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Allopregnanolone reduces neuroendocrine response to acute stressful stimuli in sheep

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Abstract

The verified hypothesis assumed that centrally administered neurosteroid, allopregnanolone (AL), could affect basal and/or stress-induced activity of the hypothalamic-pituitary-adrenal (HPA) axis in sheep. Four groups ($n = 6$ each) of luteal-phase sheep were intracerebroventricularly infused for 3 days with a vehicle without stress (control); a vehicle treated with stressful stimuli (isolation and partial movement restriction) on the third day; AL ($4 \times 15 \mu\text{g}/60 \mu\text{L}/30 \text{ min}$, at 30-min intervals) treated with stressful stimuli, and AL alone. Simultaneously, the push-pull perfusion of the infundibular nucleus/median eminence and plasma sample collection were performed. After the experiment, the sheep were killed to collect the hypothalamic and anterior pituitary (AP) tissues. Stressful stimuli evoked an increase in the expression of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) mRNA in the hypothalamic paraventricular nucleus (PVN), and AVP receptor (V1b) and proopiomelanocortin (POMC) mRNA in the AP; the concentrations of perfusate CRH, and plasma adrenocorticotrophic hormone (ACTH) and cortisol compared to controls. Conversely, the expression of the CRH receptor (CRHR1) mRNA in the AP was downregulated. AL decreased the expression of CRH and AVP mRNA in the PVN, and AVPRV1b and POMC mRNA in the AP in stressed sheep, compared to only stressed ones. There was also a reduction in perfusate CRH, and plasma ACTH and cortisol concentrations. AL alone decreased the expression of CRHR1 mRNA in the AP, and plasma cortisol concentration at the beginning of the collection period compared to controls. In conclusion, AL may function centrally as a suppressor of HPA axis activity in stressed sheep.

Key Words

- ▶ allopregnanolone
- ▶ intracerebroventricular infusion
- ▶ stress response
- ▶ HPA axis

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Introduction

The neuroendocrine response of the hypothalamic-pituitary-adrenal (HPA) axis to stress allows the animal to adapt to adverse conditions, restoring physiological homeostasis (McEwen 2007). Conversely, prolonged exposure to stressful stimuli may cause pathological changes in the organism (Vyas *et al.* 2016). The main central neurohormones that initiate a stress response include corticotropin-releasing hormone (CRH) and

arginine vasopressin (AVP), which are synthesized primarily in the hypothalamic paraventricular nucleus (PVN). Both neuropeptides are involved in the regulation of adrenocorticotrophic hormone (ACTH) secretion from the anterior pituitary (AP). The actions of CRH and AVP in the AP corticotrophs are mediated by specific G protein-coupled receptors: CRHR1 and V1b respectively (Nikodemova *et al.* 2002, Jasnic *et al.* 2013). ACTH then

stimulates the adrenal glands to synthesize and release glucocorticoids (GCs) and mineralocorticoids, which may act at multiple targets in the body to facilitate the necessary metabolic and behavioral adaptations (McEwen 2007). In addition, GCs provide negative feedback control of the HPA axis at the central and pituitary levels, terminating the stress response. Moreover, the activity of CRH neurons in the PVN is tightly regulated by inhibitory γ -aminobutyric acid (GABA)ergic synaptic input (Decavel & Van den Pol 1990, Herman *et al.* 2004) that, to a large extent, originates in adjacent interneuron populations (Boudaba *et al.* 1996). Recent studies have shown that GCs may also influence the GABAergic control of CRH neurons by the upregulation of GABA_A receptors (Colmers & Bains 2018).

In the mammalian brain, several neuroendocrine factors are formed that are able to reduce the stress response in itself or limit its negative effects. Their action is particularly important in a situation of acute stress, as well as during pregnancy and lactation, protecting the fetus or newborn from excess GC (Brunton 2016, Hasiec & Misztal 2018). The results of previous studies on rodents indicated a specific role of brain-synthesized steroids, which are a component of the mechanism that allows stress coping (McEwen 2007). Synthesis of these compounds occurs in numerous glial and nerve cells of the central nervous system (CNS) through conversion of blood-derived precursors or *de novo* from cholesterol (Baulieu 1991, Paul & Purdy 1992). The most recognized neurosteroid is allopregnanolone (AL), a product of progesterone conversion by 5α -reductase and 3α -hydroxysteroid dehydrogenase (Melcangi *et al.* 1993). The results of previous studies on rats and mice have indicated the inhibitory properties of AL in relation to the HPA axis (Brunton *et al.* 2009, Gunn *et al.* 2013). These are reached by potent neurosteroid binding to membrane-bound GABA_A receptors, where they enhance GABA-mediated inhibitory action on PVN neurons (Sarkar *et al.* 2011). Moreover, AL activated and maintained endogenous opioid inhibition of HPA axis responses to stress in late-pregnant rats (Brunton *et al.* 2009).

Studies on AL function in adult farm animals are rare, although they could have a great impact on humans. Some studies performed on sheep focused mainly on the role of this compound in the developing brain of the fetus and neonate. In general, it was shown that neuroactive steroids had potent suppressive effects on the CNS of fetal sheep (Nicol *et al.* 1999). The results of another study indicated that AL modulated the constitutive rate of apoptosis and proliferation in the hippocampus and

cerebellum of the fetal brain (Yawno *et al.* 2009). It was also found that stress evoked by the lipopolysaccharide administration induced an increase in AL concentration in the newborn brain, which subsequently contributed to somnolence (Billiards *et al.* 2002). In the present study, which belongs to the field of research on the effects and prevention of stress, we verified the hypothesis that a centrally administered AL could affect basal and/or stress-induced activity of the HPA axis in sheep. As sheep are herd animals, a few hours of isolation from the herd in combination with partial restriction of movement were used as the acute stress stimuli (Hasiec *et al.* 2014).

Materials and methods

Animal management

All animal procedures were conducted in accordance with the Polish Act on the Protection of Animals Used for Scientific or Educational Purposes (2015) and were approved by the 2nd Local Ethics Committee for Animal Experiments, Warsaw University of Life Sciences – SGGW, Warsaw, Poland (Resolution No. WAW2-24/2016). Twenty-four Polish Longwool sheep (a breed representing reproduction seasonality), aged 2 years and weighing 55 ± 3 kg were used in the experiment. They were farmed at the Sheep Breeding Center of the Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences. Throughout the experiment they were kept indoors in individual pens under natural lighting conditions (52°N , 21°E). The animals were fed twice a day with a diet based on pelleted concentrate according to the recommendations of the National Research Institute of Animal Production (Krakow–Balice, Poland) – National Institute for Agricultural Research (France) (Strzetelski 2009). All sheep had free access to hay, water and mineral licks.

Brain surgery

Sheep were bilaterally implanted with two stainless steel guide cannulae: one into the infundibular nucleus/median eminence (IN/ME), (outer diameter 1.6 mm, positions: frontal 29.5–30.5 mm and sagittal 1.0 mm) and the second into the third brain ventricle (IIIv), (outer diameter 1.2 mm, positions: frontal 30.5–31.5 mm and sagittal 1.0 mm), in accordance with the stereotaxic coordinate system for the sheep hypothalamus (Welento *et al.* 1969). The implantation was performed under general anesthesia

(xylazine: 40 mg/kg of body mass, intravenously; xylapan and ketamine: 10–20 mg/kg of body mass, intravenously; Bioketan, Vetoquinol Biowet, Pulawy, Poland) through a hole drilled in the skull, in accordance with the procedure described by Traczyk & Przekop (1963). Guide cannulae were fixed to the skull with stainless steel screws and dental cement. The external opening of the canal was closed with a stainless steel cap. After the surgery, ewes were injected daily with antibiotics for 5 days (1 g streptomycin and 1,200,000 IU benzylpenicillin; Polfa, Warsaw, Poland) and with analgesics for 4 days (metamizole sodium 50 mg/animal; Biovetalgin, Biowet Drwalew, Drwalew, Poland, or meloxicam 1.5 mg/animal; Metacam, Boehringer Ingelheim, Ingelheim am Rhein, Germany). Placement of the cannulae into the IIIv was confirmed by the outflow of CSF during surgery, and placement into the IN/ME was confirmed after slaughtering. The sheep used in the present study had the correctly localized cannulae.

Drug preparation

A day before infusion, AL (Sigma-Aldrich) was dissolved in a mixture (1:1) of DMSO (Blirt, DNA-Gdańsk, Gdańsk, Poland) and 20% 2-hydroxypropyl- β -cyclodextrin (Sigma-Aldrich). After 24 h, the mixture was diluted in a Ringer-Locke (RL) solution and divided into portions for individual sheep. The mixture without AL was diluted in RL and used as a control vehicle. Each portion of the infusion mixture contained 2% DMSO.

Experimental design

The experiment was conducted from mid-October to mid-December, during the reproductive season. Estrus synchronization was performed using the Chronogest-CR method. More specifically, polyester polyurethane sponges containing 20 mg flugestone acetate (Intervet, Boxmeer, The Netherlands) were inserted in the sheep intravaginally for 14 days and then removed at the time of the pregnant mare's serum gonadotropin (PMSG) injection (500 i.u., Folligon, Intervet). Estrus occurred within 24–36 h of PMSG administration, and its duration was additionally controlled using a teaser ram. The sheep were randomly divided into four groups ($n=6$ each) and all treatments were conducted over three consecutive days of the late luteal phase (days 12–14) of the estrous cycle. The treatments were as follows: 1) intracerebroventricular (i.c.v.) infusion of a vehicle for 3 days (C group); 2) i.c.v. infusion of a vehicle and use of stressful stimuli in the 3rd day (CS group); 3) i.c.v. infusion of AL and use of stressful

stimuli in the 3rd day (AS group); and, 4) i.c.v. infusion of AL alone for 3 days (A group). All infusions were performed in a series of four 30-min infusions, at 30-min intervals (from 10:00 to 14:00 h). The dose of AL, $4 \times 15 \mu\text{g}/60 \mu\text{L}/30 \text{ min/day}$ was selected on the basis of our preliminary study (unpublished data, Grant No. 2015/19/B/NZ9/03706). A BAS Bee microinjection pump (Bioanalytical Systems, West Lafayette, IN, USA) and calibrated 1.0-mL gas-tight syringes were used. Stressful stimuli included isolation from other sheep and partial movement restriction in the experimental cage (protection against escaping from the cage). The duration of stressful stimuli covered the period of infusions (4 h).

Simultaneously, the IN/ME was perfused with Ringer-Locke solution using the push–pull method with a rate of flow of $10 \mu\text{L}/\text{min}$. The total perfusion time was 5 h including 1 h preperfusion (from 09:00 to 10:00 h) to eliminate CRH release artifacts caused by the insertion of the cannula. The proper 4-h long perfusion (from 10:00 to 14:00 h) allowed the gathering of eight perfusates (every 30 min). Each $300 \mu\text{L}$ perfusate was collected in a separate tube, which contained $30 \mu\text{L}$ aprotinin (5–10 TIU per mL, Sigma-Aldrich) to prevent peptide degradation. Immediately after the tubes were filled, they were frozen in liquid nitrogen and kept at -80°C until the CRH concentration was assayed. All perfusions were executed with the CMA 402 pump (CMA, Stockholm, Sweden). The combination of two experimental techniques: the i.c.v. infusion and push–pull perfusion of the IN/ME, allows to observe direct changes in the secretory activity of the hypothalamic-pituitary axis that occur as a result of the central administration of the test substance (Hasiec *et al.* 2014). During the treatments, sheep were kept in comfortable experimental cages, where they could lie down and to which they were previously adapted.

Blood sample and brain tissue collection

Blood samples were collected during the 4-h long period (from 10:00 to 14:00 h) through a catheter that was inserted into the jugular vein a day before collection. Five milliliters of blood were taken every 10 min (total 125 mL) and immediately divided into two portions: one with heparin ($30 \mu\text{L}$, 500 units/mL; Polfa) to measure cortisol concentration and the other with EDTA ($100 \mu\text{L}$, 0.5 M, Sigma-Aldrich) to measure ACTH concentration. After centrifugation, the plasma was stored at -20°C until the hormones were assayed.

Immediately after the experiment, sheep were slaughtered and the brains, along with pituitaries,

were rapidly removed from the skull. After separation of the ME, each brain was sectioned sagittally into the cerebral hemispheres. The isolated blocks of the hypothalamus (cutting to a depth of 2 mm) were dissected into three parts: preoptic area, anterior hypothalamus (AHA) and mediobasal hypothalamus (Ciechanowska *et al.* 2009), according to the stereotaxic atlas of the ovine brain (Welento *et al.* 1969). Landmarks included the optic chiasm, thalamus and mammillary body. Subsequently, the PVN was isolated from the AHA by point cut-out to a depth of 1–1.5 mm. In addition, tissue of the anterior pituitary (AP) was collected. All tissue cuts were performed on sterile glass plates placed on ice, and the collected structures were frozen immediately in liquid nitrogen and then stored at -80°C .

Relative abundance of mRNA analysis

Total RNA from the hypothalamic and AP tissues was isolated using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) 4×4500, according to the manufacturer's protocol. The concentration and purity of isolated RNA were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). RNA integrity was electrophoretically verified using 1.5% agarose gel stained with ethidium bromide. cDNA was synthesized using the TransScriba Kit (A&A Biotechnology, Gdynia, Poland) 10×1000 according to the manufacturer's instructions. For this synthesis, 1 µg of total RNA was used in a reaction volume of 20 µL.

Quantitative polymerase chain reaction (qPCR) was performed with 5× HOT FIREPol® EvaGreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia). The PCR

amplification mix contained 2 µL cDNA template, 1 µL primers (0.5 µL each, concentration of 10 pmol/mL), 3 µL buffer PCR Master Mix and 9 µL dd H₂O. Reaction conditions were as follows: initial denaturation at 95°C for 15 min, denaturation at 95°C for 15 s, annealing at 60°C for 20 s, and elongation at 72°C for 20 s (40 cycles). Specific primers for determining the expression of genes of interest (*CRH* and *AVP* in the PVN and *CRHR1*, *AVPR1b* and *POMC* in the AP), as well as endogenous control genes (glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and peptidylprolyl isomerase C (*PPIC*)) were designed using Primer3 software (The Whitehead Institute, Boston, MA, USA). Respective sequences of the primers are listed in Table 1. In order to confirm that single amplification products were amplified, the samples were stained with ethidium bromide and subjected to electrophoresis on an agarose gel before visualization under a UV light camera.

Data were analyzed with the Rotor Gene 6000 v. 1.7 software (Qiagen) using the comparative quantification option and determined using the Relative Expression Software Tool (2008) according to Pfaffl *et al.* (2002), based on a PCR efficiency correction algorithm developed by Pfaffl *et al.* (2004). The levels of gene expression were normalized using the geometrical means of two reference gene expressions *GAPDH* and *PPIC*. Both endogenous control genes were assayed in each sample to compensate for cDNA concentration variation and PCR efficiency between individual tubes.

Hormone concentration assay

The CRH concentration in the CSF was determined using a commercial ELISA kit CSB-EL005963SH

Table 1 Sequences of specific primers.

Gene	Primers (5'–3')	GenBank Acc. No.	Amplicon size
<i>CRH</i>	F: CAGAAGCACCTCAGAAGCGC R: GCGGCTGGAAGAACTCAGG	XM_015097787.1	176
<i>AVP</i>	F: TCCGACCTGGAGCTGAGA R: GGCAGGTAGATCTCCTCTTGG	NM_001126341.1	146
<i>CRHR1</i>	F: AATGAGAAGTGCTGGTTGGC R: GGTCATGAGGATGCGAACGA	NM_001009727.1	126
<i>AVPR1B</i>	F: GACATAGTCAGCGACGGGAG R: GGAGTGAGATAGACGGCAGC	NM_001246237.1	138
<i>POMC</i>	F: GTAACCTGCTGGCGTGAT R: GAAGCTGCTGCTACCATTC	NM_001009266.1	167
<i>PPIC</i>	R: TGGAAAAGTCGTGCCAAGA R: TGCTTATACCACAGTGCCA	XM_004008676.1	158
<i>GAPDH</i>	F: GGGTCATCATCTCTGCACCT R: GGTCATAAGTCCCTCCACGA	NM_001190390.1	131

The real-time PCR amplification efficiencies of target and reference genes was 96–101%.

AVP, arginine vasopressin; AVPR1B, AVP receptor 1B; CRH, corticotropin-releasing hormone; CRHR1, CRH receptor 1; F, forward primer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; POMC, proopiomelanocortin; PPIC, peptidylprolyl isomerase C (cyclophilin C); R, reverse primer.

(Cusabio, Wuhan, China) according to the manufacturer's protocol. The minimum detectable dose of CRH was less than 14 pg/mL, and the intra- and inter-assay coefficients of variation (CVs) were <15 and <15% respectively.

The plasma ACTH concentration was determined using a commercial ELISA kit CSB-EQ027618SH (Cusabio) according to the manufacturer's protocol. The minimum detectable dose of ACTH was less than 1 pg/mL, and the intra- and inter-assay CVs were <8 and <10% respectively.

The plasma cortisol concentration was measured using a radioimmunoassay (RIA), according to Kokot & Stupnicki (1985), using rabbit anti-cortisol antisera (R/75) and a cortisol standard (Sigma-Aldrich). The assay sensitivity was 0.95 ng/mL, and the intra- and inter-assay CVs were <10 and <12% respectively.

Statistical analysis

Initially, all data were tested for normality by the Shapiro-Wilk normality test and then grouped into parametric and non-parametric. Perfusate CRH, as well as plasma ACTH and cortisol concentrations over time were analyzed using one-way ANOVA (STATISTICA, Stat Soft, Tulsa, OK, USA) or, where appropriate (hourly estimation), by the two-way ANOVA with repeated measures, with time and treatment as factors of repeated measurements. Each analysis was followed by the *post hoc* least significant difference test. CRH data from the preperfusion period were not included in the statistical analysis.

Statistical evaluations of differences in CRH and AVP mRNA expression in the PVN, as well as in CRHR1, AVPRV1b and POMC mRNA expression in the AP between treatment groups were carried out using non-parametric statistics: the Kruskal-Wallis test, followed by multiple comparisons of average ranks and then the Mann-Whitney *U* test for particular groups. Differences were considered significant at $P < 0.05$ and all data are presented as a mean $\pm$ S.E.M.

Results

Relative abundances of mRNA in the hypothalamus and AP tissues

The relative abundances of both CRH and AVP mRNA in the PVN were compared between control and treatment groups (Fig. 1). The abundance of *CRH* gene transcripts increased ($P < 0.01$) in the CS group, compared to the control group. Likewise, the content of *AVP* gene transcripts

Figure 1

Relative mRNA expression (mean $\pm$ S.E.M.) of corticotropin-releasing hormone (CRH, top) and arginine vasopressin (AVP, bottom) in the hypothalamic paraventricular nucleus of sheep treated with: vehicle (C), vehicle and stressful stimuli (CS), allopregnanolone and stressful stimuli (AS) and allopregnanolone alone (A). Significance of differences: * $P < 0.05$, ** $P < 0.01$.

was higher ($P < 0.01$) in the CS group than in controls. In the AS group, there was a reduction in abundance of both the *CRH* ($P < 0.01$) and *AVP* ($P < 0.01$) transcripts in comparison with the CS group. Interestingly, the relative abundance of *CRH* mRNA in the AS group was higher ($P < 0.01$), whereas that of *AVP* mRNA was lower ($P < 0.01$), compared to controls. AL infused alone caused a decrease in the relative abundance of *CRH* mRNA in the PVN, compared to controls ($P < 0.05$) and had no effect on *AVP* mRNA content. This latter, however, was less compared to the CS group ($P < 0.01$) and higher compared to the AS group ($P < 0.05$).

The relative abundances of *CRHR1*, *AVPRV1b* and *POMC* mRNA in the AP of sheep from all treatment groups are shown in Fig. 2. A decrease ($P < 0.01$) was observed in the abundance of *CRHR1* mRNA in the CS, AS and A groups, compared to controls. On the contrary, the expression of *AVPRV1b* mRNA increased ($P < 0.01$) in response to stressful stimuli. In the AS group, there was reduction in the abundance of *AVPRV1b* mRNA ($P < 0.01$), in comparison with the CS group, while AL alone was without effect, compared to controls.

The relative abundance of *POMC* mRNA in the AP increased ($P < 0.05$) in response to stressful stimuli

Figure 2

Relative mRNA expression (mean ± S.E.M.) of corticotropin-releasing hormone receptor 1 (CRHR1, top), arginine vasopressin receptor V1b (AVPRV1b, middle) and proopiomelanocortin (POMC, bottom) in the anterior pituitary of sheep treated with: vehicle (C), vehicle and stressful stimuli (CS), allopregnanolone and stressful stimuli (AS) and allopregnanolone alone (A). Significance of differences: * $P < 0.05$, ** $P < 0.01$.

(CS group), compared to controls. In the AS group, there was reduction in the abundance of *POMC* gene transcripts in comparison with the CS group ($P < 0.01$), reaching a level similar to controls. The abundance of *POMC* mRNA in the A group was also less than in the CS group ($P < 0.01$) but similar to those observed in the C and AS groups.

Hormone concentration

The mean perfusate CRH concentrations in sheep from all groups are shown in Fig. 3 and profiles of changes in mean CRH concentrations in consecutive perfusates during the collection period are shown in Fig. 4. Mean CRH concentration in the CS group was higher ($P < 0.001$) than in the control (non-stressed) group. The increased CRH

Figure 3

Perfusate corticotropin-releasing hormone (CRH) concentrations (mean ± S.E.M.) in sheep treated with: vehicle (C), vehicle and stressful stimuli (CS), allopregnanolone and stressful stimuli (AS) and allopregnanolone alone (A). Significance of differences: * $P < 0.05$, *** $P < 0.001$.

concentration in stressed sheep persisted for 3 h ($P < 0.01$ – $P < 0.001$ up to the sixth perfusate) of the collecting period compared to controls. The CRH concentration in the AS group was also higher ($P < 0.001$) than in the C group: from $P < 0.05$ to $P < 0.001$ up to the fourth perfusate. There was, however, a decrease ($P < 0.05$) in mean CRH concentration in the AS group compared to the CS group: the highest difference ($P < 0.05$) was noted in the sixth perfusate. Interestingly, the second rise in CRH concentration in the AS group was observed in the seventh perfusate ($P < 0.05$, compared to controls). In the A group, perfusate CRH concentration was similar to controls, whereas it was less ($P < 0.001$) compared to the concentrations in the CS and AS groups.

Due to the characteristics of hormone response to acute stress (long-term irregular surges with high amplitudes), plasma concentrations of ACTH and cortisol were compared among groups in 1-h periods, without

Figure 4

Corticotropin-releasing hormone (CRH) concentrations (mean ± S.E.M.) in the consecutive perfusates (eight perfusates) over a period of collection from 10:00 to 14:00 (every 30 min) in sheep treated with: vehicle (C), vehicle and stressful stimuli (CS), allopregnanolone and stressful stimuli (AS) and allopregnanolone alone (A). Significance of differences: ** $P < 0.01$, and *** $P < 0.001$ CS group vs C group; # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ AS group vs C group; † $P < 0.05$ AS group vs CS group.

calculating the frequency and amplitude of the pulses. The comparisons are depicted in Figs 5 and 6 respectively. There were increases in both plasma ACTH ($P < 0.001$) and cortisol ($P < 0.05$ – $P < 0.001$) concentrations in the CS group, which persisted until the end of the experiment in comparison with the C group. In the AS group, plasma concentration of ACTH began to decrease ($P < 0.001$) from the second hour of the experiment onward compared to the CS group. The concentration in the AS group was, however, still higher during the subsequent hours than concentrations noted in the C group ($P < 0.001$ in the 2nd, $P < 0.01$ in the 3rd, and $P < 0.05$ in the 4th hour). The mean plasma concentration of cortisol in the AS group decreased ($P < 0.05$) during the first hour of the experiment compared to the CS group, remaining at the same level as in controls to the end of the experiment ($P < 0.05$ – $P < 0.001$ vs CS group). AL alone had no effect on plasma ACTH concentration at any time of the treatment compared to controls. In the case of cortisol, the only decrease ($P < 0.001$) in plasma concentration occurred in the first hour of the AL treatment in comparison to the control group.

Discussion

The results of the present study showed several significant changes in the activity of the HPA axis in sheep exposed to acute stressful stimuli. The principal finding of this study, however, was that allopregnanolone, functioning centrally, suppressed the expression of the majority of stress response events. This indicated the important

Figure 5

Adrenocorticotrophic hormone (ACTH) concentrations (mean $\pm$ s.e.m.) in 1-h periods in sheep treated with vehicle (C), vehicle and stressful stimuli (CS), allopregnanolone and stressful stimuli (AS) and allopregnanolone alone (A). Significance of differences: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ treatments groups vs C group; ### $P < 0.001$ treatment groups vs CS group; † $P < 0.05$, and †† $P < 0.001$ A group vs AS group.

Figure 6

Cortisol concentrations (mean $\pm$ s.e.m.) in 1-h periods in sheep treated with: vehicle (C), vehicle and stressful stimuli (CS), allopregnanolone and stressful stimuli (AS) and allopregnanolone alone (A). Significance of differences: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ treatment groups vs C group; # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ treatment groups vs CS group; ††† $P < 0.001$ A group vs AS group.

protective role of this neurosteroid in the reaction to acute stressors in ruminants.

The results of previous studies indicated that isolation from the flock is a strong stressful stimulus for sheep causing numerous expressive neurosecretory and hormonal changes at the level of the CNS and peripheral blood respectively (Hasiec *et al.* 2014). In this study, the isolation stress was further enhanced by partial restriction of movement to prevent the animal escaping from the experimental cage. In response to these stimuli, a marked increase in CRH neuron activity in the PVN was observed, expressed by an increase in the abundance of CRH and AVP gene transcripts, and CRH release into the extracellular spaces of the IN/ME. This brain region is rich in nerve terminals, where numerous hypothalamic neurotransmitters and neuropeptides are released into the hypophyseal portal system affecting the secretory activity of the AP. According to Hewitt *et al.* (2009), acute stress causes alterations in chloride homeostasis within PVN, which releases the HPA axis from GABAergic inhibition, initiating the neuroendocrine stress response. The time-concentration profile of CRH in stressed sheep, based on the averaged concentrations of CRH in individual perfusates, indicated that the releasing activity of CRH neurons persisted for several hours during the stimulation period. The consequence of stress-induced activity of CRH neurons was a significant ACTH surge from the AP, lasting to the end of the experiment. The results of studies on rats and humans indicated that CRH is a more potent ACTH secretagogue than AVP, but that the latter is essential for a full ACTH response when subjects are challenged by a variety of stressors (Roper *et al.* 2011). It is suggested that the interaction between the signaling pathways of

these two neuropeptides causes a variety of biochemical alterations within the corticotrophs, which are in turn responsible for the synergistic effect on ACTH (Liu *et al.* 1990). In parallel with the secretory response of AP corticotrophs, there was a marked and long-lasting rise in plasma cortisol concentration. Considering the role of GC negative feedback (Keller-Wood & Dallman 1984), cortisol may have initiated the inhibition of the stress response, which was reflected by decreased CRH concentration in perfusates during the fourth hour of collection.

The pattern of the neuroendocrine stress reaction is complemented in the present study by data that show changes in the abundance of CRH and AVP receptors mRNA, as well as POMC mRNA in the AP of stressed animals. Both CRHR1 and AVPRV1b are co-expressed in corticotrophs and participate in the HPA axis activation in response to different stressful stimuli (Nikodemova *et al.* 2002, Jasnic *et al.* 2013) forming homo- and heterodimers in a ligand-independent manner (Young *et al.* 2007). In the present study there was a decrease in the relative abundance of CRHR1 transcripts in response to acute stressors. This finding is in agreement with the results of earlier studies on rodents, which also indicated that CRH receptors mRNA level in the AP is affected by changing concentrations of CRH and GC during alterations of HPA axis activity (Aguilera 1998, Young *et al.* 2007, Greetfeld *et al.* 2009). It was shown that both CRH and GC may downregulate the CRH receptors in the pituitary corticotrophs implicating these receptors in the mechanism of stress response regulation (Hauger *et al.* 1987, Kasagi *et al.* 2002, Klenerova *et al.* 2018). The number of active CRH receptors in the cell membrane, however, does not depend on the levels of mRNA and full ACTH responses can be achieved with partial receptor occupancy (Aguilera 1994, Nikodemova *et al.* 2002). Moreover, post-receptor events probably contribute to sustained ACTH responses in the presence of reduced CRH binding (Aguilera *et al.* 2004). In the present study, an interesting relationship can also be observed in response to acute stress: strong CRH mRNA expression/weak CRHR1mRNA expression vs. weak AVP mRNA expression/strong AVPV1b mRNA expression. Thus, an increase in the abundance of AVPRV1b mRNA could compensate for the smaller amount of CRHR1 mRNA to enhance the secretion of ACTH. It was possible that sustained high plasma ACTH concentrations, followed by increased cortisol secretion, resulted from the activation of different signal transduction pathways and/or post-transcriptional processing of CRHR1 and AVPRV1b mRNA. This is further

supported by the increased abundance of POMC mRNA in the AP of stressed animals. Transcriptional stimulation of the pituitary POMC gene has been observed previously in rats after a single restraint challenge (Autelitano 1998). Due to the multiple role of the POMC gene transcript, its protein products may contribute either to the maintenance of the stress response (ACTH) or to its completion (β -endorphin).

In general, neurosteroids are synthesized in the brain and modulate neuronal excitability by rapid non-genomic actions (Baulieu 1991, Paul & Purdy 1992, Zheng 2009). In particular, AL was shown to be a highly selective modulator of GABA_A receptor-mediated neurotransmission (Gunn *et al.* 2011, Sarkar *et al.* 2011). Previous studies on rats and mice indicated that activation of the HPA axis by acute stressors, such as swim stress, CO₂ inhalation or LPS treatment is associated with increases in neuroactive steroid concentrations in plasma and the brain (Purdy *et al.* 1991, Barbaccia *et al.* 1994, Billiards *et al.* 2002, Sze *et al.* 2018). Purdy *et al.* (1991) showed that in the rat brain (cerebral cortex and hypothalamus) AL concentration increased for an hour after initiation of stress, prior to peaking in the plasma. Moreover, stress had a greater impact on central AL concentrations in females, than in males (Sze *et al.* 2018). The results of other studies confirmed that stress could regulate neurosteroid biosynthesis via the hormones CRH and ACTH (Torres *et al.* 2001). It is thought that the concentration of neurosteroids increases to have, in turn, stress-protective effects (Purdy *et al.* 1991, Gunn *et al.* 2011). In the present study, a reduction of the neuroendocrine stress-response of the HPA axis following AL treatment was observed at all tested compartments: central, pituitary and consistently peripheral. Although the difference in mean CRH concentrations between the CS and AS groups reached a significance at only $P < 0.05$, the time-concentration profile according to the individual perfusates clearly showed a lowering CRH concentration during AL treatment. The reduction of CRH response to stress in the AS group was continued for 3 h, after which the concentration of CRH increased again. Interestingly, the second increase in CRH concentration in the AS group occurred at the same time as a decrease in CRH concentration in the CS group. These events are difficult to explain, but they may indicate changes in stimulatory or inhibitory neural signaling in response to prolonged stressful stimuli and/or AL treatment. For example, the changes in GABA_A receptors sensitization and/or plasticity in CRH neurons could be taken under consideration (Miklos & Kovacs 2002, Kim *et al.* 2019).

Moreover, AL reduced the abundance of CRH and AVP mRNA in the PVN. The difference in the inhibition of the stress-induced expression of *CRH* and *AVP* genes in the PVN may confirm the differential role of both neuropeptides in stimulating ACTH secretion, especially in response to acute stressors (Engler *et al.* 1989, Familiar *et al.* 1989, Roper *et al.* 2011). The potency of CRH mRNA expression, induced by stress, despite the inhibitory action of AL, was much greater in the AS group than for AVP mRNA. This may indicate a dominant role for CRH at least in initiating a stress response in sheep. Furthermore, a decrease in CRH mRNA expression following administration of AL alone may suggest a preventive silencing effect on HPA axis activity at the molecular level. Budziszewska *et al.* (2010) found that AL inhibited CRH gene promoter activity in the differentiated Neuro-2A cells and suggested that the inhibition of *CRH* gene transcription may be a molecular mechanism responsible, at least partly, for its stress-protective effects. Similarly, such a situation may be related to the reduced expression of CRH and AVP receptors mRNA, as well as POMC mRNA in the AP, since some data point also to the involvement of other possible AL targets, such as intracellular nuclear receptors, protein kinases and microtubule-associated protein (Plassart-Schiess & Baulieu 2001).

Another interesting observation in the present study concerns the changes in the concentrations of ACTH and cortisol in peripheral circulation in the AS group, which indicate faster return of adrenal cortisol to control levels. This may not be accidental, because a peripheral mechanism that prevents excessive supply of GC, including function of adrenal and/or ovary originated AL (Corpechot *et al.* 1993), cannot be ruled out. It was possible that increased secretion of progesterone in the luteal phase of the estrous cycle promotes peripheral production of allopregnanolone. Furthermore, there were initial increases in plasma concentrations of ACTH and cortisol observed in the control group caused probably by the handling and insertion of cannulas into the CNS. On the contrary, in sheep from group A, a clear anti-stress effect of AL was observed, at least in the case of cortisol secretion, during the first hour of the infusion.

The suppressive effects of neurosteroids on neuroendocrine responses to stress were extensively studied in pregnant females, fetuses and newborns (Brunton & Russell 2011, Brunton 2015, Hirst *et al.* 2016). The results of the present study form part of research into the preventive and anti-stress action of AL and simultaneously show that adult sheep are a convenient model for stress research at the CNS level.

In conclusion, allopregnanolone may function centrally as a suppressor of HPA axis activity in adult stressed sheep.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Data statement

The datasets analyzed during the current study are available from the corresponding author, given appropriate justification.

Author contribution statement

T M developed the original concept and experimental design. T M, P M, E M and A M performed the experiment and laboratory analyses. T M and P M analyzed the data, edited and wrote the manuscript. All authors approved the final version.

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