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Developmental Changes in the Pituitary-Adrenocortical Axis and Plasma Met-Enkephalin Concentration in Response to Isolation Stress in Growing Lambs

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Original article

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Hypothalamic-pituitary-adrenal (HPA) axis function may change over the course of aging, and altered stress-induced secretion of cortisol could predispose older animals to negative health outcomes. The main questions arise about the HPA maturity and sensitivity/resilience to stressors as well as the role of endogenous opioid peptides in the modulation of HPA hormones responses. Thus, the aim of the study was twofold: 1. to determine the development of the pituitary-adrenocortical axis in growing sheep by examining responses to the isolation stress, and 2. to measure the effect of endogenous opioid peptide – Met-enkephalin on the HPA hormones. The experiment was carried on 3, 6 and 9 months old male and female sheep divided into control and isolated from the herd for 30 min. Blood was taken 10 min before and 10, 20, 30 and 60 min after the onset of isolation. This stress uniformly increased plasma concentrations of adrenocorticotrophic hormone (ACTH) and cortisol in sheep at each of 3, 6 and 9 months old. Peak plasma concentrations of ACTH were observed with isolation for 20 minutes. In contrast, the peak concentrations of cortisol were observed with 30 minutes of isolation stress. The ACTH response was greater in male than female lambs. Moreover, there was a large increase in the magnitude of the ACTH response between 3 and 6/9 months of age. There was a developmental increase in the response in both female and male lambs. Isolation stress also influenced plasma concentrations of Met-enkephalin with declining responses with the lambs aging. These results suggest modulating effect of endogenous opioid peptides on the HPA axis under stress responses which declines with lambs aging.

Key words: Cortisol, development, ACTH, HPA, isolation stress, Met-enkephalin.

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It is well established that stressors activate the hypothalamo-pituitary-adrenal (HPA) with increased release of corticotrophin releasing hormone (CRH), adrenocorticotrophic hormone (ACTH) and cortisol in human and animals. Many experiments showed that plasma concentrations of ACTH and cortisol in sheep are elevated by insulin induced hypoglycemia, haemorrhage (OWENS *et al*. 1988), handling (HASIEC *et al*. 2014), isolation stress coupled with restraint (TILBROOK *et al*. 2006), exercise, endotoxin and wetting (TURNER *et al*. 2010).

Met-enkephalin is one of the most important opioid peptides involved in modulation of pain sensitivity, analgesia, hunger, emotion and fear. Met-enkephalin is also a putative constituent of the HPA axis with plasma concentrations of Met-enkephalin influenced by some stressors. On the other hand, there is considerable evidence that Met-enkephalin influences the HPA axis. Met-enkephalin analogue depressed ACTH release and lowered both circulating concentrations of cortisol and ACTH in humans (DEL POZO *et al*. 1980). *In vitro*

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ACTH release from rat pituitary tissue was also decreased by Met-enkephalin analogue (LAMBERTS *et al*. 1983).

In adult sheep, neither Met-enkephalin nor naloxone influenced circulating concentrations of ACTH (BROOKS & CHALLIS 1988, 1989). However, centrally administered Met-enkephalin depressed plasma concentrations of ACTH in conscious sheep (WANG *et al*. 1988). Met-enkephalin containing neurons were found in sheep hypothalamus, particularly in the lateral hypothalamus (MARSON *et al*. 1987). It was also found that Met-enkephalin synthesis, secretion and activation of opioid receptors in the lamb HPA axis change with age (PIERZCHAŁA-KOZIEC & KĘPYS 2000) and CRH treatment (PIERZCHAŁA-KOZIEC *et al*. 2017).

However, most of the studies were performed on adult animals. Major remaining issues include HPA maturity and sensitivity/resilience to stressors as well as the role of different physiological systems, for example endogenous opioid peptides, in the modulation of HPA responses to stressors.

The main aim of the study was to promote the well-being of growing lambs, and to increase our overall understanding of physiological development. It is known that stress factors present during the growth of animals can affect psychobiological responses to stress, resilience to stress, and subsequent effects on health in older animals. Recently, the psychological aspects of stress responses in human and animals have been discussed. However, most of the published results are fragmentary, usually focused on one or two parameters. Thus, the aim of the study was twofold: (1) to determine the development of the pituitary-adrenocortical axis in growing sheep by examining responses to isolation stress, and (2) to estimate the potential impact of stress evoked changes in the endogenous opioid peptide- Met-enkephalin on HPA hormones. Effects of isolation stress on plasma concentrations of ACTH, cortisol and Met-enkephalin were examined during growth/post-natal development in lambs.

We planned a comprehensive study on sheep as an animal model at different ages: at 3 months when lambs are still with mothers and isolation is a very severe stressor; at 6 months when lambs are more independent (with feed and herd); at 9 months when animals are fully developed.

Materials and Methods

Animals

The study employed the multipurpose breed, the Polish Mountain Sheep. This is one of the native breeds of the Polish Carpathians and is thought to be derived from Wallachian Zackel sheep. The breed is considered as very hardy, living in adverse mountain conditions (KAWĘCKA & KRUPIŃSKI 2014); these being presumed to be stressful.

We used Polish Mountain sheep (female and male – 3, 6, 9 months old) at the National Institute of Animal Sciences-Mountain Sheep Station. The protocol was approved by the First Local Ethical Committee on Animal Testing in Krakow (64/OP/2005/I LKE). The sheep were maintained under standard herd conditions at a temperature of 22^{o}C with a photoperiod 12L: 12D (lights on from 7 a.m to 7 p.m). Feed (grass and hay) and water were provided *ad libitum.* Young lambs (3 and 6 months old) were kept with the dams. In contrast, 9 month old lambs had been weaned and were maintained separately from dams.

Isolation stress

At 3, 6, 9 months old, female (F) and male (M) lambs were subjected to isolation stress (removal from herd and without visual or acoustic contact with other sheep) for 30 minutes. Control animals stayed with the herd. Protocol: 3 month old lambs -control groups: F =6 and M=6 (total 12 animals), isolated groups: F=6 and M=6 (total 12 animals), the same for 6 and 9 month animals. In three experiments (performed on 3, 6 and 9 month old animals) 36 F and 36 M lambs were divided into 12 groups with six animals per group. All animals were included into the final analysis.

Blood sampling

Blood samples were obtained from the right jugular vein five times: 10 min before and 10, 20, 30 and 60 min after the onset of isolation. Lambs were without catheter, blood was taken by venipuncture, each sampling took less than 20 sec. Animals from control and experimental groups were bled, only two persons had contact with lambs. Before the main experiment we performed a pilot study and we compared the blood cortisol levels in animals with catheter and without. The plasma basal level of cortisol in lambs with catheter was similar to that measured in blood taken by venipuncture in spite of a longer bleeding time and flushing of the catheter. Also, because isolation from the herd was the stressor, lambs were kept together before and after stress and catheters were very difficult to maintain, causing more emotional stress than quick bleeding. Taking this into consideration we decided to take the blood by venipuncture. The blood (2 ml) was divided into three polypropylene tubes containing respectively, ethylene diamino-tetra acetic acid (5% of EDTA) for ACTH estimation, heparin (1000 IU) for cortisol measurement and for Met-enkephalin estimation [5% EDTA plus 17.7 nmol/ml of citric acid and aprotinin (Trasylol, 200 KIU/ml)]. Blood was centrifuged for 30 min at 4ºC and 4000xg and plasma samples were stored at -80 °C.

ACTH and cortisol concentrations were determined by commercial radioimmunoassays, respectively, Diagnostic Products Corporation, Los Angeles, CA (USA) and ORION Diagnostica (Finland). These had limits of detection – 8 pg/ml and 4.7 nmol/l; intra-assay coefficient of variation of 5.8% and 3.1%; and inter-assay coefficient of variation – 9.3% and 5.8%

Plasma Met-enkephalin concentrations

Biologically active Met-enkephalin concentration in the plasma was estimated by the radioimmunoassay method of PIERZCHAŁA & VAN LOON (1990). Met-enkephalin was purified on Porapak Q (Waters,100-120 mesh) in 2 ml of absolute ethanol, lyophilized and assayed after reconstitution in 100 µl of 0.06 M phosphate buffer (pH 6.5, 0.2% bovine serum albumin, 0.002% sodium azide). The assay entailed the addition of 50 µl antiserum (rabbit, 1:10,000) and 50 µl of ^{125}I-Met-enkephalin (~1500 cpm) and incubation at 4°C for 24 h. Bound and free was separated after 24 h by the addition of 50 µl of rabbit γ-globulin (1%), incubation for 30 min at 4°C, addition of 250 µl of 25% polyethylene glycol (PEG 8000), incubation for 30 min of incubation samples and finally centrifugation (2000×g, 4°C, 20 min). The supernatants were discarded and the pellets were counted in a γ-counter (1470 Wizard, Turku, Finland).

Statistical analysis

Data are presented as means ± SEM according to ALTMAN & BLAND (2005) that the standard error of the sample mean (SEM) depends on both the standard deviation (SD) and the sample size by the relation SEM=SD/ √ (sample size). This measure was used as a means of calculating a confidence interval. There were no outliers. The analysis was performed using the O' Dell Statistica, ver.13, (1984-2016 Dell Inc.). Hormone concentrations in repeated blood samples were analyzed by repeated measures ANOVA followed by Least Significant Difference (LSD) test.

Results

Isolation stress and plasma concentrations of ACTH

The effects of isolation stress on plasma concentrations of ACTH in 3-, 6- and 9- month old female and male lambs are summarized in Table 1. Plasma concentrations of ACTH were increased (P<0.05) in lambs subjected to isolation stress in female and male lambs at 3, 6 and 9 months old. The effect was seen as soon as after 10 minutes of isolation stress. The peak response was observed with a duration of isolation stress of 20-30 minutes. A response to stress was observed after 10 minutes in 9 month old female and 3, 6 and 9 month old male lambs and after 20 minutes in 3 and 6 month old female lambs. The effect of stress on plasma concentrations of ACTH was still uniformly present after 30 minutes of isolation stress in females (3 and 6 month old) and males (6 month old). Similarly, increases in plasma ACTH concentrations after 10 minutes of stress were greater in males than females and were progressively larger with age; the increases being: + 0.7, +0.6 and 15.4 pg/ml in females and +4.0, +23.3 and 54.1 pg/ml in males (Table 1).

Figure 1A presents the maximal response to isolation stress (Δ ACTH between control and stressed lambs) during the 30 min of stress. There were marked age and sex differences in the ACTH response to isolation stress with the response being earlier and larger in males (Fig. 1A). The Δ ACTH concentrations were greater (P<0.01) in males than females at 6 and 9 months of age (Fig. 1A). Moreover, there were large increases in the Δ ACTH with age increasing (P<0.01) between 3 and 6 month old females and between 6 and 9 months in male lambs.

Isolation stress and plasma concentrations of cortisol

The effects of isolation stress on plasma concentrations of cortisol in 3-, 6- and 9- month old female and male lambs are summarized in Table 2. Plasma concentrations of cortisol were increased (P<0.05) with 10 minutes of isolation stress in both female and male lambs at each of the age groups. Peak plasma concentrations of cortisol were observed after 30 minutes of isolation stress in female lambs at 3, 6 and 9 months of age and males at 3 and 9 months old. Plasma concentrations of cortisol declined between 30 and 60 minutes after isolation stress in female lambs at all three ages and in male lambs at 3 and 9 months old.

There were no differences in the overall basal plasma concentrations of cortisol between female and male lambs (females: 62.8 ± 2.35 nmol/l; males: 67.5 ± 2.82 nmol/l). The increases in plasma concentrations of cortisol in female lambs were either consistently greater or tended to be greater than in males.

Table 1

Effect of isolation stress on plasma concentrations of ACTH (pg/ml) in female and male lambs (3, 6 and 9 months old). Data are shown as mean ± SEM, each group n=6

Treatments	Plasma ACTH concentration (pg/ml) before and after isolation stress				
	-10	+10	+20	+30	+60
FEMALE					
3 months					
Control	5.9 ± 0.3	4.2 ± 0.2	5.8 ± 0.4	5.6 ± 0.5	5.5 ± 0.5
Stressed	6.1 ± 0.3^{a}	6.8 ± 0.4^{ab}	7.9 ± 0.3^{b}**	9.8 ± 0.4^{c}***	9.4 ± 0.4^{bc}***
6 months					
Control	11.1 ± 0.7	10.1 ± 0.6	9.8 ± 0.5	10.1 ± 0.5	10.2 ± 0.7
Stressed	11.7 ± 0.5^{a}	12.3 ± 0.8^{a}	44.1 ± 3.4^{c}***	28.3 ± 2.9^{b}***	15.9 ± 1.1^{a}**
9 months					
Control	13.3 ± 1.2	18.5 ± 1.6	14.3 ± 1.5	17.9 ± 1.9	15.0 ± 0.9
Stressed	12.7 ± 1.1^{a}	28.1 ± 2.1^{b}**	12.6 ± 0.9^{a}	36.2 ± 2.6^{c}***	12.0 ± 0.8^{a}*
MALE					
3 months					
Control	1.9 ± 0.2^{a}	3.0 ± 0.3^{ab}	5.7 ± 0.7^{b}	5.8 ± 0.6^{b}	5.7 ± 0.9^{b}
Stressed	2.1 ± 0.2^{a}	6.1 ± 0.6^{b}**	5.6 ± 0.7^{b}	13.3 ± 1.1^{c}***	4.3 ± 0.3^{ab}
6 months					
Control	9.8 ± 0.8	9.3 ± 0.9	9.5 ± 1.0	9.7 ± 1.1	9.8 ± 1.2
Stressed	8.9 ± 0.9^{a}	32.2 ± 1.9^{b}***	126 ± 9.8^{d}***	65.3 ± 5.6^{c}***	41.2 ± 4.7^{b}***
9 months					
Control	9.1 ± 0.9	10.0 ± 1.1	9.8 ± 0.9	10.3 ± 1.2	10.2 ± 0.9
Stressed	11.2 ± 1.1^{a}	65.3 ± 4.5^{b}***	209.4 ± 15.1^{c}***	51.3 ± 3.9^{b}***	11.2 ± 1.3^{a}

* Difference between stressed and control lambs * P<0.05, ** P<0.01 and *** P<0.001

^{a,b,c} Different letter in a row indicates difference P<0.05.

Table 2

Effect of isolation stress on plasma concentrations of cortisol (nmol/l) in female and male lambs (3, 6 and 9 months old). Data are shown as mean ± SEM, each group n=6

Treatments	Plasma cortisol concentration (nmol/l) before and after isolation stress				
	-10	+10	+20	+30	+60
FEMALE					
3 months					
Control	68 ± 5	60 ± 6	67 ± 5	70 ± 8	55 ± 6
Stressed	70 ± 4^{a}	125 ± 9^{b}***	195 ± 14^{c}***	210 ± 17^{c}***	101 ± 8^{b}***
6 months					
Control	58 ± 7	59 ± 8	60 ± 6	62 ± 7	63 ± 5
Stressed	60 ± 6^{a}	175 ± 13^{c}***	178 ± 12^{c}***	168 ± 13^{c}***	90 ± 7^{b}*
9 months					
Control	60 ± 7	62 ± 6	60 ± 7	59 ± 7	60 ± 7
Stressed	61 ± 6^{a}	148 ± 11^{b}***	199 ± 15^{c}***	252 ± 18^{d}***	180 ± 15^{bc}***
MALE					
3 months					
Control	60 ± 7	67 ± 6	70 ± 7	66 ± 5	58 ± 5
Stressed	52 ± 6^{a}	102 ± 8^{b}**	95 ± 8^{b}*	110 ± 9^{b}**	60 ± 7^{a}
6 months					
Control	75 ± 6	72 ± 8	70 ± 6	72 ± 7	71 ± 8
Stressed	73 ± 7^{a}	125 ± 11^{b}**	162 ± 13^{c}***	140 ± 12^{bc}***	120 ± 11^{b}**
9 months					
Control	75 ± 6	70 ± 7	69 ± 8	71 ± 6	70 ± 5
Stressed	70 ± 7^{a}	140 ± 12^{b}***	152^{bc} ± 14***	181 ± 17^{c}***	140 ± 11^{b}***

* Difference between stressed and control lambs * P<0.05, ** P<0.01 and *** P<0.001

^{a,b,c} Different letter in a row indicates difference P<0.05.

Table 3

Effect of isolation stress on plasma concentrations of met-enkephalin (pmol/l) in female and male lambs (3, 6 and 9 months old). Data are shown as mean ± SEM, each group n=6

Treatments	Plasma met-enkephalin concentration (pmol/l) before and during isolation stress				
	-10	+10	+20	+30	+60
FEMALE					
3 months					
Control	244 ± 19.2	248 ± 20.9	246 ± 17.4	209 ± 19.2	253 ± 22.7
Stressed	209 ± 15.7	253 ± 20.9	249 ± 17.4	261 ± 20.9	270 ± 22.0
6 months					
Control	253 ± 22.7	261 ± 20.9	244 ± 17.4	227 ± 15.7	223 ± 19.1
Stressed	244 ± 19.2[b]	314 ± 24.4[c]	165 ± 13.9[a]**	305 ± 24.4[bc]*	242 ± 20.9[b]
9 months					
Control	218 ± 15.7	223 ± 12	227 ± 14.0	227 ± 19.2	223 ± 15.7
Stressed	200 ± 15.7[b]	218 ± 20.9[b]	139 ± 12.2[a]***	235 ± 17.4[b]	202 ± 15.7[b]
MALE					
3 months					
Control	218 ± 15.7	227 ± 19.2	209 ± 17.4	218 ± 19.2	220 ± 20.9
Stressed	235 ± 20.9[b]	401 ± 27.9[c]***	150 ± 13.9[a]*	540 ± 33[d]***	265 ± 20.9[b]
6 months					
Control	305 ± 24.4	296 ± 22.7	314 ± 24.4	340 ± 22.7	296 ± 20.9
Stressed	296 ± 19.2[b]	261 ±20.9[ab]	227 ± 15.7[a]*	418 ± 20.9[c]*	305 ± 19.2[b]*
9 months					
Control	261 ± 20.9	279 ± 19.2	244 ± 17.4	279 ± 22.7	261 ± 20.9
Stressed	235 ± 12.2[b]	165 ± 13.9[a]***	227 ± 19.2[b]	244 ± 22.7[b]	235 ±19.7[b]

* Difference between stressed and control lambs * $P<0.05$, ** $P<0.01$ and *** $P<0.001$

[a,b,c] Different letter in a row indicates difference $P<0.05$.

Figure 1B presents the maximal response to isolation stress (Δ cortisol between control and stressed lambs) during the 30 min of stress.

The maximum responses in plasma concentrations of cortisol (Fig.1B) were greater ($P<0.01$) in female than male lambs (Δ cortisol: females – 198 ± 6.2; males – 110 ± 4.8 nmol/l, 9 months old). Moreover, there was a greater response for 3 month old females (Δ cortisol =140 ± 8.8 nmol/l) compared to an increase of cortisol plasma concentration in males of the same age ($P<0.01$, Δ cortisol =44.0 ± 4.8 nmol/l).

Isolation stress and plasma concentrations of Met-enkephalin

Table 3 summarizes the effects of isolation stress on plasma concentrations of Met-enkephalin in 3-, 6- and 9- month old female and male lambs. Basal (time -10 minutes) plasma concentrations of Met-enkephalin were 11.7 pmol/l lower ($P<0.05$) in female than male lambs (females 228 ± 7.7; males 258 ± 9.1 pmol/l). Plasma concentrations of Met-enkephalin appeared to exhibit multiphasic responses to isolation stress. There were decreases ($P<0.05$) in plasma concentrations of Met-enkephalin with 20 minutes of stress for both female and male lambs (female: 6 and 9 months old; males: 3 and 6 months old) and in 9 month old males with 10 minutes of isolation stress. Conversely, there were either rebounds ($P<0.05$ compared to preceding time point) or increases ($P<0.05$ compared to controls and pre-treatment) in the plasma concentrations of Met-enkephalin with 30 minutes of stress (rebound: females – 9 months old, males 9 months old; increases: females – 6 months old, males – 3 and 6 months old). There were no changes of plasma concentrations of Met-enkephalin at the 60 minutes time point in any of the six age/sex groups.

Figure 1C presents the maximal response to isolation stress (Δ Met-enkephalin between control and stressed lambs) during the 30 min of stress.

The maximal decrease of plasma Met-enkephalin (Δ Met-enkephalin) was at 20 min of stress in 6, 9 month old females and at 10 min of isolation in 9 month old males ($P<0.01$). In contrast, maximal increase of plasma Met-enkephalin level was observed at 30 min in 6 month old females (Δ Met-enkephalin = 78 ± 6.1 pmol/l) and at 30 min in 3 month old males (Δ Met-enkephalin = 322 ± 22.5 pmol/l, Fig. 1C).

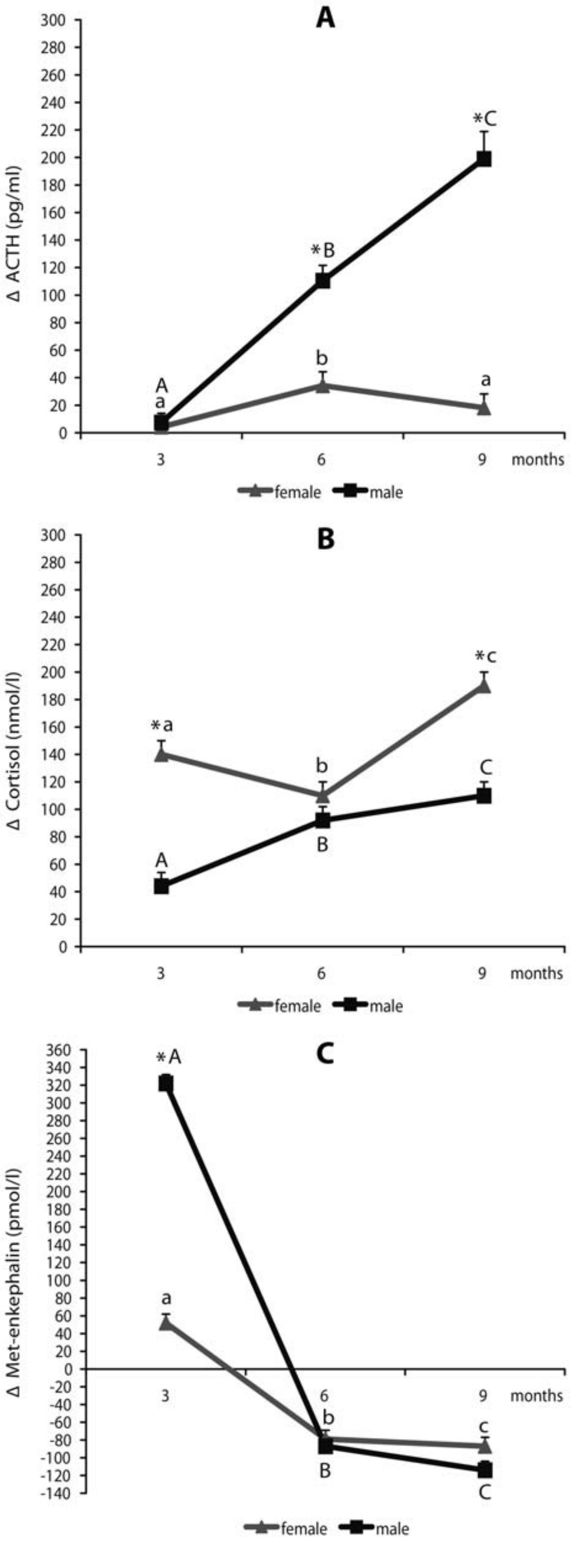

Fig. 1. Effect of isolation stress on plasma of ACTH (A), cortisol (B) and Met-enkephalin (C) maximal responses (Δ differences between control and stressed lambs), x±SEM, n=6.
*P<0.01 indicates differences between females and males. a,b,c: P<0.01 indicates differences between 3, 6, 9 months old female lambs, A,B,C: P<0.01 indicates differences between 3,6,9 months old male lambs.

Discussion

The HPA of sheep is similar to other mammals with ACTH release stimulated by corticotrophin releasing hormone (CRH) and vasopressin (SENN *et al*. 1995; SLOBODA *et al*. 2007). However, most results demonstrating the response of the HPA axis to different stressors were obtained from experiments performed on adult animals. Release of cortisol from the adrenal cortex is stimulated by ACTH in a dose dependent manner. The experiment of FULKERSON & JAMISON (1982) showed that the peak concentrations of cortisol were at 8 minutes with the 0.01 and 0.1 IU ACTH but extended 40 minutes with 1 IU and to 50 minutes with 10 IU ACTH.

In the present study, plasma ACTH and cortisol reached maximal concentrations evoked by isolation stress between 20 and 30 min of stress. The magnitude of the HPA response to emotional stress increased with the age of female and male lambs. This was probably the effect of increased sensitivity of ACTH receptors in the adrenal cortex cells and maybe decreased/ smaller activity of the opioid system at the central and peripheral levels. It could also be speculated that during the maturation of the hypothalamo-pituitary-gonadal axis (HPG), gonadotrophin releasing hormone and gonadotrophins additively interact with the HPA axis hormones.

Plasma concentrations of cortisol in adult sheep were similarly increased by stressors such as shearing, placing into a novel yard (FULKERSON & JAMISON 1982), a barking dog (CANNY *et al*. 1990), myiasis in the skin due to blowfly larvae (SHUTT *et al*. 1988) and insulin induced hypoglycemia (TURNER *et al*. 2002). Psychological stress such as isolation induced increases in the circulating concentrations of cortisol in adult sheep (isolation stress alone: TURNER *et al*. 2002; combined isolation and restraint stress: e.g TILBROOK *et al*. 2006, HASIEC *et al*. 2014). The hypothalamo-pituitary-adrenocortical axis of adult sheep also responded to other stressors such as insulin induced hypoglycemia, hemorrhage, exercise, endotoxin and wetting with increased circulating concentrations of cortisol (OWENS *et al*. 1988; TURNER *et al*. 2010). The results of our experiments clearly show that the sensitivity and activity of the HPA axis in growing lambs changes with age.

There were marked differences in the ACTH and cortisol responses to isolation stress between male and female lambs and with age. The increases in plasma concentrations of ACTH were of a greater magnitude in male than female lambs. In contrast, increases in the plasma concentrations of cortisol were larger in females than in male lambs. This would suggest that the adrenocortical response to ACTH is greater in the females as is the sensitivity to cortisol negative feedback at the hypothalamic and/or pituitary levels. There are reports of differences in HPA axis functioning with gender and reproductive state. There was a larger increase in circulating concentrations of cortisol in female than male adult sheep subjected to isolation stress (TURNER *et al*. 2002). Similarly, increased circulating concentrations of cortisol were reported in adult female rather than male sheep challenged with ACTH (VAN LIER *et al*. 2003). Moreover, circulating concentrations of cortisol tended to be lower in male than female adult sheep irrespective of whether they were basal or in response to combined isolation and restraint stress (RIVALLAND *et al*. 2007). In addition, the ACTH response to CRH challenge is greater in female lambs (CHADIO *et al*. 2007). In contrast, insulin induced hypoglycemia evoked a smaller increase in circulating concentrations of cortisol in female than male sheep irrespective of whether they were gonadectomized or intact (TURNER *et al*. 2002). There was a larger response of male fetal lambs compared to females to the stressor hypoxia, due to greater adrenocortical sensitivity to ACTH (GIUSSANI *et al*. 2011). There is other evidence for reproductive status influencing the HPA axis. Moreover, the cortisol response to ACTH was reduced in castrated males but not in females subjected to oophorectomy (VAN LIER *et al*. 2003). In addition, the ACTH and cortisol responses to stress were attenuated in lactating sheep compared to anestrous sheep (HASIEC *et al*. 2014).

Post-natal development of responsiveness of the HPA axis to external stimuli was similar in mice (SCHMIDT *et al*. 2003).

There are age differences in the ACTH response to CRH challenge with both the ACTH and cortisol responses being greater at 2.2 months than either 5.5 or 10 month old sheep (CHADIO *et al*. 2007). The present data together with the report of CHADIO and colleagues (2007) supports the concept that the basis of the development of the HPA axis in lambs is the acquisition of the ability to respond to specific external stressors. This would place a precocial species, the sheep, in a similar situation to altricial species such as mice and rats. It is recognized that fetal lambs readily respond to another stressor, hypoxia, with increases in plasma concentrations of both ACTH and cortisol (GARDNER *et al*. 2001; GIUSSANI *et al*. 2011).

There was a multiphasic response of plasma concentrations of Met-enkephalin in lambs to isolation stress. An analogous multiphasic response to restraint stress has been reported in rats (PIERZCHALA & VAN LOON 1990). Similarly, Met-enkephalin stress responses to other stresses have been reported. For instance, insulin induced hypoglycemia increases the circulating concentrations of Met-enkephalin in conscious sheep (OWENS *et al*. 1988) while streptozotocin (STZ) influences both circulating and tissue concentrations of met-enkephalin in rats (KOLTA *et al*. 1992). Asphyxia/hypoxia/hypoxemia increase plasma concentrations of Met-enkephalin and Met-Enkephalin-arg6-phe7 (COULTER *et al*. 1990; SIMONETTA *et al*. 1996). However, there is no effect of adrenalectomy on plasma concentrations of Met-enkephalin or of Met-enkephalin-arg6-phe7 in hypoxemia (SIMONETTA *et al*. 1993; 1996) suggesting that the adrenal gland is not a major source of the Met-enkephalin released in hypoxemia. Similarly, there is an effect of hemorrhage on the circulating concentrations of Met-enkephalin in plasma or cerebrospinal fluid in dogs (OTA *et al*. 1988) but not in sheep (OWENS *et al*. 1988). It was also found that Met-enkephalin synthesis, secretion and activation of opioid receptors in the lamb HPA axis change with age (PIERZCHAŁA-KOZIEC & KĘPYS 2000) and exogenous CRH treatment (PIERZCHAŁA-KOZIEC *et al*. 2015, 2017).

The present data on plasma concentrations of Met-enkephalin are consistent with an initial decrease followed by a homeostatic increase with an overshoot followed by a return to basal. Moreover, plasma concentrations of Met-enkephalin may be under tight physiological control. An alternative is that plasma concentrations of Met-enkephalin reflect "leakage" from sites of release and autocrine/paracrine action. The novelty of our extensive experiments is determination of parallel changes/interaction between opioid peptide Met-enkephalin and hormones of the hypothalamo-pituitary-adrenal cortex during physiological and stressful situations in lambs (female and male) at three ages.

Summary

Our results clearly showed increasing sensitivity of the HPA axis to emotional stress in growing lambs of both sexes. There are marked increases in plasma concentrations of both ACTH and cortisol in response to isolation stress in growing lambs with greater ACTH response in males and greater cortisol response in female. The ACTH response increases with lamb age. Plasma concentrations of Met-enkephalin exhibit a biphasic response to stress returning to basal by 60 minutes after onset of isolation stress. Also, maximal responses of opioids to isolation stress, manifested by changes of plasma Met-enkephalin, decrease with age (6 and 9 month old female and male lambs), in contrast with increasing responses of pituitary and adrenal cortex at these ages. These results suggest a modulating effect of endogenous opioid peptides on the HPA axis under stress responses which fades with lamb age. What is not known is whether stress influences tissue synthesis and concentrations of enkephalin and how basal plasma concentrations of enkephalin are homeostatically regulated.

In conclusion, the present data demonstrated that the activity of the pituitary-adrenal cortex to emotional stress increases with lamb age. Our results provide further support for the interaction between Met-enkephalin and HPA axis under stress.

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Author Contributions

Research concept and design: K.P.-K.; Collection and/or assembly of data: K.P.-K., B.K.; Data analysis and interpretation: K.P.-K., B.K., C.G.S.; Writing the article: K.P.-K., C.G.S.; Critical revision of the article: K.P.-K., P.O., C.G.S.; Final approval of article: K.P.-K., C.G.S.

Conflict of Interest

The authors declare no conflict of interest.

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