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Effect of caffeine on adenosine and ryanodine receptor gene expression in the hypothalamus, pituitary, and choroid plexus in ewes under basal and LPS challenge conditions

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ABSTRACT. Caffeine is a plant alkaloid that stimulates the central nervous system. It easily crosses the blood-brain barrier located in the endothelial cells of brain microvessels and the blood-cerebrospinal fluid barrier located in the epithelial cells of the choroid plexus (ChP). Caffeine exerts most of its biological effects by antagonising adenosine receptors (ADORs), but is also an agonist of ryanodine receptors (RYRs). A recent study in a sheep model has suggested that the effect of caffeine on the expression of many genes may depend on the animal's immune status. Therefore, the aim of the study was to determine the effect of caffeine administration (iv, 30 mg/kg) in ewes, under basal and acute inflammatory conditions induced by lipopolysaccharide injection (iv, 400 ng/kg), on the expression of *ADOR* and *RYR* genes in the mediobasal hypothalamus (MBH), anterior pituitary (AP), and ChP. Our study showed that among caffeine-interacting receptors, *ADORA1* was the most highly expressed in the AP and ChP, while *ADORA3* in the MBH. Caffeine reduced ($P < 0.05$) the inhibitory effect of inflammation on *ADORA1*, but only in the MBH, and decreased ($P < 0.05$) the stimulatory effect of endotoxin treatment on *ADORA2B* in the MBH and ChP. In contrast to *ADORs*, the expression of *RYRs* was less sensitive to the effects of inflammation and caffeine. We showed that caffeine influenced the expression of its receptor genes in the brain, but this effect seemed to be tissue-dependent and could be affected by the immune status of the animals. However, the physiological implications of these results require further detailed studies.

Introduction

Caffeine (1,3,7-trimethylxanthine, CAF), a plant alkaloid structurally related to adenosine, exerts numerous physiological and pharmacological effects on many organs, including the cardiovascular, renal, respiratory, and central nervous systems (Persad, 2011). Caffeine readily crosses the blood-brain barrier located in cerebral microvascular endothelial cells and the blood-cerebrospinal fluid barrier located in the epithelial cells of the choroid plexus (ChP) (McCall et al., 1982; Ikeda-Murakami et al., 2022). In the brain, caffeine exerts most of its biological effects by antagonising adenosine receptors (ADORs) and, like adenosine, affects neurons and glial cells of all brain areas (Ribeiro and Sebastião, 2010). Four subtypes of ADORs are currently distinguished: A1, A2A, A2B, and A3, which are members of the rhodopsin-like family of 7-transmembrane receptors and couple primarily through either inhibitory G_i (A1 and A3 receptors) or stimulatory G_s (A2A and A2B) heterotrimeric G proteins

(Fredholm et al., 2011; Van Eps et al., 2018). Ca^{2+} release from intracellular storage sites as an agonist of ryanodine receptors (RYRs) is another mechanism of the caffeine-mediated signalling that may affect brain cell function (Kong et al., 2008). All three RyR isoforms, i.e. RYR1, RYR2, and RYR3 are expressed in the brain (Abu-Omar et al., 2018).

Since these receptors trigger different regulatory processes, changes in their expression may significantly influence the action of caffeine in the target tissues. Recently, using a female sheep model, we demonstrated that a single administration of caffeine attenuated the inflammatory response at the hypothalamic level and partially affected the ChP response to lipopolysaccharide (LPS)-induced immune/inflammatory challenges (Szczepkowska et al., 2021). The present study also suggests that the effect of caffeine on the expression of some genes may be dependent on the immune status of the animal.

Therefore, the present study aimed to determine the effect of a single caffeine administration to ewes, under basal and LPS-induced acute inflammatory conditions, on the expression of ADORs and RYRs in the mediobasal hypothalamus (MBH) and anterior pituitary (AP), which control the function of several endocrine glands, i.e. adrenals, thyroid, and gonads, as well as the ChP, which participates in the regulation of caffeine access to the brain.

Material and methods

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Second Local Ethics Committee of the Warsaw University of Life Sciences (authorization No. WAW2/023/2018; February 2, 2018).

Animals and experimental design

The study was performed on frozen samples obtained from 2-year-old Blackhead ewes (n = 24, follicular phase of the oestrous cycle), as described in the study of Szczepkowska et al. (2021). The ewes were divided into four groups receiving intravenously the following treatments: 1. saline (0.9% w/v NaCl), 2. LPS (from *Escherichia coli* 055:B5, at a dose of 400 ng/kg body weight (bw); Sigma Aldrich, Saint Louis, MO, USA), 3. caffeine (CAF at a dose of 30 mg/kg bw, Sigma Aldrich, Saint Louis, MO, USA) and 4. LPS/CAF (400 ng/kg bw, and 30 mg/kg bw, respectively). Judging by the measurments of body temperature and blood plasma cortisol levels, all ewes responded to LPS treatment as indicated previously (Szczepkowska et al., 2021). Animals were sacrificed 3 h after treatments and the MBH, AP, and ChP were rapidly removed from the brain and snap-frozen in liquid nitrogen.

Gene expression analysis

Relative gene expression assays were performed as described by Szczepkowska et al. (2021). Briefly, frozen samples were homogenized and lysed in lysis buffer (RA1) from the NucleoSpin® RNA kit (MACHEREY-NAGEL GmbH and Co. Düren, Germany), and then total RNA was isolated using the NucleoSpin® RNA kit (MACHEREY-NAGEL). Total RNA concentration and purity were quantified (NanoDrop 1000, NanoDrop Technologies Inc., Wilmington, NC, USA), and its integrity was verified electrophoretically. The synthesis of cDNA was conducted using 1000 ng of total RNA and components of the Maxima™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) for reverse transcription. Real-time PCR reactions for brain samples were carried out using primers (Genomed, Warszawa, Poland) listed in Table 1, and a Rotor-Gene 6000 instrument (Qiagen, Dusseldorf, Germany) and HOT FIREPol EvaGreen® qPCR Mix Plus (Solis BioDyne, Tartu, Estonia), or a Viia7 instrument (Applied Biosystems by Life Technologies, Waltham, MA, USA) for ChP samples and the DyNAmo SYBR Green qPCR kit with ROX (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Data and statistical analysis

Relative gene expression was calculated using the comparative quantification option (Rasmussen, 2001) of the Rotor-Gene 6000 software 1.7. (Qiagen, Dusseldorf, Germany) for brain samples (MBH, AP) and Real-time PCR Miner (available online: http://ewindup.info/miner/) based on the algorithm described by Zhao and Fernald (2005) for ChP samples, with reference to the mean expression of three housekeeping genes: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), actin beta (*ACTB*), and histone deacetylase 1 (*HDAC1*). All data are presented as mean ± SEM, and statistical significance was set at $P < 0.05$. Gene expression data were normalised to the mean relative mRNA expression level of this gene in the control group or *ADORA1* expression in the comparison of all tested receptors in the MBH, AP, and ChP separately, which were set to 1.0. Statistical analyses were performed using Graph Pad PRISM 8 (Graph Pad Software, San Diego, CA, USA). Raw data were analysed after verification of normality assumptions (Shapiro-Wilk test). The results were analysed using one-way analyses of variance (ANOVA), followed by post-hoc Fisher's least significance test comparing groups with each other.

Table 1. Oligonucleotide primer sequences for real-time PCR reactions

Gene	(Forward / Reverse) Sequence 5'→3'	Amplicon size, bp	Accession no./ Reference
ADORA1	F: CCT CAC TCA GAG CTC CAT CC R: AGA ATC CAG CAG CCA GTG AT	135	XM_042256875.1
ADORA2A	F: CGA TGA CGA AGG ACA GCA C R: CTC ACG CAG AGC TCC ATC TT	148	XM_015101442.3
ADORA2B	F: TCC CGC TCA GGT ATA AGA GTC R: AAG AGA CAC CTG ATG AGG CA	190	XM_027974823.2
ADORA3	F: CCT GGG TGT CAC AAT CCA CT R: TCT GTA TCT GAC TGT GAG CTT GA	139	NM_001009775.2
RYR1	F: GGC GAT GAC CTC ATT CTT GT R: GAG CAG ATG GGG TTC ATG TT	106	XM_042230932
RYR2	F: GGT GAA GCT TGT TGG TGG AT R: AAG CTG CCG TTA CCG TAA GA	140	XM_027962380.2
RYR3	F: ACA AAT TGC TCG CTG CTC TT R: GAT GCA GTG CAA AAC TTC CA	134	XM_015096914.3
ACTB	F: CTT CCT TCC TGG GCA TGG – MBH, AP R: GGG CAG TGA TCT CTT TCT GC	168	Kowalewska et al., 2017
	F: GCC AAC CG TGA GAA GAT GAC – ChP R: TCC ATC ACG ATG CCA GTG	122	Szczepkowska et al., 2021
GAPDH	F: AGA AGG CTG GGG CTC ACT – MBH, AP R: GG CAT TGC TGA CAA TCT TGA	134	Haziak et al., 2014
	F: TGA CCC CTT CAT TGA CCT TC – ChP R: GAT CTC GCT CCT GGA AGA TG	143	Kowalewska et al., 2017
HDAC1	F: CTG GGG ACC TAC GGG ATA TT R: GAC ATG ACC GGC TTG AAA AT	115	Kowalewska et al., 2017

ADORA1 – adenosine A1 receptor, *ADORA2A* – adenosine A2 receptor, *ADORA2B* – adenosine B2 receptor, *ADORA3* – adenosine A3 receptor, *RYR1* – ryanodine receptor 1, *RYR2* – ryanodine receptor 2, *RYR3* – ryanodine receptor 3; reference genes: *ACTB* – actin beta, *GAPDH* – glyceraldehyde-3-phosphate dehydrogenase, *HDAC1* – histone deacetylase 1

Results

Expression of *ADOR* and *RYR* genes in the ovine MBH, AP, and ChP

Of the four ADOR subtypes, only *ADORA1*, *A2B,* and *A3* were detected in the ovine MBH and AP (Figure 1A and B), while all subtypes were expressed in the ChP (Figure 1C). The order of their expression level in the studied structures was as follows: *ADORA3* > *A1* > *A2B* in the MBH, *ADORA1* > *A2B* = *ADORA3* in the AP, and *ADORA1* > *A3* > *A2A* = *A2B* in the ChP. Real-time RNA analysis detected the expression of RYRs (*RYR1*, *RYR2,* and *RYR3*) in all examined tissues (Figure 1A, B, C), however, their expression level varied only in the MBH (*RYR1* > *RYR2* = *RYR3*), while the differences in the AP and ChP were not significant.

Effect of caffeine on *ADOR* gene expression in the ovine MBH, AP, and ChP under basal and LPS challenge conditions

As shown in Figures 2 and 3, a single LPS injection significantly reduced ($P < 0.05$) mRNA levels of *ADORA1* and *A3* in the MBH (Figures 2A, D, respectively), and AP (Figure 2D, F, respectively) and *ADORA2A* in the ChP (Figure 3B) compared to control animals. Moreover, acute inflammation increased ($P < 0.05$) *ADORA2B* expression in all tissues tested. There was no effect of LPS treatment only in case of *ADORA1* and *A3* expression in the ChP.

Single injection of caffeine affected *ADORA1* in the ChP (Figure 3A) and *ADORA2B* in both AP (Figure 2E) and ChP (Figure 3C), and their expression was higher ($P < 0.05$) in C/CAF compared to control. The effect of caffeine on *ADORA2A* in the ChP was the opposite, as its expression was lower ($P < 0.05$) in C/CAF compared to controls (Figure 3B). No effect of caffeine alone was observed on *ADOR* expression in the MBH (Figures 2A, B, C), *ADORA3* in the AP and ChP (Figure 2F and 3D, respectively), and *ADORA1* in the AP (Figure 2D).

Caffeine injection after prior LPS treatment attenuated the effect of LPS on *ADORA1* in the MBH (Figure 2A) and *ADORA2B* in the MBH and ChP (Figure 2B and 3C, respectively). This effect was indicated by higher expression ($P < 0.05$) of *ADORA1* and lower ($P < 0.05$) of *ADORA2B* in the LPS/CAF group than in the LPS/C group. In contrast, caffeine applied in combination with LPS did not affect the expression of *ADORs* in the ovine AP.

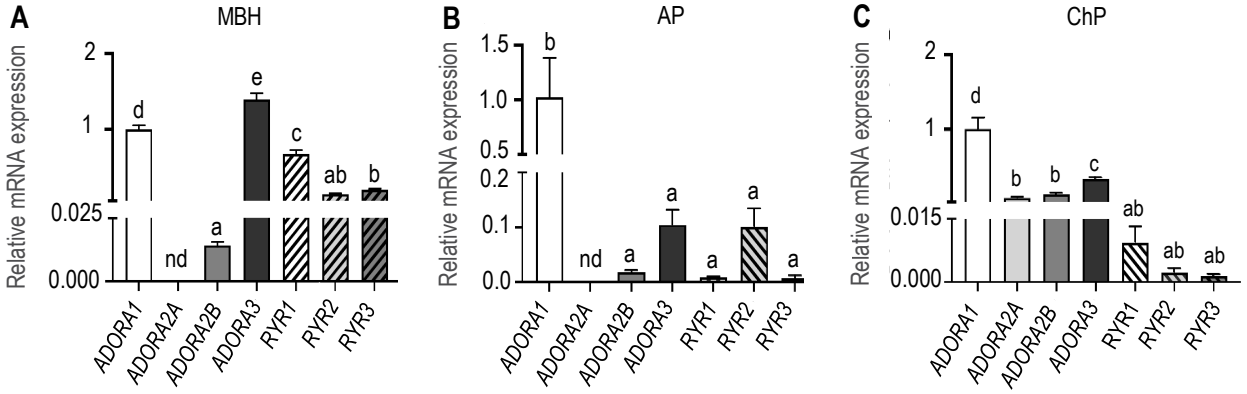

Figure 1. Relative mRNA expression of adenosine receptors in control ewes in the follicular phase of the oestrous cycle: *ADORA1* (white bars), *ADORA2A* (light grey bars), *ADORA2B* (medium grey bars), *ADORA3* (dark grey bars) and ryanodine receptors: *RYR1* (white hatched bars), *RYR2* (light grey hatched bars) and *RYR3* (medium grey hatched bars), in the mediobasal hypothalamus (MBH – A), anterior pituitary (AP – B) and choroid plexus (ChP – C). Data are presented as mean value ± SEM. Different letters indicate significant differences at $P < 0.05$, according to one-way ANOVA followed by Fisher's post hoc test comparing groups with each other.

ADORA1 – adenosine A1 receptor, *ADORA2A* – adenosine A2 receptor, *ADORA2B* – adenosine B2 receptor, *ADORA3* – adenosine A3 receptor, *RYR1* – ryanodine receptor 1, *RYR2* – ryanodine receptor 2, *RYR3* – ryanodine receptor 3, nd – not detected

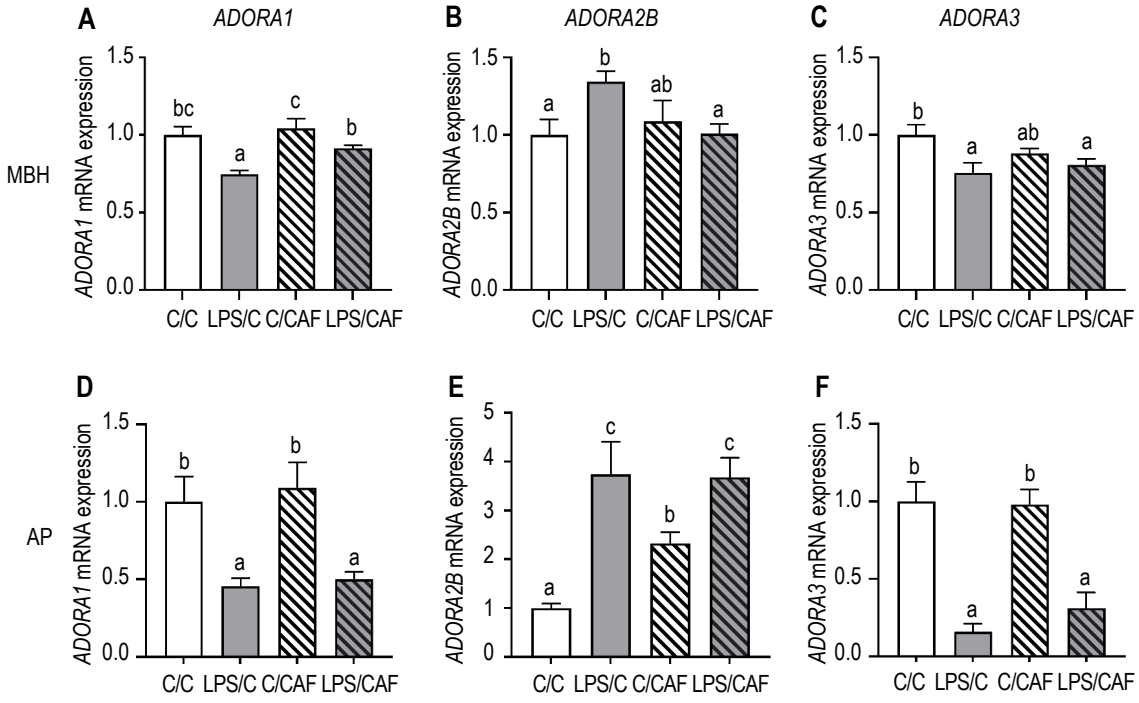

Figure 2. Relative mRNA expression of adenosine receptors: *ADORA1* (A and D), *ADORA2B* (B and E), *ADORA3* (C and F) in the mediobasal hypothalamus (MBH – top panels) and anterior pituitary (AP – bottom panels) in female sheep treated with saline (C/C, 0.9% NaCl, iv; white bars), lipopolysaccharide (LPS/C, 400 ng/kg body weight (bw), iv; grey bars), caffeine (C/CAF, 30 mg/kg bw, iv; white hatched bars) and LPS/CAF (grey hatched bars). Data are presented as mean value ± SEM. Different letters indicate significant differences at $P < 0.05$, according to one-way ANOVA followed by Fisher's post hoc test comparing groups with each other.

ADORA1 – adenosine A1 receptor, *ADORA2B* – adenosine A1 receptor, *ADORA3* – adenosine A3 receptor

Effect of caffeine on the expression of *RYR* genes in the ovine MBH, AP and ChP under basal and LPS challenge conditions

Single LPS injection decreased ($P < 0.05$) mRNA expression of *RYR2* in the MBH (Figure 4B) and did not affect *RYR* expression in the AP and ChP (Figure 4D–F and G, respectively), wherein the ChP, *RYR2,* and *RYR3* were not analysed due to their very low expression in these structures (Figure 1C).

Single caffeine injection increased ($P < 0.05$) *RYR1* expression in the AP (Figure 4D), and did

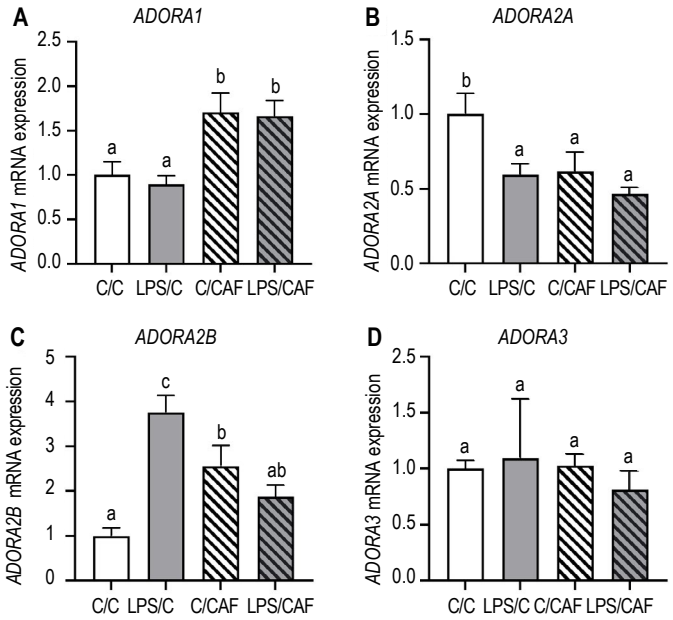

Figure 3. Relative mRNA expression of adenosine receptors: *ADORA1* (A), *ADORA2A* (B), *ADORA2B* (C), and *ADORA3* (D) in the choroid plexus in female sheep treated with saline (C/C, 0.9% NaCl, iv; white bars), lipopolysaccharide (LPS/C, 400 ng/kg body weight (bw), iv; grey bars), caffeine (C/CAF, 30 mg/kg bw, iv; white hatched bars) and LPS/CAF (grey hatched bars). Data are presented as mean value ± SEM. Different letters indicate significant differences at $P < 0.05$, according to one-way ANOVA followed by Fisher's post-hoc test comparing groups with each other.
ADORA1 – adenosine A1 receptor, *ADORA2A* – adenosine A2 receptor, *ADORA2B* – adenosine B2 receptor, *ADORA3* – adenosine A3 receptor

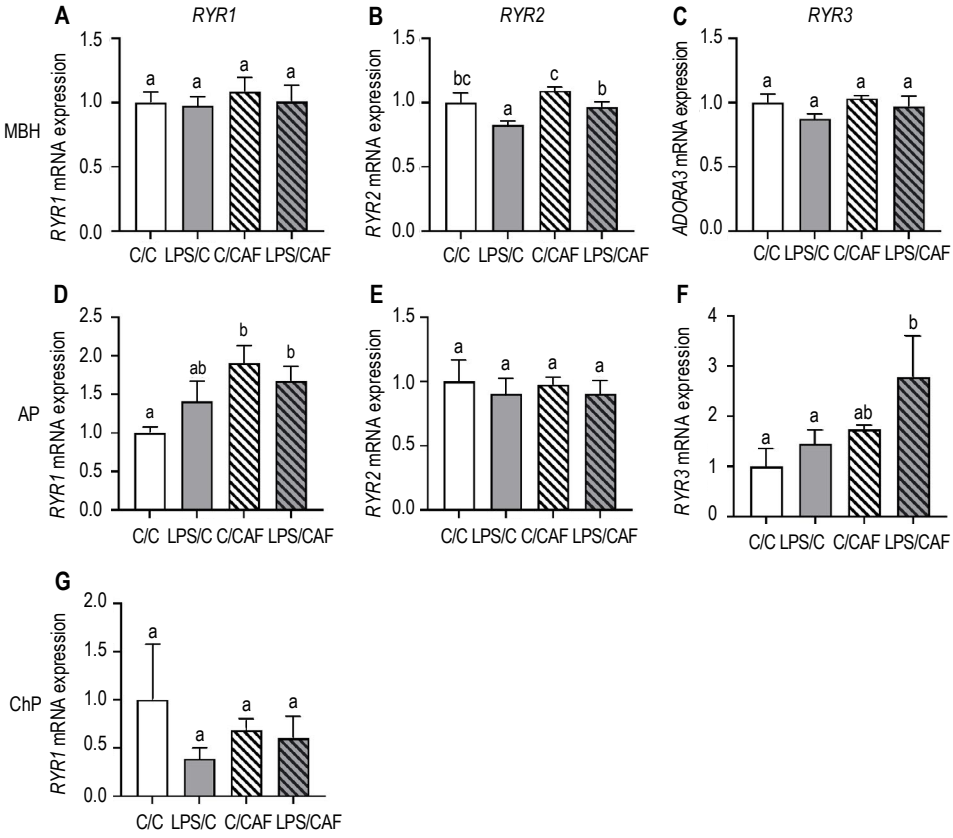

Figure 4. Relative mRNA expression of ryanodine receptors: *RYR1* (A, D and G), *RYR2* (B and E) and *RYR3* (C and F) in the mediobasal hypothalamus (MBH – top panels), anterior pituitary (AP – middle panels) and choroid plexus (ChP – bottom panel) in female sheep treated with saline (C/C, 0.9% NaCl, iv; white bars), lipopolysaccharide (LPS/C, 400 ng/kg body weight (bw), iv; grey bars), caffeine (C/CAF, 30 mg/kg bw, iv; white hatched bars) and LPS/CAF (grey hatched bars). Data are presented as mean value ± SEM. Different letters indicate significant differences at $P < 0.05$, according to one-way ANOVA followed by Fisher's post hoc test comparing groups with each other.
RYR1 – ryanodine receptor 1, *RYR2* – ryanodine receptor 2, *RYR3* – ryanodine receptor 3

not affect the *RYR* genes in the MBH and ChP (Figure 4A–C and G, respectively).

In contrast, the effect of LPS on *RYR2* in the MBH was attenuated by co-treatment with caffeine, as indicated by its higher ($P < 0.05$) expression in the LPS/CAF group than the LPS/C group (Figure 4B). Moreover, LPS in the co-treatment with caffeine elevated ($P < 0.05$) *RYR3* expression in the AP to a level higher than in the C/C and C/LPS groups, but not the C/CAF group (Figure 4F).

Discussion

Our study showed that mRNA encoding ADORs and RYRs are transcribed in the ovine MBH, AP, and ChP, but with different expression patterns. It should be noted that our experiments were conducted in tissues collected during the same time of day, which is important due to circadian changes in ADOR expression observed in the work of Jagannath et al. (2021). Among the examined *ADOR* genes, the highest expression was determined for *ADORA1* in the ovine ChP and AP, which was consistent with previous observations by Hackett et al. (2015). The latter authors reported that ADORA1 was generally the most abundant form of ADORs in the brain. Only in the MBH, the most highly expressed *ADOR* was *ADORA3*, but it should be noted that both ADORA1 and A3 belong to the same type of G protein-coupled receptors, G_i (Fredholm et al., 2011), hence they may be functionally complementary. In addition to the predominant expression of *ADORA3* in the ovine MBH, we found that *ADORA1* was still highly expressed in relation to the other analysed receptors. For further discussion, we referred to the data available in The Human Protein Atlas (HPA), an online database (https://www.proteinatlas.org; Sjöstedt et al., 2020) that compiles RNA sequencing data provided as nTPM units, i.e. number of transcripts per million (Zhao et al., 2021). According to the HPA, *ADORA1* is not expressed in the human ChP, but these results were based only on 4 ChPs from the elderly (67–91 years old); importantly, according to Modic et al. (1980), the ChP becomes significantly calcified with age. In contrast, both *ADORA1* and *A2A* were reported by another database: the Harmonizome (https://maayanlab.cloud/Harmonizome; Rouillard et al., 2016) to be expressed in the ChP, with a predominance of the second receptor subtype. Other sources also indicated that ADORA2A was most abundant in the human ChP (Schiffmann et al., 1991); however, no data are available on *ADORA1* and *A2A* mRNA in non-human or rodent ChP tissues. With respect to the hypothalamus and pituitary, the HPA data indicated species-specific variation in *ADORA1* expression, with hypothalamic expression at 23 nTPM, 16.7 nTPM and 47.3 nTPM, while in the pituitary gland at 1.9 nTPM, 0.6 nTPM and 6.6 nTPM in human, pig, and mouse, respectively (https://www.proteinatlas.org).

The absence of *ADORA2A* transcript in the ovine MBH observed in the present study was consistent with the results of *in situ* hybridization of Schiffmann et al. (1991). However, due to the development of research techniques, we considered the HPA data, showing that ADORA2A is expressed in the hypothalamus, but at different levels depending on the area, as relevant. This source reported that the highest expression of ADORA2A protein in the mouse hypothalamus was recorded in the suprachiasmatic nucleus, while the lowest in the preoptic area. Moreover, the same HPA source demonstrated significant interspecies differences in the average expression levels of *ADORA2A* mRNA in the human (7.3 nTPM), pig (0.7 nTPM), and mouse (3.7 nTPM) hypothalamus. Therefore, we suggest that the lack of *ADORA2A* mRNA in the ovine MBH may be associated with the hypothalamic areas, or may be species-related. Moreover, the same absence of *ADORA2A* observed in the ovine AP seems to be species-related, while the HPA source demonstrates its very low levels in the pig (0.4 nTPM) and mouse (0.6 nTPM) pituitary, and approx. 10-fold higher expression in humans (6.7 nTPM) (https://www.proteinatlas.org). As regards *ADORA2B* and *A3* expression in the ovine ChP, MBH and AP, it can be considered consistent with the data presented in the HPA. However, as in the previous cases, the HPA shows interspecies differences between humans, pigs, and mice.

All three RYR isoforms were shown to be present in the mammalian brain, with RyR2 being the most abundant (Abu-Omar et al., 2018), but these data did not distinguish between different brain regions. We found confirmation of RYR expression in the hypothalamus in the HPA database, but again with significant interspecies differences. For example, in humans, the nTPM values for RYR1, RYR2, and RYR3 were 20.5, 1.5, and 29.3, while in mice 0.1, 15, and 1.6, respectively; the data for RYR1 in pigs is missing and RYR2 and RYR3 expression was recorded at the level of 9.1, and 3.4 nTPM. Therefore, the highest expression of *RYR1* in the ovine MBH may be species-specific. According to Sundaresan et al. (1997), only type 2 and 3, but not type 1 RYRs were expressed in the rat pituitary; however, these results were acquired using regular PCR, while we detected the presence of mRNA of all 3 RYR types in the ovine AP using more sensitive real-time PCR. It is possible that once more these discrepancies were caused by interspecies

differences, as the HPA indicated that *RYR1* was expressed in the human pituitary (1.5 nTPM) but not in the mouse counterpart (0 nTPM). We observed an interesting situation regarding *RYR2* and *RYR3*, whose expression in the ChP was at such a low level that we did not consider these genes in further analysis. Although the HPA indicated a lack of *RYR2* expression (0 nTPM) in the ChP, it reported a high level of *RYR3* expression (33 nTPM). However, the HPA data related to ChP were again derived only from humans and based on 4 samples. Therefore, our results point to species-specific expression. It should be noted that the RYR expression pattern in the tissue could be also affected by the time of day this tissue was collected, as RYR expression undergoes circadian fluctuations, and generally, RYR protein levels are elevated during the day and highest at dusk (Gamble and Ciarleglio, 2009).

Our experiment showed that LPS-induced inflammation influenced *ADOR* gene expression, however, this effect seemed to be tissue-dependent. Endotoxin treatment suppressed *ADORA1* mRNA expression in the MBH and AP, whereas it did not affect this gene expression in the ChP. The inhibitory effect of LPS treatment on the *ADORA1* gene expression has been previously found in the study on human lung macrophages (Buenestado et al., 2010). However, a low-dose endotoxin conditioning experiment increased the expression of macrophage *ADOR* genes, including *ADORA1* (Murphy et al., 2017). ADORA1 plays an inhibitory role and is involved in the reduction of metabolic and secretory activity of many cells. Stimulation of ADORA1 leads to the inhibition of adenyl cyclase and reduction of cAMP production, as well as the inhibition of cAMP-dependent kinase in neurons to modulate the release of neurotransmitters and neuropeptides (Chen et al., 2014). Although the involvement of ADORA1 during inflammatory processes is not completely clear, the study on mice showed that animals with *ADORA1* knockout were characterized by reduced oxidative stress and inflammatory responses, including IL-1β, IL-6, and TNFα production (Yang et al., 2015). Therefore, the reduction of this receptor gene expression could be a mechanism protecting against the excessive production of pro-inflammatory cytokines and reactive oxygen species in the brain tissue during inflammation. Although *ADORA2A* expression was not found in the brain tissues tested, administration of LPS was shown to reduce the expression of this gene in the ChP. ADORA2A seems to be the most studied in terms of its anti-inflammatory functions and actions. Anti-inflammatory properties of ADORA2A have been demonstrated in many cells, including macrophages, neutrophils, T cells, NK T cells, dendritic cells, and T regulatory cells, where ADORA2A signalling has been associated with inhibition of proliferation, secretion of inflammatory cytokines, and stimulation of anti-inflammatory cytokines synthesis (Karmouty-Quintana et al., 2013). ADORA2A expression has also been found to be regulated by several inflammatory stimuli. It has been shown to be enhanced by factors stimulating activation of such factors as TNF, IL-1, or endotoxin (Cronstein and Sitkovsky, 2017). Previously mentioned studies on human lung macrophages (Buenestado et al. 2010) and mice macrophages (Murphy et al., 2017) also reported a stimulatory effect of LPS treatment on *ADORA2A* gene expression. Therefore, elucidating the mechanism leading to the downregulation of *ADORA2A* gene expression in the ChP observed in our study requires further in-depth research. On the other hand, we found that endotoxin-induced inflammation increased *ADORA2B* gene expression in all examined tissue. This receptor was also demonstrated to play important anti-inflammatory functions in many tissues. The study on transgenic *ADORA2B* knockout mice showed that these animals were characterized by increased production of pro-inflammatory cytokines and reduced synthesis of anti-inflammatory cytokines during the inflammatory and physiological state (Yang et al., 2006). Since these receptors play anti-inflammatory and tissue-protective roles (Karmouty-Quintana et al., 2013), increased *ADORA2B* gene expression in the ovine tissue found in the present study could be another mechanism against excessive development of the inflammatory response. It was also determined that inflammation suppressed *ADORA3* gene expression in the MBH, and particularly strongly in the AP, while it showed no effect in the ChP. ADORA3 was also shown to be involved in anti-inflammatory response, and it was found that the activation of this receptor inhibited the production of inflammatory cytokines, including IL-1, IL-6, and TNFα by downregulating nuclear factor-kappa B signalling (Cohen and Fishman, 2019; Ren et al., 2020). In contrast to the results of the aforementioned ADORAs, the observed downregulation of *ADORA3* gene expression in the AP could result in increased local production of inflammatory cytokines in brain tissue.

Peripheral administration of caffeine did not affect *ADOR* gene expression in the hypothalamus, whereas it increased *ADORA2B* mRNA levels in the AP and ChP. Moreover, caffeine treatment also increased *ADORA1*, but reduced *ADORA2A* gene expression in the ChP. The ability of caffeine

to modulate the expression of *ADOR* genes has been previously described in the lungs of rat pups (Endesfelder et al., 2020). However, in the present study, caffeine increased the expression of adenosine receptors, i.e. *ADORA1*, *2A*, and *2B*. The effect of caffeine on the expression of *ADOR* genes may be dependent on the tissue and caffeine dose used in the study. The experiment in human parathyroid cells showed that low caffeine doses did not influence *ADORA1* mRNA levels in these cells, but higher caffeine doses downregulated the expression of this receptor gene. It should be noted that *ADORA2A* gene expression in this study did not change regardless of the dose of caffeine applied (Lu et al., 2013).

Our study demonstrated that caffeine administration reduced the inhibitory effect of caffeine on *ADORA1* mRNA expression and completely abolished the LPS-dependent increase in *ADORA2B* gene expression in the MBH; caffeine also reduced the stimulatory effect of inflammation on *ADORA2B* mRNA levels in the ChP. The latter effect of caffeine on the expression of these genes in the ChP during endotoxin-induced inflammation could be partly due to its ability to modulate the expression of pro-inflammatory cytokines. Our previous study in sheep showed that caffeine treatment inhibited IL-1β and TNFα synthesis in the hypothalamus, and *TNF* gene expression in the ChP (Szczepkowska et al., 2021). This in turn could affect mRNA levels of *ADOR* genes because many reports suggested an important role of pro-inflammatory cytokines in modulating the expression of these receptors (Stamp et al., 2012). However, a better understanding of caffeine's modulating effect on ADOR expression during inflammation certainly requires further comprehensive studies.

In contrast to ADORs, the expression of another group of caffeine-interacting receptors – RYRs – was significantly more stable and less sensitive to the effects of inflammation. Of the tissues studied, only *RYR2* gene expression in the MBH was found to be altered, as it decreased during the endotoxin-induced inflammation. Although the regulatory role of inflammation on RYR2 expression is not well described, a study in rats showed that the reduction of active IL-6 by anti-IL-6 antibodies increased RYR2 expression in atrial tissue (Liao et al., 2021). Furthermore, the increased expression of *RYR1* mRNA found in our study only in the AP after caffeine treatment has suggested that short-term exposure could affect RYR1 expression in a tissue-dependent manner. Moreover, our study has indicated that inflammatory conditions may influence the effect exerted by caffeine on *RYR3* gene expression in the AP, as caffeine administration did not affect *RYR3* mRNA levels in intact ewes, whereas caffeine in LPS-treated animals increased the expression of this receptor gene. Since caffeine is a potent RYR3 agonist (Kong et al., 2008), this result suggests that caffeine may more strongly induce Ca^{2+} release from intracellular storage sites in AP cells during inflammation. However, due to the novel nature of these findings, and the lack of data in the available literature on the effect of the immune status on RYR expression, a comprehensive explanation of these observations is not yet possible.

Conclusions

In summary, our experiment has shown that caffeine can affect mRNA expression of ADOR and RYR receptors in brain tissues, but this effect seems to be tissue-dependent and perhaps also related to the immune status of the animals. However, the physiological implications of these results require further detailed studies.

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Conflict of interest

The Authors declare that there is no conflict of interest.

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