e-ISSN 1734-9168
http://www.isez.pan.krakow.pl/en/folia-biologica.html

Folia Biologica (Kraków), vol. 71 (2023), No 4
https://doi.org/10.3409/fb_71-4.19

Effects of hexarelin and isolation stress on the Met-enkephalin system in young lambs

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Accepted November 29, 2023 *Published online December 29, 2023* *Issue online December 29, 2023*

Original article

Koziec K., Scanes C.G., Zubel-Łojek J., Gajewska A. 2023. Effects of hexarelin and isolation stress on the Met-enkephalin system in young lambs. Folia Biologica (Kraków) **71**: 195-206.

Stress stands out as the primary instigator of numerous diseases – ranging from cardiovascular and gastrointestinal to diabetes and nervous disturbances – in most developed nations. Endogenous opioid peptides (EOP), particularly Met-enkephalin, play a crucial role in mitigating the up-regulation of the hypothalamo-pituitary-adrenal axis during stress responses, consequently reducing the risk of serious diseases. Hexarelin, a synthetic analog of Met-enkephalin, has been predominantly investigated for its impact on growth hormone (GH) release in both human subjects and rodent models. This study was undertaken to evaluate the influence of isolation stress and/or hexarelin administration on various Met-enkephalin-related parameters in a novel animal model – 3-month-old lambs. Four distinct groups were established: a control group, a group intravenously injected with hexarelin, a group subjected to 60 min of isolation stress from the herd, and a group treated with both hexarelin and stress. Blood and hypothalamus samples were collected to analyze cortisol and Met-enkephalin profiles, proenkephalin (PENK) gene expression, Met-enkephalin concentration, *in vitro* Met-enkephalin secretion, and opioid receptor binding. The findings revealed a significant impact of stress on all assessed parameters. Hexarelin alone led to a decrease in cortisol levels and Met-enkephalin synthesis, release, and receptor binding in the hypothalamus. When administered prior to stress, hexarelin potentiated the responses of opioid parameters to isolation. These results, for the first time, demonstrate that hexarelin interacts with Met-enkephalin, modulating the stress response at both central and peripheral levels in growing lambs. It is suggested that hexarelin plays a crucial role during stress responses; however, further research on its effects should be conducted concurrently with the examination of opioid profiles.

Key words: opioids, opioid receptors, PENK synthesis, growth hormone secretagogue.

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Stress impacts the interaction of nervous, endocrine, and immune systems in both humans and animals, leading to the onset of various serious diseases such as cardiovascular issues, gastrointestinal problems, insulin resistance, and growth retardation. Endogenous opioid peptides (EOP), particularly Met-enkephalin, play a vital role in reducing the risk of disruptions in physiological systems during stress responses. They achieve this by attenuating the activity of the hypothalamo-pituitary-adrenal (HPA) axis, while also stimulating the secretion of growth hormone, thyroxine, and ghrelin. This simultaneous action increases the availability of energy sources, as highlighted in the study by Pierzchala-Koziec *et al.* (2018).

Among EOP, enkephalins exhibit the broadest distribution, being naturally produced as small molecules in various organs. These organs include the

brain (Hughes *et al.* 1977; Pierzchala-Koziec *et al.* 2019); spinal cord, dorsal root ganglion (Pohl *et al.* 1997; Sapio *et al.* 2020), myenteric plexus (Pierzchala *et al.* 1987), pituitary gland, small intestine (Pierzchala *et al.* 1987), pancreas (Timmers *et al.* 1986; Pierzchala *et al.* 1987), and adrenal gland (Jackson *et al.* 1985; Pierzchala *et al.* 1987). Met-enkephalin exists in both the blood and tissues in two forms: the native form with five amino acid residue fragments and the total form (precursor) either in or associated with large proteins. These forms are then processed by proteases to yield active Met-enkephalin and Leu-enkephalin (Pierzchala and Van Loon 1990).

Met-enkephalin is found within the hypothalamus, where it engages with corticotropin-releasing hormone (CRH), growth hormone-releasing hormone (GHRH), and somatostatin neurons. Its secretion serves the purpose of modulating the activity of the hypothalamo-pituitary-adrenal (HPA) axis and the GHRH/growth hormone (GH)/insulin-like growth factor (IGF) axis in mammals (Cabral *et al.* 2016).

The inherent enkephalin possesses an exceedingly brief half-life (less than 1 min), making it challenging to utilize as a medicinal peptide. Subsequent in-depth research has revealed that enkephalin operates through specific opioid receptors, particularly delta receptors. Furthermore, it functions as a ligand for growth hormone secretagogue receptors (GHS-Rs), thereby capable of stimulating the release of GH. The identification of GHS-R has led to the invention of several synthetic peptidyl and nonpeptidyl molecules with potent growth hormone-releasing activity, including GHRP-2, GHRP-6, and hexarelin. Hexarelin, a synthetic analog of Met-enkephalin, has undergone extensive investigation for its impact on GH release, both in peripheral endocrine and non-endocrine tissues. Additionally, it has been explored for its effects on ACTH, cortisol, prolactin, heart cell survival, and responses to nutrient deprivation (Arvat *et al.* 1999; Mao *et al.* 2014; Mosa *et al.* 2017).

Hexarelin serves as a ligand for the growth hormone secretagogue receptor 1a (GHSR-1a), also known as the ghrelin receptor. In humans, hexarelin has been observed to elevate circulating concentrations of both ACTH and cortisol, as reported in studies by Massoud *et al.* (1996), Ghigo *et al.* (1997), and Korbonits *et al.* (1999). Similarly, in rats, another ligand for GHSR-1a, GH-releasing peptide-6, has demonstrated an increase in circulating concentrations of corticosterone, as shown in the research conducted by Thomas *et al.* (1997).

It is noteworthy that the endogenous ligand for the GH secretory receptor (GHSR)-1a, ghrelin, exerts an influence on the HPA axis. Both ghrelin and/or desacyl ghrelin have been shown to enhance adrenal glucocorticoid production, as indicated by circulating concentrations of ghrelin (mice: Cabral *et al.* 2016; Stark *et al.* 2016; humans: Lambert *et al.* 2011). Additionally, ghrelin has been demonstrated to stimulate glucocorticoid production from rat adrenal cortical cells (Rucinski *et al.* 2009). Similarly, other GHSR agonists such as L-692,585 (Jacks *et al.* 1994) and L-692,429 (Hickey *et al.* 1994) were found to elevate serum concentrations of cortisol in dogs and pigs (Glavaski-Joksimovic *et al.* 2002).

Rats exhibited elevated circulating concentrations of corticosterone in response to another ligand for GHSR-1a, GH-releasing peptide-6 (Rucinski *et al.* 2009). What remains unknown is whether hexarelin stimulates the hypothalamo-pituitary-adrenocortical axis in sheep and/or has an impact on Met-enkephalin physiology in any species. The current study aims to explore the effects of hexarelin in the presence of isolation stress on circulating concentrations of cortisol and Met-enkephalin, along with other Met-enkephalin parameters, in young lambs. The Met-enkephalin system components under examination include plasma concentrations of Met-enkephalin, hypothalamic concentrations of Met-enkephalin, *in vitro* Met-enkephalin release from hypothalamic tissue, and expression in the hypothalamus of the proenkephalin gene, PENK. Furthermore, the study delves into the effects of isolation stress on Met-enkephalin in 3-month-old lambs receiving hexarelin administration or not, extending previous investigations into the impact of isolation stress on Met-enkephalin in sheep.

Materials and Methods

Materials

Most of the chemicals were acquired from Sigma-Aldrich, while others are explicitly mentioned in the text. The specific substances include Sodium chloride (S9888), Potassium chloride (P3911), Naltrexone chloride (N3136), RNAlater (R090), Tween 20 (P1379), Sodium carbonate (S7795), Krebs (K3753), Heparin sodium (H3149), Hexarelin (80666), Tris-HCl (T5941), and the Bicinchoninic Acid kit (BCA1).

Animal model

The study utilized 24 three-month-old female lambs of the Polish Mountain Sheep breed from the National Research Institute of Animal Production in

Kraków. The animal study protocol 64/OP/2005 received approval from the Institutional Review Board and the First Local Ethical Committee on Animal Testing in Kraków, Poland, under approval code 65/OP/2005. The approval was granted in July 2005 and confirmed in November 2007.

The lambs were kept within a herd alongside the ewes in a controlled environment (photoperiod 12L:12D, with lights on from 7 a.m. to 7 p.m.) and maintained at a room temperature of 20°C. The sheep had unrestricted access to feed and water. The animals acclimated to these conditions for a period of 7 days before the commencement of the experiment. Lambs, ensuring no siblings were grouped together, were randomly assigned to the experimental groups.

Experimental design

After obtaining pretreatment blood samples in heparinized tubes, lambs were intravenously injected with either 0.9% saline (treatment group A, control, and treatment group C, to induce stress) or hexarelin (0.5 µg/kg b.w.; Peptides, USA; treatment group B, hexarelin, and treatment group D, to induce stress). Lambs in treatment groups B and D were exposed to isolation stress for 60 min without visual or acoustic contact with other lambs/mothers, covering the 0- to 60-min time points. In accordance with a prior experiment (Pierzchala-Koziec *et al*. 2017, 2018), 60 min of isolation serves as a potent stressor, activating the HPA axis responses and resulting in significant changes in the plasma levels of catecholamines, opioids, and glucocorticoids.

Blood sampling and organotypic slice culture

Blood samples (each totaling 2 ml) were extracted from the right external jugular vein and collected in heparinized tubes at specific intervals: 15 min before the onset of stress, and at 15, 30, 45, 60, and 90 min from the initiation of stress. At the 90-min time point, lambs were euthanized through intravenous injection of pentobarbital (Euthanimal, 0.25 mg/kg b.w.). The blood collection at the 90-min time point commenced at 9 a.m.

Hypothalamus slice culture

The hypothalami were dissected and placed in oxygenated Krebs-Ringer media 60 min after the termination of the stress. Met-enkephalin secretion from the hypothalamus was assessed using a modified method based on Kowalski and Giraud (1993). In summary, coronally sliced tissue sections (50 µm) were positioned on filter inserts within 24-well plates containing 1 ml of Krebs-Ringer bicarbonate medium. Following a 20-min preincubation period, the tissues underwent incubation at 37°C for five consecutive 20-min intervals in 1 ml medium, following this sequence: (1) basal medium; (2) stimulating medium with 100 nM of naltrexone; (3, 4) basal media; (5) stimulating medium with 56 mM KCl (to verify tissue survival). The concentrations of Met-enkephalin in the basal media did not exhibit significant differences, leading to the pooling of results and their presentation as Met-enkephalin release under basal conditions.

Hormones assay

Met-enkephalin concentrations in the plasma, hypothalamus, and in *in vitro* studies were assessed using radioimmunoassay, as detailed in a prior publication (Pierzchala-Koziec *et al*. 2018). Cortisol concentrations were determined through radioimmunoassay employing commercial kits from DRG, Germany. The interassay and intraassay coefficients of variance were as follows: 7% and 11% for Met-enkephalin, and 6% and 12% for cortisol.

PENK expression

The expression of PENK was assessed through quantitative PCR using the following primers: 18 rRNA: F 5′-CTTTGGTCGCTCGCTCCTC-3′, R 5′-CTGACCGGGTTGGTTTTGAT-3′;
PENK: F 5′-CAGCTCTTTGGCTTCATCT-3′, R 5′-AGAGGCCAATGGAAGTGAGA-3′ (Genomed S.A., Warszawa, Poland). Tissues were extracted with TRIzol reagent (Invitrogen; Thermo Fisher Scientific), and the concentration and purity of total RNA were determined by measuring absorbance at 260 and 280 nm (A260/A280=1.60/1.89). Single-strand cDNA was synthesized through reverse transcription with a High Capacity RNA-to-cDNA Kit (Thermo Fisher, USA). Quantitative PCR (qPCR) was conducted using a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) with TaqMan® Gene Expression Master Mix (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) and TaqMan chemistry as recommended by the protocol: 50°C for 5 min and 95°C for 15 min, followed by 45 cycles at 95°C for 15 sec, 62°C for 20 sec, and 72°C for 20 sec. The expression levels of the target genes were calculated relative to that of the housekeeping gene 18S rRNA using the StepOne Software v2.1 (Applied Biosystems) with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

In vitro studies

Opioid receptor binding

Opioid receptor binding was determined using the method reported by Hytrek *et al*. (1996) with modifications by Pierzchała-Koziec *et al.* (2017). In brief, the dissected tissues were homogenized in ice-cold buffer (50 mM TRIS-HCl, pH = 7.4), and the homogenate was centrifuged at 20,000× g for 15 min. Cell membranes (1 ml, 1 mg of protein) were incubated at 30°C for 30 min with tritiated agonists specific to each type of opioid receptor: 26.0 nM ^{3}H-DAGO ([D-Ala2,MePhe4,Gly(ol)^{5}]enkephalin) for mu receptors, 6.80 nM ^{3}H-DPDPE ([D-Ala2, N-Me-Phe4, Gly-ol]) for delta receptors, and 59.18 nM ^{3}H-EKC (ethylketocyclazocine) for kappa receptors. Radioligands were obtained from Amersham International (^{3}H-DAGO and ^{3}H-DPDPE) and New England Nuclear (^{3}H-EKC). Nonspecific binding was assessed with 10 µM of unlabeled ligands: Met-enkephalin for delta, Leu-enkephalin-Arg for kappa, and morphine for mu receptors. The separation of free ligand from membrane-bound radioligand was achieved by filtration under reduced pressure through GF/B Whatman glass filters. Protein concentrations were determined using the bicinchoninic acid (BCA) method (Olson 2016).

Statistical analysis

The results were presented as means ± standard error of the mean (SEM). The normality of the data was assessed using the Shapiro-Wilk test. The area under the curve (AUC) was determined utilizing the trapezoidal method. Analysis of data for the four treatment groups (control, isolation stress, hexarelin injection, and isolation plus hexarelin) was conducted through two-way ANOVA. Multiple plasma samples were subjected to one-way ANOVA for repeated measures, followed by Tukey's post hoc tests, with significance set at $p < 0.001$. If both a general treatment effect and an interaction between time and treatment were identified during ANOVA, data were further analyzed using linear regression (Met-enkephalin). Statistical analysis was performed using SigmaPlot_V_13 (Systat Software Inc., San Jose, CA, USA).

Results

Plasma and tissue of hormones concentration

Effects of hexarelin and/or isolation stress on plasma concentrations of cortisol

To validate the induction of a stress response by isolation, plasma concentrations of cortisol were assessed. Lambs subjected to isolation stress exhibited a significant rise in plasma cortisol concentrations (Table 1, Fig. 1). Conversely, the administration of hexarelin, either alone or 15 min before the onset of isolation, resulted in a reduction in plasma cortisol concentrations (Table 1, Fig. 1).

The impact of hexarelin and/or isolation stress became more evident when the plasma concentrations of cortisol were expressed as the AUC. This confirmed that lambs exposed to isolation stress exhibited an elevation in plasma cortisol concentrations, while hexarelin administration resulted in a decrease (Fig. 2).

Table 1

Effect of hexarelin and/or isolation stress on plasma concentrations of cortisol in 3-month-old lambs (n = 6, nmol/l, X ± SEM)

Treatments	Plasma concentrations of cortisol (nmol/l, X ± SEM)					
	Time in minutes					
	-15′	+15′	30′	45′	60′	90′
Group A Control	41.2 ± 1.08^{b}	35.8 ± 1.01^{a}	41.2 ± 1.08^{b}	44.2 ± 1.08^{c}	36.8 ± 1.05^{a}	43.0 ± 1.06^{bc}
Group B Hexarelin[1]	43.2 ± 0.94^{c}	40.0 ± 0.89^{c}	34.2 ± 0.73^{b}	25.8 ± 0.85^{a}	36.8 ± 0.98^{b}	36.8 ± 1.05^{b}
Group C Stress[2]	40.2 ± 1.01^{a}	43.0 ± 0.77^{a}	58.3 ± 1.77^{b}	100.0 ± 2.52^{e}	88.8 ± 1.62^{d}	76.8 ± 1.08^{c}
Group D Hexarelin[1]+ Stress[2]	43.0 ± 1.03^{c}	30.8 ± 1.08^{a}	33.7 ± 0.82^{ab}	35.0 ± 0.89^{b}	84.0 ± 1.15^{d}	102.8 ± 1.94^{e}

[1] Lambs received hexarelin immediately after the 15-min blood sampling
[2] Lambs were subjected to isolation stress between 0 and 60 min
[a,b,c,d,e] Different superscript letters in the row (treatment) indicate differences $p < 0.05$.

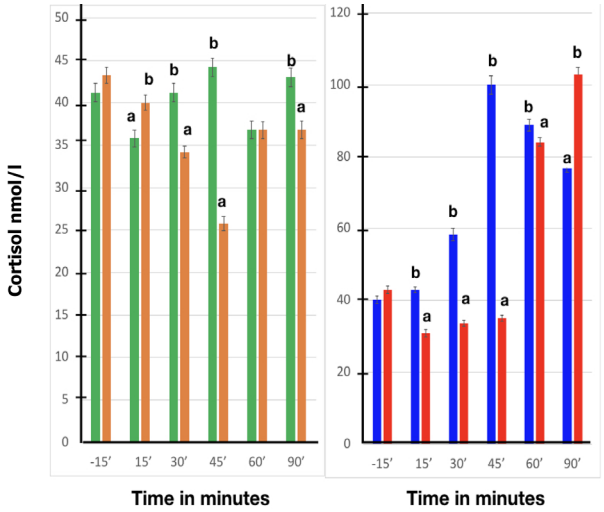

Fig. 1. Effect of hexarelin (1) and/or isolation stress (2) on plasma concentrations of cortisol in 3-month-old lambs, as indicated. (1) Lambs received hexarelin immediately after the 15-min blood sampling, (2) Lambs were subjected to isolation stress between 0 and 60 min. Data are presented as means (n = 6, pmol/l ± SEM). Vertical lines indicate SEM. Different letters at the same time indicate differences $p < 0.001$ between lambs treated with hexarelin or not. Control (green), orange (hexarelin administered), blue (isolation stress), and red (isolation stress plus hexarelin administered).

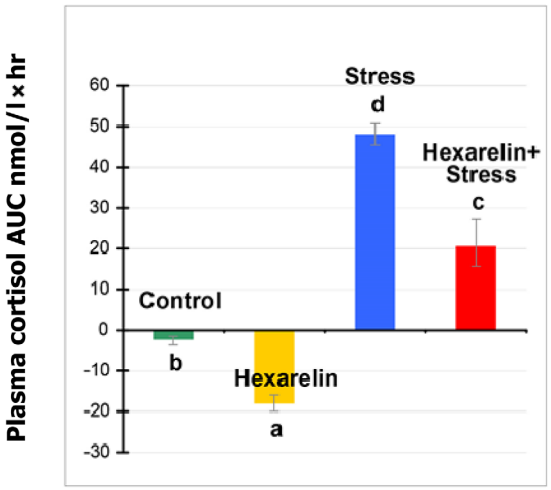

Fig. 2. Effect of isolation stress and/or hexarelin on plasma concentrations of cortisol, as indicated by the area under the curve (AUC), in 3-month-old lambs. Data are presented as means (n = 6, pmol/l x hr ± SEM). Vertical lines indicate SEM. Different letters at the same time indicate differences $p < 0.001$ between treatments (control in green, hexarelin administered in orange, isolation stress in blue, and isolation stress plus hexarelin administered in red).

Effect of hexarelin and/or isolation stress on plasma concentrations of Met-enkephalin

Table 2 and Fig. 3 present the plasma concentrations of Met-enkephalin in lambs undergoing hexarelin administration and/or isolation stress. Hexarelin administration led to a transient decline ($p < 0.001$), reaching a nadir at the 30-min time point in plasma concentrations of Met-enkephalin compared to pretreatment, followed by a rebound increase ($p < 0.001$) with a peak at the 60-min time point (Table 2, Fig. 3). In control lambs, plasma concentrations of Met-enkephalin exhibited an increase (linear regression – adjusted $R^2 = 0.671$; Fig. 3, Table 2). Lambs subjected to isolation stress for 15 min experienced a transient elevation ($p < 0.001$) in plasma concentrations of Met-enkephalin by 33.9%, and for 30 min, it increased by 48.1% compared to pretreatment levels (Fig. 3, Table 2). Subsequently, plasma concentrations of Met-enkephalin displayed a decline (linear regression – adjusted $R^2 = 0.905$) between the 30- and 90-min time points (Fig. 3, Table 2).

Table 2

Effect of hexarelin and/or isolation stress on plasma concentrations of Met-enkephalin in 3-month-old lambs (n = 6, pmol/l, X ± SEM)

Treatments	Plasma concentrations of Met-enkephalin (pmol/l, X ± SEM)					
	Time in minutes					
	-15′	+15′	30′	45′	60′	90′
Group A Control	172.0 ± 1.95^{b}	151.1 ± 1.46^{a}	200.8 ± 1.76^{c}	204.8 ± 1.76^{c}	204.8 ± 2.34^{c}	226.2 ± 2.18^{d}
Group B Hexarelin[1]	182.1 ± 2.65^{c}	154.3 ± 1.32^{b}	125.0 ± 1.39^{a}	246.6 ± 2.06^{e}	313.7 ± 2.04^{f}	209.7 ± 2.00^{d}
Group C Stress[2]	188.2 ± 3.80^{a}	252.0 ± 2.79^{c}	278.9 ± 1.92^{d}	244.5 ± 1.60^{c}	200.8 ± 2.63^{b}	176.9 ± 1.95^{a}
Group D Hexarelin[1] +Stress[2]	175.3 ± 1.62^{a}	259.7 ± 1.88^{d}	314.1 ± 2.42^{e}	261.4 ± 2.46^{d}	226.9 ± 2.28^{c}	209.2 ± 2.32^{b}

[1] Lambs receive hexarelin immediately after the 15-minute blood sampling
[2] Lambs were subjected to isolation stress between 0 and 60 min
[a,b,c,d,e,f] Different superscript letters in the row (treatment) indicate differences $p < 0.05$.

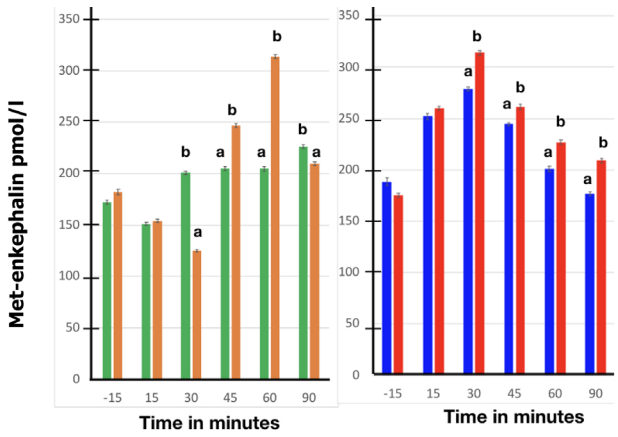

Fig. 3. Effect of hexarelin (1) and/or isolation stress (2) on plasma concentrations of Met enkephalin in 3-month-old lambs. (1) Lambs received hexarelin immediately after the 15-min blood sampling, (2) Lambs were subjected to isolation stress between 0 and 60 min. Data are presented as means (n = 6, pmol/l ± ± SEM). Vertical lines indicate SEM. Different letters at the same time indicate differences $p < 0.001$ between lambs treated with hexarelin or not. Control (green), orange (hexarelin administered), blue (isolation stress), and red (isolation stress plus hexarelin administered).

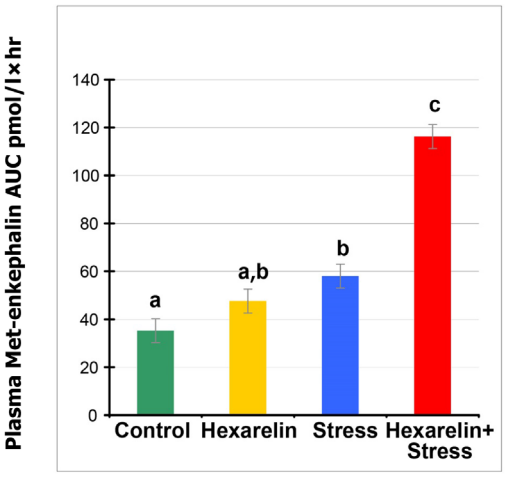

Fig. 4. Effect of isolation stress and/or hexarelin on plasma concentrations of Met enkephalin as the area under the curve (AUC) in 3-month-old lambs. Data are presented as means (n = 6, pmol/l x hr ± SEM). Vertical lines indicate SEM. Different letters at the same time indicate differences $p < 0.001$ between treatments. Control (green), orange (hexarelin administered), blue (isolation stress), and red (isolation stress plus hexarelin administered).

The data regarding plasma concentrations of Met-enkephalin were subsequently analyzed as the AUC minus the initial concentration. These findings are depicted in Fig. 4 (refer also to Table 3). Plasma concentrations of Met-enkephalin, expressed as the AUC, remained unaffected by hexarelin treatment alone. However, they showed an increase ($p < 0.001$) in lambs exposed to isolation stress (by 64.8%) and were further elevated (3.3-fold) when hexarelin treatment was combined with isolation stress (Fig. 4, Table 3).

Table 3

Effect of isolation stress and/or hexarelin on plasma (AUC, pmol/l x hr) and hypothalamic concentrations of native and total Met-enkephalin (n = 6, pmol/mg protein, X± SEM) in 3-month-old lambs

Group/hormone	Plasma Met-enkephalin concentration	Hypothalamic concentrations	
	Area under curve pmol/l x hr	Native Met-enkephalin pmol/mg protein	Total Met-enkephalin pmol/mg protein
Group A Control	35.2 ± 3.61^{a}	0.81 ± 0.010^{b}	6.62 ± 0.045^{b}
Group B Hexarelin	47.6 ± 3.92^{ab}	1.23 ± 0.070^{d}	8.41 ± 0.034^{c}
Group C Stress	58.0 ± 5.46^{b}	0.64 ± 0.006^{a}	4.32 ± 0.038^{a}
Group D Hexarelin + Stress	116.2 ± 2.46^{c}	1.00 ± 0.156^{c}	7.22 ± 0.016^{bc}
Two way ANOVA	p =	p =	p =
Hexarelin	$2.13E^{-8}$	$8.59E^{-7}$	$3.74E^{-9}$
Stress	$2.84E^{-10}$	$1.13E^{-17}$	$2.37E^{-7}$
Interaction	$1.12E^{-5}$	$1.33E^{-11}$	0.00544

[a, b, c, d] Different superscript letters indicate differences between treatments $p < 0.001$.

Effect of hexarelin and/or isolation stress on hypothalamic concentrations of native and total Met-enkephalin

Significant effects ($p < 0.001$) were observed for both hexarelin and stress on the hypothalamic concentrations of Met-enkephalin, with a notable interaction ($p < 0.001$). Hypothalamic concentrations of native Met-enkephalin and total Met-enkephalin were reduced ($p < 0.001$) by 21.0% and 34.7%, respectively, after 60 min of isolation stress and a subsequent 30-min recovery period in 3-month-old lambs (Table 3). Furthermore, hypothalamic concentrations of native Met-enkephalin and total Met-enkephalin increased ($p < 0.001$) by 51.9% and 27.0%, respectively, 60 min and 30 min into the recovery period following hexarelin administration in 3-month-old lambs (Table 3).

The study on Met-enkephalin secretion and opioid receptors binding

Effect of stress and/or hexarelin treatment *in vivo* on Met-enkephalin *in vitro* release from the hypothalamus

The *in vitro* release of Met-enkephalin from the hypothalamus varied based on the treatment of the lambs before harvesting the hypothalamic tissue (Table 4). The basal release of Met-enkephalin *in vitro* was significantly higher ($p < 0.001$) with tissue from stressed lambs, showing an increase of 20.1% (Table 4). Likewise, Met-enkephalin release *in vitro* was higher from tissue from stressed lambs that received hexarelin administration compared to the other treatments (Table 4).

The variation (Δ Met-enkephalin release) in the *in vitro* release of Met-enkephalin in the presence of naltrexone showed differences based on the treatment of the tissue sources (Table 4). The alteration in Met-enkephalin release from the hypothalami was 2.96 times higher ($p < 0.001$) in tissue from lambs administered hexarelin compared to control lambs (Table 4). Furthermore, the change in hypothalamic Met-enkephalin release was 44.4% greater ($p < 0.001$) in tissue from stressed lambs compared to control lambs (Table 4).

Table 4

Effect of stress and/or hexarelin treatment *in vivo* on Met-enkephalin release from the hypothalamus *in vitro* (n = 6, fmol/mg protein, X ± SEM)

Treatments	Met-enkephalin release *in vitro* (fmol/mg protein)	Δ Met-enkephalin release *in vitro* in response to naltrexone (fmol/mg protein)
Group A Control	4.02 ± 0.042^{a}	1.08 ± 0.048^{b}
Group B Hexarelin	3.90 ± 0.017^{a}	3.20 ± 0.023^{d}
Group C Stress	4.83 ± 0.012^{b}	1.56 ± 0.040^{c}
Group D Hexarelin+Stress	6.00 ± 0.013^{c}	-3.53 ± 0.023^{a}
Two way ANOVA	p =	p =
Hexarelin	$2.98E^{-20}$	$8.18E^{-18}$
Stress	$3.05E^{-14}$	$5.64E^{-23}$
Interaction	$1.34E^{-14}$	$5.92E^{-24}$

^{a,b,c,d} Different superscript letters indicate differences between treatments $p < 0.001$.

Effect of hexarelin treatment and/or stress *in vivo* on hypothalamic opioid receptors binding

Both hexarelin administration and isolation stress influenced the concentrations of opioid receptors in the hypothalamus. Hexarelin administration significantly reduced ($p < 0.001$) hypothalamic concentrations of delta opioid receptors by 87.0% in unstressed lambs and by 63.9% in stressed lambs (Table 5). However, hexarelin alone had no effect on either mu-opioid receptors or kappa receptors (Table 5). Isolation stress increased ($p < 0.001$) hypothalamic concentrations of mu-opioid receptors (by 2.95-fold), delta opioid receptors (by 34.0%), and kappa receptors by 105.2% (Table 5). In stressed lambs, hypothalamic binding of mu and delta opioid receptors was diminished ($p < 0.001$) in lambs receiving hexarelin injections by 56.3% and 44.0%, respectively (Table 5).

Proenkephalin (PENK) gene expression

Effect of stress and/or hexarelin on PENK expression in hypothalamus

The expression of PENK in the hypothalamus was impacted by both hexarelin administration and isolation stress. Hexarelin administration resulted in a 55.5% decrease ($p < 0.001$) in PENK expression (Table 6). In contrast, isolation stress led to a 3.72-fold increase ($p < 0.001$) in the expression of PENK in the hypothalamus (Table 6).

Table 5

Effect of stress and/or hexarelin treatment *in vivo* on hypothalamic opioid receptors binding (n = 6, fmol/mg protein, X ± SEM)

Treatments	Hypothalamic opioid receptors (fmol/mg protein)		
	Mu	Delta	Kappa
Group A Control	5.12 ± 0.070^{a}	55.2 ± 0.91^{c}	19.4 ± 0.81^{a}
Group B Hexarelin	4.80 ± 0.097^{a}	7.17 ± 0.115^{a}	21.6 ± 0.51^{a}
Group C Stress	15.1 ± 0.119^{c}	72.0 ± 1.63^{d}	39.8 ± 1.25^{b}
Group D Hexarelin+Stress	6.6 ± 0.073^{b}	26.0 ± 1.03^{b}	22.3 ± 0.88^{a}
Two way ANOVA	p =	p =	p =
Hexarelin	$4.28E^{-22}$	$2.26E^{-21}$	$2.14E^{-8}$
Stress	$1.33E^{-24}$	$3.36E^{-13}$	$8.54E^{-11}$
Interaction	$1.88E^{-21}$	0.361	$3.03E^{-10}$

^{a,b,c,d} Different superscript letters indicate differences between treatments $p < 0.001$.

Table 6

Effect of stress and/or hexarelin on PENK expression in the hypothalamus (n = 6, RQ, X ± SEM)

Treatments	PENK RQ
Group A Control	3.82 ± 0.111^{b}
Group B Hexarelin	1.70 ± 0.091^{a}
Group C Stress	14.2 ± 0.283^{d}
Group D Hexarelin+Stress	5.12 ± 0.075^{c}
Two way ANOVA	p =
Hexarelin	$2.25E^{-13}$
Stress	$1.90E^{-14}$
Interaction	$6.02E^{-11}$

^{a,b,c,d} Different superscript letters indicate differences between treatments $p < 0.001$.

Discussion

The results obtained demonstrated the acute effects of hexarelin on the synthesis, secretion, receptor binding, and concentration of native and total Met-enkephalin in growing lambs.

Table 7 compiles the effects of hexarelin administration and/or isolation stress on various Met-enkephalin endpoints along with plasma concentrations of cortisol, providing a comprehensive overview of the entire scenario.

Plasma concentrations of cortisol in sheep were elevated due to isolation stress (Table 7, Figs 1 and 2), consistent with findings from previous studies (Pierzchala-Koziec *et al.* 2018). In contrast to humans, where hexarelin has been shown to increase circulating concentrations of cortisol (Massoud *et al.* 1996; Ghigo *et al.* 1997; Korbonits *et al.* 1999), the present study revealed a reduction in plasma concentrations of cortisol following hexarelin administration in 3-month-old lambs (Figs 1 and 2). Furthermore, hexarelin administration significantly mitigated the increase in plasma concentrations of cortisol in lambs subjected to isolation stress (Figs 1 and 2).

Reports on the effect of GHSR on the circulating concentration of cortisol are inconsistent, with increases observed in some studies and no effect in others. This variability is likely attributed to the diverse experimental protocols used, including variations in acute/chronic hexarelin treatment, route of administration, the choice of human or animal models, gender, age, and the clinical status of humans (Massoud *et al.* 1996; Ghigo *et al.* 1997). In the present study, conducted on growing lambs receiving an intravenous injection of only 0.5 µg/kg b.w. of hexarelin, it may be speculated that this dosage was too small to stimulate adrenal cortisol secretion. A previous study (Massoud *et al.* 1996) demonstrated that a dose of 0.5 µg/kg b.w. or lower did not alter basal cortisol levels in healthy adult males. Addi-

Table 7

Summary of the effects hexarelin and/or isolation stress on the cortisol and Met-enkephalin system in lambs. No change →, increase ↑, decrease ↓

	Hexarelin	Stress	Hexarelin & Stress
Plasma concentration of cortisol	↓↓↓	↑↑↑	↓
Plasma concentration of Met-enkephalin	↑	↑↑	↑↑↑↑
Hypothalamic concentrations			
Native Met-enkephalin	↑↑	↓	↑
Total Met-enkephalin	↑↑	↓	↑
In vitro Met-enkephalin release from hypothalamic tissue			
Basal	→	↑	↑↑
In presence of naltrexone	↑↑	↑	↓↓
PENK expression	↓↓	↑↑↑↑	↑↑
Hypothalamic Mu opioid receptors	→	↑↑↑	↑
Hypothalamic Delta opioid receptors	↓↓↓	↑	↓↓
Hypothalamic Kappa opioid receptors	→	↑↑	→

tionally, the effects of hexarelin's different half-life (35-70 min) must be considered when interpreting the obtained results.

In contrast to a prior study where no effect of isolation stress was observed on plasma concentrations of Met-enkephalin in 3-month-old female lambs (Pierzchała-Koziec *et al*. 2019), isolation stress increased plasma concentrations of Met-enkephalin in the present study. This discrepancy can be explained by the multiple sampling times in the present study, in contrast to the single sampling time in the previous study (Pierzchała-Koziec *et al.* 2019).

Hexarelin administration elevated plasma concentrations of Met-enkephalin (Tables 2 and 3, Figs 3 and 4). This represents a novel finding across all species, to the best of our knowledge. However, notable interactions were observed between the effects of hexarelin and isolation stress on Met-enkephalin-related parameters (Tables 2 and 3, Figs 3 and 4).

Hypothalamic concentrations of Met-enkephalin, which increased with hexarelin and decreased with isolation stress (Table 3), mirrored the plasma concentrations of cortisol (decreased by hexarelin and increased by isolation stress). The elevated plasma concentrations of Met-enkephalin in lambs subjected to isolation stress were accompanied by increases in the release of Met-enkephalin, *in vitro* hypothalamic PENK gene expression (Table 6), and opioid receptor binding (Table 5). Concurrently, with the increase in plasma concentrations of Met-enkephalin in lambs receiving hexarelin (Tables 2 and 3, Figs 3 and 4), there were increases in the hypothalamic concentration of both native and total Met-enkephalin (Table 3), along with hypothalamic *in vitro* release of Met-enkephalin in the presence of naltrexone (Table 4).

It is likely that the effects of hexarelin are mediated by GHSR-1a alone or in conjunction with other receptors. GHSR-1a has been demonstrated to form heterodimers with various receptors, including melanocortin receptor 3 (MC3 receptor), GPR83, dopamine receptor 2, serotonin receptor 2c, and another GPCR (Al Massadi *et al*. 2017). Alternatively, hexarelin may act through a different receptor, specifically CD36, a scavenger receptor class B member 3 (Mao *et al.* 2014). CD36 has widespread expression in various cell types in peripheral organs and the nervous system, including endothelial cells, pericytes, astrocytes, and microglia. It has been proposed that CD36 mediates endothelial and mitochondrial dysfunctions, oxidative stress, and inflammation, which are implicated in numerous central nervous system diseases such as stroke, Alzheimer's disease, Parkinson's disease, and spinal cord injury (Feng *et al.* 2023). Hexarelin has demonstrated neuroprotective and anti-apoptotic effects, and it can protect Neuro-2a cells from H_2O_2-induced injury through a molecular pathway involving mitogen-activated protein kinase (MAPK). For example, it has been suggested that the effects of hexarelin on the heart are not mediated by a GH/GHSR-1a mechanism (Locatelli *et al.* 1999; Tivesten *et al.* 2000), but rather through the presence of a hexarelin-specific receptor subtype

in the heart and other nonendocrine tissues identified as CD36. Hexarelin-mediated activation of CD36 in perfused hearts increased coronary perfusion pressure in a dose-dependent manner. In *in vitro* studies, hexarelin has demonstrated cardioprotective activity in cardiovascular conditions such as cardiac fibrosis, ischemic heart disease, cardiac dysfunction, and atherosclerosis. These beneficial effects appear to be mediated through the direct binding and activation of its cardiac receptors CD36 and GHSR 1a. However, since current evidence is mainly derived from experimental animal models or *in vitro* cell lines, clinical application of hexarelin in human subjects to observe its efficacy and potential side effects is necessary (Mao *et al*. 2014). What remains unclear is whether the effects of hexarelin observed in the present study are *via* GHSR-1a or not.

Conclusions

The current data showcase the acute effects of hexarelin on the synthesis, secretion, receptor binding, and concentration of native and total Met-enkephalin in growing lambs. These results offer additional backing for the cross-talk between hexarelin, Met-enkephalin, and the HPA axis. Moreover, the evidence put forth supports the capacity of the growth hormone secretagogue, hexarelin, to modulate some of the effects of stress on the EOP activity in growing lambs.

Acknowledgments

The authors express gratitude to Professor M. Dziedzicka-Wasylewska for her assistance with the opioid binding assay.

Funding source

This research was funded by the Ministry of Science and Higher Education of the Republic of Poland (Grant No. 020002-D-015).

Author Contributions

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

Conflict of Interest

The authors declare no conflict of interest.

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