding IL6 was increased in all LPS-treated groups. The *IL6R* mRNA level did not change in all research groups. The mRNA level of *IL6ST* was increased by LPS injection in groups 3 h and 9 h. The expression of the gene encoding TNF has been changed after endotoxin administration only in the 9 h group, in which it increased the number of *TNF* mRNA. The administration of LPS also decreased *TNFRSF1A* mRNA expression in the 1.5 h group, while increased *TNFRSF1B* mRNA level in the 1.5 h and 3 h groups (Table 2).

It was found that in the MBH, the expression of the gene encoding IL1B was increased in groups 1.5 and 3 h after LPS treatment but in animals sacrificed 9 h after the endotoxin injection, *IL1B* expression

Table 2. The relative expression of genes encoding the following proteins: IL1B, IL1R1, IL1R2, IL6, IL6R, IL6ST, TNF, TNFRSF1A, TNFRSF1B at the MBH and DMH level

		MBH			DMH		
Gene	Time, h	Control	LPS	*P*	Control	LPS	*P*
IL1B	1.5	1 ± 0.13	4.46 ± 0.11↑	0.004	1 ± 0.06	1.85 ± 0.31↑	0.009
	3	1 ± 0.17	5.75 ± 0.70↑	0.002	1 ± 0.12	3.12 ± 0.36↑	0.002
	9	1 ± 0.13	0.99 ± 0.20	0.485	1 ± 0.07	0.72 ± 0.07↓	0.015
IL1R1	1.5	1 ± 0.10	0.62 ± 0.06↓	0.009	1 ± 0.13	0.87 ± 0.11	0.429
	3	1 ± 0.16	1.16 ± 0.09	0.394	1 ± 0.20	1.13 ± 0.23	0.699
	9	1 ± 0.05	2.10 ± 0.10↑	0.002	1 ± 0.11	1.46 ± 0.26	0.178
IL1R2	1.5	1 ± 0.08	0.88 ± 0.05	0.247	1 ± 0.13	1.02 ± 0.10	0.792
	3	1 ± 0.17	1.86 ± 0.27↑	0.015	1 ± 0.13	0.96 ± 0.08	0.818
	9	1 ± 0.19	1.96 ± 0.12↑	0.009	1 ± 0.09	1.47 ± 0.04↑	0.002
IL6	1.5	1 ± 0.10	6.63 ± 1.16↑	0.004	1 ± 0.11	3.57 ± 0.55↑	0.004
	3	1 ± 0.15	30.77± 5.56↑	0.002	1 ± 0.14	4.59 ± 0.65↑	0.002
	9	1 ± 0.04	1.67 ± 0.18↑	0.004	1 ± 0.05	0.72 ± 0.04↓	0.004
IL6R	1.5	1 ± 0.03	1.04 ± 0.05	0.329	1 ± 0.12	1.46 ± 0.07↑	0.043
	3	1 ± 0.11	0.96 ± 0.06	0.485	1 ± 0.17	1.13 ± 0.21	0.699
	9	1 ± 0.07	0.91 ± 0.07	0.699	1 ± 0.07	0.82 ± 0.15	0.589
IL6ST	1.5	1 ± 0.07	1 ± 0.05	0.931	1 ± 0.15	1.01 ± 0.13	1
	3	1 ± 0.11	1.67 ± 0.17↑	0.009	1 ± 0.14	1.93 ± 0.13↑	0.004
	9	1 ± 0.04	1.20 ± 0.04↑	0.009	1 ± 0.06	1.46 ± 0.1↑	0.009
TNF	1.5	1 ± 0.08	1.18 ± 0.09	0.429	1 ± 0.07	1.01 ± 0.07	0.662
	3	1 ± 0.07	1 ± 0.17	0.937	1 ± 0.13	0.81 ± 0.11	0.132
	9	1 ± 0.06	0.78 ± 0.04↓	0.015	1 ± 0.05	0.83 ± 0.05↓	0.026
TNFRSF1A	1.5	1 ± 0.02	0.82 ± 0.04↓	0.004	1 ± 0.07	1.58 ± 0.05↑	0.004
	3	1 ± 0.09	0.96 ± 0.06	0.700	1 ± 0.10	1.40 ± 0.14	0.132
	9	1 ± 0.06	1.11 ± 0.07	0.132	1 ± 0.02	1 ± 0.08	0.818
TNFRSF1B	1.5	1 ± 0.08	1.36 ± 0.06↑	0.009	1 ± 0.02	1.53 ± 0.09↑	0.004
	3	1 ± 0.07	1.49 ± 0.15↑	0.009	1 ± 0.12	1.76 ± 0.13↑	0.004
	9	1 ± 0.02	1.20 ± 0.1	0.240	1 ± 0.05	1.34 ± 0.09↑	0.025

IL1B – interleukin 1 beta, *IL1R1* – interleukin 1 receptor type 1, *IL1R2* – interleukin 1 receptor type 2, *IL6* – interleukin 6, *IL6R* – interleukin 6 receptor, *IL6ST* – interleukin 6 family signal transducer, *TNF* – tumour necrosis factor, *TNFRSF1A* – TNF receptor superfamily member 1A, *TNFRSF1B* – TNF receptor superfamily member 1B, MBH – mediobasal hypothalamus, DMH – dorsomedial hypothalamus, control – control group, LPS – lipopolysaccharide treated group, *P* – *P*-value; $P \leq 0.05$ indicates the significance of the change. The data are presented as mean ± standard error of the mean. The arrows represent significant changes – ↑ for increase and ↓ for decrease

was at the control level. The administration of LPS influenced *IL1R1* mRNA level in the MBH in a more complex way, it decreased *IL1R1* gene expression only in the group 1.5 h, whereas increased it only in the 9 h group. The *IL1R2* mRNA level was increased after endotoxin injection in 3 h and 9 h groups. The expression of the gene encoding IL6 was increased in all LPS-treated groups. The *IL6R* mRNA level did not change in all research groups. The mRNA level of *IL6ST* was increased by LPS injection in groups 3 h and 9 h. The expression of the gene encoding TNF has been changed after endotoxin administration only in the 9 h group, in which it increased the number of *TNF* mRNA. The administration of LPS also decreased the *TNFRSF1A* mRNA level in the 1.5 h group, while increasing the *TNFRSF1B* mRNA level in the 1.5 h and 3 h groups (Table 2).

In the DMH, LPS administration exerted a temporally specific modulatory effect on the transcriptional profiles of cytokines and their cognate receptors. Specifically, LPS exposure significantly up-regulated *IL1B* mRNA expression at 1.5 and 3 h post-administration, with a subsequent down-regulation observed at the 9 h time point. Notably, mRNA levels of *IL1R2* exhibited an up-regulation exclusively at the 9 h mark, implicating LPS as the causative agent. *IL6* mRNA expression was uniformly augmented across all examined time points following LPS administration. At 1.5 h post-LPS treatment, a discernible elevation in *IL6R* mRNA levels was observed, while *IL6ST* mRNA levels experienced up-regulation at both 3 h and 9 h. *TNF* mRNA expression was specifically enhanced at 9 h following LPS exposure. Interestingly, LPS elicited an increase in *TNFRSF1A* mRNA levels solely at the 1.5 h time point, while inducing a ubiquitous stimulatory effect on *TNFRSF1B* mRNA levels across all investigated time points (Table 2).

Anterior pituitary

In the AP, the administration of LPS had a clear impact on the expression of cytokines and their receptors. Specifically, LPS increased *IL1B* mRNA levels at 1.5 h and 3 h. It also decreased *IL1R1* mRNA at 1.5 h but increased it at 3 h and 9 h. *IL1R2* mRNA levels increased across all time points after LPS treatment. *IL6* mRNA levels were consistently elevated in all groups treated with LPS. Likewise, *IL6R* mRNA levels increased at 3 h and 9 h. *TNF* mRNA increased at 1.5 h but decreased at 9 h, while *TNFRSF1A* mRNA levels increased at 3 h and 9 h. *TNFRSF1B* mRNA levels increased in all groups treated with LPS. (Table 3).

Table 3. The relative expression of genes encoding the following proteins: IL1B, IL1R1, IL1R2, IL6, IL6R, IL6ST, TNF, TNFRSF1A, TNFRSF1B at the AP level

		AP		
Gene	Time, h	Control	LPS	*P*
IL1B	1.5	1 ± 0.09	4.92 ± 0.26↑	0.004
	3	1 ± 0.08	5.52 ± 0.47↑	0.002
	9	1 ± 0.11	0.87 ± 0.05	0.699
IL1R1	1.5	1 ± 0.12	0.60 ± 0.1↓	0.030
	3	1 ± 0.17	3.34 ± 0.19↑	0.002
	9	1 ± 0.07	1.63 ± 0.18↑	0.004
IL1R2	1.5	1 ± 0.11	1.72 ± 0.16↑	0.009
	3	1 ± 0.21	4.08 ± 0.19↑	0.002
	9	1 ± 0.04	2.17 ± 0.30↑	0.004
IL6	1.5	1 ± 0.03	6.22 ± 1.17↑	0.004
	3	1 ± 0.13	27.47 ± 3.84↑	0.002
	9	1 ± 0.03	1.71 ± 0.19↑	0.015
IL6R	1.5	1 ± 0.07	0.98 ± 0.13	0.329
	3	1 ± 0.13	2.14 ± 0.22↑	0.004
	9	1 ± 0.11	1.4 ± 0.07↑	0.025
IL6ST	1.5	1 ± 0.10	0.70 ± 0.07↓	0.030
	3	1 ± 0.11	3.02 ± 0.13↑	0.002
	9	1 ± 0.10	1.22 ± 0.07↑	0.041
TNF	1.5	1 ± 0.08	1.35 ± 0.09↑	0.017
	3	1 ± 0.06	1.01 ± 0.09	0.937
	9	1 ± 0.06	0.75 ± 0.05↓	0.009
TNFRSF1A	1.5	1 ± 0.1	1.02 ± 0.14	0.931
	3	1 ± 0.13	4.44 ± 0.24↑	0.002
	9	1 ± 0.06	1.71 ± 0.17↑	0.004
TNFRSF1B	1.5	1 ± 0.04	2.10 ± 0.25↑	0.004
	3	1 ± 0.09	7.06 ± 0.35↑	0.002
	9	1 ± 0.1	1.44 ± 0.13↑	0.015

IL1B – interleukin 1 beta, *IL1R1* – interleukin 1 receptor type 1, *IL1R2* – interleukin 1 receptortype 2, *IL6* – interleukin 6, *IL6R* – interleukin 6 receptor, *IL6ST* – interleukin 6 family signal transducer, *TNF* – tumour necrosis factor, *TNFRSF1A* – TNF receptor superfamily member 1A, *TNFRSF1B* – TNF receptor superfamily member 1B, AP – anterior pituitary, control – control group, LPS – lipopolysaccharide treated group, *P* – *P*-value; $P \leq 0.05$ indicates the significance of the change. The data are presented as mean ± standard error of the mean. The arrows represent significant changes – ↑ for increase and ↓ for decrease

Liver

LPS treatment significantly modulated cytokine and receptor gene expression in the liver over time. Specifically, LPS elevated *IL1B* gene expression at 1.5 h and later caused a decline at the 9 h mark. *IL1R1* and *IL1R2* mRNA levels were increased only at 9 h due to LPS treatment. *IL6* gene expression consistently increased across all tested groups under the effect of LPS. Correspondingly, *IL6R* mRNA levels surged at the 3 h time point following LPS

treatment. *IL6ST* mRNA levels, influenced by LPS, decreased at 1.5 h and increased at 9 h. *TNF* gene expression showed a notable increase at 1.5 h under LPS, while *TNFRSF1A* and *TNFRSF1B* mRNA levels increased only at the 9 h mark, clearly indicating LPS's impact (Table 4).

Table 4. The relative expression of genes encoding the following proteins: IL1B, IL1R1, IL1R2, IL6, IL6R, IL6ST, TNF, TNFRSF1A, TNFRSF1B at the liver level

		Liver		
Gene	Time, h	Control	LPS	*P*
IL1B	1.5	1 ± 0.14	6.68 ± 0.82↑	0.002
	3	1 ± 0.17	1.05 ± 0.25	0.937
	9	1 ± 0.23	0.44 ± 0.09↓	0.041
IL1R1	1.5	1 ± 0.09	0.96 ± 0.08	0.589
	3	1 ± 0.19	1.20 ± 0.17	0.394
	9	1 ± 0.06	2.37 ± 0.42↑	0.004
IL1R2	1.5	1 ± 0.23	0.92 ± 0.13	0.937
	3	1 ± 0.26	1.52 ± 0.23	0.093
	9	1 ± 0.16	7.59 ± 1.90↑	0.002
IL6	1.5	1 ± 0.20	23.18 ± 1.53↑	0.002
	3	1 ± 0.29	39.8 ± 7.35↑	0.002
	9	1 ± 0.27	6.14 ± 0.59	0.002
IL6R	1.5	1 ± 0.09	0.88 ± 0.20	0.485
	3	1 ± 0.18	0.53 ± 0.08↓	0.041
	9	1 ± 0.13	1.18 ± 0.20	0.589
IL6ST	1.5	1 ± 0.10	0.93 ± 0.08	0.818
	3	1 ± 0.04	0.73 ± 0.07↓	0.004
	9	1 ± 0.07	1.01 ± 0.12	1
TNF	1.5	1 ± 0.20	3.52 ± 0.46↑	0.002
	3	1 ± 0.24	2.88 ± 1.23	0.394
	9	1 ± 0.29	1.54 ± 0.16	0.240
TNFRSF1A	1.5	1 ± 0.06	0.97 ± 0.14	0.699
	3	1 ± 0.12	1.34 ± 0.19	0.240
	9	1 ± 0.16	1.99 ± 0.28↑	0.009
TNFRSF1B	1.5	1 ± 0.09	1.39 ± 0.24	0.394
	3	1 ± 0.1	0.93 ± 0.1	0.699
	9	1 ± 0.12	1.42 ± 0.07↑	0.0260

IL1B – interleukin 1 beta, *IL1R1* – interleukin 1 receptor type 1, *IL1R2* – interleukin 1 receptor type 2, *IL6* – interleukin 6, *IL6R* – interleukin 6 receptor, *IL6ST* – interleukin 6 family signal transducer, *TNF* – tumour necrosis factor, *TNFRSF1A* – TNF receptor superfamily member 1A, *TNFRSF1B* – TNF receptor superfamily member 1B, control – control group, LPS – lipopolysaccharide treated group, *P* – *P*-value; $P \leq 0.05$ indicates the significance of the change. The data are presented as mean ± standard error of the mean. The arrows represent significant changes – ↑ for increase and ↓ for decrease

Discussion

Our study showed that endotoxin-induced inflammation modulates the gene expression of proinflammatory cytokines and their corresponding receptors in the MBH and DMH which are hypothalamic structures responsible for regulation of HPS axis activity. However, the inflammatory-dependent changes in the gene expression of proinflammatory cytokines and their receptors were largely similar, but in some cases, significant differences in this gene expression were observed between studied structures.

The significant increase in *IL1B* gene expression in the MBH was stated at 1.5 h and 3 h after LPS injection, but 9 h after the treatment the gene expression of this cytokine was at the control level. On the other hand, endotoxin-induced inflammation increased *IL1B* mRNA expression in the DMH at 1.5 h and 3 h after LPS administration but 9 h after the treatment transcription of this cytokine was reduced. The stimulatory effect of systemic inflammation on the *IL1B* gene in the hypothalamus was not a surprise, because it was previously found in sheep (Herman et al., 2010; Szczepkowska et al., 2021) and rodents (Gabellec et al., 1995; Layé et al., 2000). Our experiment showed that in acute endotoxin-induced inflammation, increased expression of *IL1B* mRNA persists in the hypothalamus only in the first hours after the appearance of the inflammatory signal. Increasing the local synthesis of IL1B may significantly affect the activity of the HPS axis. The results of an *in vitro* study on rat hypothalamic explants showed that IL1 caused an increase in GHRH release and even more pronounced dose-dependent stimulation of SST (Honegger et al., 1991). It is worth mentioning that acting centrally IL1B is considered as a stimulator of the HPS axis activity. The *in vivo* studies on rats showed that intracerebroventricular injection of IL1B stimulates GH secretion by stimulation of hypothalamic GHRH (Payne et al., 1992). In contrast, the role of IL1B in the regulation of SST seems to be more ambiguous. Although the earlier *in vitro* research showed that IL1B stimulates the SST expression at the hypothalamus of rats, such results were not confirmed *in vivo* (Taishi et al., 2004). It is postulated that IL1B plays a complex role in SST regulation having the ability to stimulate (minutes), inhibit (hours), or not alter (days) SST release (Tittle, 1998). The current study showed that the effect of inflammation on the *IL1R* gene expression, which is considered to be primarily responsible for transmitting the effects of IL1, in the hypothalamus may depend on the hypothalamic region and duration of the inflammatory signal. Upon binding of IL1B to IL1R1, this receptor forms a complex with an accessory protein known as interleukin-1

receptor accessory protein (IL-1RAcP). This initiates intracellular signalling cascades involving kinases like the mitogen-activated protein kinases (MAPKs) and transcription factors such as NF-κB (Mordret, 1993; Dinarello, 2009). The role of IL1R1 is crucial in various pathological conditions associated with inflammation, including autoimmune diseases, and sepsis (O'Neill, 2008). In the MBH, *IL1R1* mRNA level was decreased at 1.5 h, which seems to be the physiologic anti-inflammatory or adaptive mechanism, which may protect the local cells from excessive stimulation by IL1. In mouse models of autoimmune diseases such as multiple sclerosis, reducing the expression of IL1R1 has been shown to lessen symptoms and inflammation (Komuczki et al., 2019). Similarly, animal studies on intestinal and cardiac conditions have demonstrated protective effects when IL1R1 expression is lowered (Nadatani et al., 2012). The authors indicate that limiting the expression of *IL1R1* in the brain could have significant therapeutic advantages for stroke and other cerebrovascular diseases (Wong et al., 2019).

In the group of animals euthanized 3 h after the treatment, *IL1R1* gene expression was at the control level, which may be associated with the strongest transduction of the IL1B signal, especially together with increased expression of gene encoding IL1B found in these animals. While in the 9 h group, the mRNA expression of *IL1R1* in the MBH was significantly increased, but the *IL1B* expression level was at the control level. Interestingly, in contrast to MBH, no inflammatory-dependent alterations in the *IL1R* gene expression were observed in the DMH in any of the examined groups. This may suggest that the changes in IL1R influence SST secretion to a lesser extent than GHRH. Our experiment also showed that *IL1R2* expression was upregulated at both the 3 h and 9 h time points in the MBH. This data, when considered alongside with the *IL1B* and *IL1R1* results, suggests that the MBH experiences a peak in IL1B-mediated pro-inflammatory activity at the 3 h time point. In contrast, observations in the DMH differed specifically, *IL1R2* expression remained unaltered at the 1.5 h and 3 h time intervals but showed a notable increase at the 9 h after the treatment. The up-regulation of *IL1R2* gene expression could be one of the central mechanisms leading to the reduction of IL1B stimulation of the analysed hypothalamic structures. IL1R2 primarily acts as a "decoy" receptor for IL1B cytokine, and such binding but does not initiate intracellular signalling upon binding, thereby acting as a negative regulator for IL1 responses (Dinarello, 2009). IL1R2 exists both as a membrane-bound form and a soluble form in plasma and is produced in two ways, by shedding by metalloproteinase and alternatively splicing (Orlando et al., 1997; Vambutas et al., 2009; Peters et al., 2013). Both forms are capable of binding IL1B and preventing its interaction with the signalling-competent IL1R1 receptor (Gabay et al., 2010). The action of this receptor is to modulate the signalling of IL1B and other IL1 family cytokines, which is critical for maintaining homeostasis and controlling pathological inflammatory response (Mantovani et al., 2019).

It is worth mentioning that in the current experiment, the expression of *TNF* remained stable during the first part of the experiment, but in the end, a significant reduction in this gene expression was determined. This result is a bit surprising because based on the results of our previous studies (Herman et al., 2014; Kowalewska et al., 2017), we expected an increase in the expression of the gene encoding TNF in the hypothalamic structures. This different response in *TNF* mRNA synthesis in the analysed hypothalamic tissue may partially explain the higher purity of the LPS used in our experiment because it was previously reported that the purity of LPS significantly determines its activity to stimulate the immune response (Parusel et al., 2017). It is also possible, that the increase in the animals differed in the pace of the inflammatory reaction and the increase in *TNF* expression in the hypothalamus took place in the period preceding the chosen time points at which the animals were euthanized.

In the MBH, we observed a consistent up-regulation of *IL6* gene expression across all time points, with the most pronounced increase manifesting at the 3 h time point. Conversely, in the DMH, *IL6* gene expression also varied across all research groups but exhibited a distinct pattern: expression levels increased at both the 1.5 h and 3 h time points, while a decrease was observed in the 9 h group. This suggests divergent temporal dynamics of IL6-mediated inflammatory signalling between the MBH and DMH. Increased local synthesis of IL6 in the MBH and DMH during inflammation may influence the secretory activity of these hypothalamic structures. The role of IL6 in regulating GHRH in the hypothalamus is not fully clear yet. Some studies suggest that IL6 affects the brain-liver-pituitary system, which in turn impacts the levels and activities of different hormones like GHRH and GH. Research on mice indicates that IL6 can boost the production of GHRH (Vijayakumar et al., 2010). There's also

evidence that IL6 has similar effects during stressful or disease conditions (Mastorakos et al., 1993). The results of *in vitro* studies showed that IL6 as well as TNF stimulate the synthesis and release in a dose- and time-dependent manner (Scarborough, 1990). On the other hand, *IL6R* gene expression remained constant across all study groups in the MBH, while IL6ST mRNA levels were elevated at both the 3 h and 9 h time groups. Comparable patterns for *IL6ST* gene expression were observed at the DMH level, albeit with a notable increase in *IL6R* mRNA levels found in the 1.5 h group. The minimal fluctuations in *IL6R* expression within the hypothalamic nuclei may be attributable to the lesser magnitude of *IL6* expression changes compared to those in peripheral tissues. Intriguingly, an increase in *IL6ST* expression was consistently determined in both the MBH and DMH at the 3 h and 9 h time points. This upregulation might be indicative of an anti-inflammatory mechanism that dampens IL6 signalling, given the role of IL6ST as a decoy receptor in this pathway (Reeh et al., 2019).

Hypothalamic neurohormones such as GHRH and SST are released to the hypophyseal portal circulation that supplies blood to the AP, which influences GH secretion generally in the opposite meaner: GHRH stimulates GH release, while SST inhibits GH release. This regulatory mechanism also involves a short-loop feedback component, by which GH acts on the hypothalamus to stimulate SST release (Hiller-Sturmhöfel and Bartke, 1998). It is worth mentioning that the research on primary pituitary cell cultures from baboons suggests that the role of the hypothalamic hormones in the regulation of GH secretion could be more complex and ambiguous. In this study, SST decreased the sensitivity of GHRH receptors to GHRH, but its effect on GH secretion was dose-dependent. High doses of SST blocked GHRH-induced GH release, whereas at low concentrations, SST dramatically stimulated GH release to levels comparable to those evoked by GHRH (Córdoba-Chacón et al., 2012). Therefore, the alteration in these neurohormone secretions in the hypothalamus may significantly influence the GH release. However, the pituitary maintains its anatomical and functional connections with the brain yet sits outside the blood-brain barrier (Nussey and Whitehead, 2001), therefore its secretory activity may be influenced by several factors reaching this gland with the circulating blood as well as by the locally produced factors which may regulate the pituitary cells activity in auto- and paracrine meaner (Arzt, 2001; Mainardi et al., 2002; Silverman et al., 2005; Wojtulewicz et al., 2020).

It is also considered that the inflammatory mediators influence the GH synthesis and/or release GH through direct action at the level of the pituitary, omitting the hypothalamic regulation of GH secretion. Previous research showed that the modulatory effect of inflammation on GH secretion seems to be dependent upon the dose of LPS used to induce the inflammatory response as well as the phase of the inflammatory response. At low dosages, LPS increases pituitary GH release, but with greater LPS dosages, a decrease in plasma GH is observed (Martín et al., 2021). Our research showed that at the AP level, the expression of gene encoding IL1B was increased in groups euthanized 1.5 h and 3 h after the LPS treatment. Previous research has demonstrated that IL1B is a key cytokine involved in the initiation and propagation of inflammatory responses, often acting in a paracrine or autocrine manner within the endocrine system (Jaffer et al., 2010). Our study suggests that locally produced IL1B may influence the secretory activity of the AP, particularly during the early inflammatory phase. The increase in local synthesis of IL1B may influence the secretory activity of the AP because it was previously found that IL1B is a potent cytokine directly modulating the pituitary hormone secretion (Herman et al., 2013b; Schettini et al., 1990). The role of IL1B in the direct regulation of GH secretion is still not entirely clear. *In vitro* study on pituitary cells from adult pigs showed that IL1B enhanced GH output, but reduced the galanin-induced GH secretion (Mainardi et al., 2002). The experiment on anterior pituitary cells of adult male rats also showed that IL1B stimulated GH-secretion through direct action on the pituitary (Niimi et al., 1994). However, another study on rat pituitary cells under serum-free conditions suggested a rather suppressive effect of IL1B on GH release (Renner et al., 1995). At least, the study on ovine pituitary cells showed that IL1B stimulated GH release from cultured pituitary cells at all doses, however, IL1B affected GHRH-stimulated GH release (Fry et al., 1998). A decrease in the *IL1R1* gene expression in the AP during the first stage of the experiment may be a mechanism preventing excessive pro-inflammatory effects of IL1B, which may lead to homeostasis disorders in the case of short increases in the level of this and other interleukins. On the other hand, an increase in this gene expression found at the later stages of the experiment suggests that the earlier-mentioned anti-inflammatory mechanism is temporary and occurs only at the beginning of the inflammatory response. Such effect was not stated in the expression of gene

encoding IL1R2 which expression was increased in all LPS-treated groups. IL1R2 plays a role of a decoy receptor and it serves as a negative regulator of IL1 signalling by competing with IL1R1 for IL1 and by complexing with IL-1RAcP once it binds IL1, thereby sequestering both the ligand and the accessory protein required for signal transduction (Peters et al., 2013).

In the AP, the expression of *TNF* mRNA was increased only at the first stage of the experiment. This increased expression of *TNF* at the beginning of the inflammatory response was not a surprise because it is one of the cytokines produced during the acute phase of the inflammatory response (Meijer et al., 1993; Gulhar et al., 2023). This increased local TNF synthesis may affect the secretion of pituitary hormones, including GH because previously conducted *in vitro* studies showed that this cytokine inhibits GRH-stimulated GH release from cultured sheep pituitary cells (Fry et al., 1998). Interestingly, although the expression of *TNF* in the AP decreases at later stages of the inflammatory reaction, the expression of genes for the receptors of this cytokine remains generally elevated. This suggests an increased sensitivity of this gland to the action of the ligand of these receptors.

Our experiment showed that endotoxin-induced inflammation stimulates *IL6* gene expression in the AP, which was raised throughout the experiment. Increased local synthesis of IL6 may also have a pronounced effect on the secretory activity of the pineal gland including GH release. Previous *in vitro* studies showed that IL6 stimulated GH synthesis and release (Zhao et al., 2014). Moreover, the study on pituitary cells from adult pigs showed that IL6 potentiated the effect of GH releasers (Mainardi et al., 2002). The stimulatory effect of IL6 on GH release was also stated in the *in vivo* study on men infused with recombinant human (rh) IL6 via an antecubital vein. Which showed that IL6 infusion led to a significant increase in GH, peaking 1 h after the beginning of the infusion (Nemet et al., 2006). Endotoxin-induced inflammation also influenced *IL6R* and its signal transducer gene expression but this influence was more clear from the 3rd hour of the experiment, when the expression of both tested genes was increased. This may suggest increased transduction of the IL6 signal in the AP cells.

It was also found that the activation of the immune system inhibits IGF1 secretion (Colson et al., 2003), which suggests that inflammatory mediators may influence its production in the liver because this organ is the main source of circulating IGF1 (Lara-Diaz et al., 2017). In the liver, the GH signal of the somatotropic axis is transduced by the GHR and results in the IGF1 expression and secretion. The GHR is a transmembrane receptor and is classified as a class I signalling molecule. Binding one molecule of GH to GHR dimer results in its activation and modification of its extracellular domains which result in the activation of tyrosine kinases and signal transduction (Brooks and Waters, 2010). The GHR activation results also in its intracellular part binding of Janus kinase 2 (JAK2) and phosphorylation of JAK tyrosines which subsequently forms binding sites for Src homology 2 domain, which enables the phosphorylation of, among others, STAT proteins, of the which STAT5β plays the most important action in the GH signal transduction (Udy et al., 1997; Teglund et al., 1998; Cui et al., 2004; Wójcik et al., 2018). The factors influencing GHR expression and signal transduction may have a pronounced effect on IGF1 secretion. It was reported that LPS reduced IGF1 expression in hepatic cells as well as the circulating concentration of this hormone (Priego et al., 2004). The experiments on rats also showed that administration of LPS at low doses increased GH secretion, whereas it decreased serum concentration of IGF1 and its expression in rat liver (Priego et al., 2003). Moreover, serum IGF1 was also found to be reduced in the acute phase of critically ill patients with sepsis (Xu et al., 2017). These all suggest that inflammation disrupts the activity of the somatotropic axis.

Our current study showed that endotoxin-induced inflammation stimulated the gene expression of all examined pro-inflammatory cytokines in the ovine liver in the first phase of the experiment, whereas only *IL6* expression increased for the entire duration of the experiment. Interestingly, the gene expression of *IL1B* and *TNF* receptors was increased only at the last hour of the experiment, whereas the expression of *IL6R* and *IL6ST* genes were reduced in the middle and at the end of the experiment, respectively. These increases in local synthesis of pro-inflammatory cytokines may significantly influence the secretion of IGF1. It was found that IL1B inhibits IGF1 production in dendritic epidermal T cells (Li et al., 2022). Previous studies also demonstrated that IL6 inhibits the secretion of IGF1 and that IL6 overproduction is a mechanism implicated in IGF1 downregulation (Barbieri et al., 2003). On the other hand, the *in vitro* study on rat aortic vascular smooth muscle cells suggested that neither IL1B nor IL6, but TNF is responsible for the suppression of *IGF1* expression (Anwar et al., 2002). It is worth

mentioning that IL1, IL6, and TNF not only influence IGF1 release but also may inhibit its activity (Lazarus et al., 1993; Hashimoto et al., 2010; Al-Shanti and Stewart, 2012; Zhao et al., 2015).

Conclusions

Our experiment showed that lipopolysaccharide-induced inflammation modulates the gene expression of pro-inflammatory cytokines such as *IL1B*, *IL6*, and tumour necrosis factor α and their corresponding receptor at the different levels of the somatotropic axis in sheep. Because these cytokines are potent modulators of the secretion of hormones and neurohormones of the somatotropic axis, the paracrine action of these locally synthesized cytokines may disturb the activity of this axis. However, further deep-in research is needed to understand the role of locally synthesized pro-inflammatory mediators in the modulation of production and activity of the somatotropic axis components.

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Conflict of interest

The Authors declare that there is no conflict of interest.

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