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ORIGINAL PAPER

QRFP43 modulates somatotrophic axis activity in female sheep

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ABSTRACT. RFamide (RFa) peptides are a large family of neuropeptides present in all groups of vertebrates. Members of this family have been shown to play many physiological roles in neuroendocrine, behavioral, sensory and autonomic functions. QRFP43 is one of the youngest members of this peptide family, which have been suggested to affect somatotrophic axis activity, mainly through changes in blood GH levels. The aim of the study was to investigate the role of QRFP43 in regulation of the somatotrophic axis hormones activity. The research hypothesis posited that QRFP43 could modulate gene expression, storage and release of somatotrophic axis hormones at the level of the hypothalamus and pituitary. The animals (n = 48) were randomly divided into three experimental groups: a control group receiving an intracerebroventricular (ICV) infusion of Ringer-Locke solution, and two experimental groups administered ICV infusions of QRFP43 at doses of 10 and 50 µg per day. All sheep received four 50-min ICV infusions with 30-min intervals between infusion, on three consecutive days. Hypothalamic structures and pituitary were collected and preserved for further analyses. The results of the present study have demonstrated that QRFP43 may exert an inhibitory effect on somatostatin expression in the hypothalamus, while increasing growth hormone releasing hormone levels in the median eminence, leading to stimulation of growth hormone synthesis in the pituitary gland of sheep. Furthermore, QRFP43 administration increased the mean GH concentration in the blood plasma. Summarizing, QRFP43 may modulate the somatotrophic axis activity in female sheep.

Introduction

Somatotrophic axis functions are primarily regulated by two antagonistic neurohormones: somatostatin (SRIF) and growth hormone releasing hormone (GHRH). Immunohistochemical studies have shown that neurons expressing SRIF are located mainly in the periventricular nucleus (PVN) of the anterior hypothalamus (AHA). From there, SRIF is transported by neurites within the medio-basal hypothalamus (MBH) to the median eminence (ME). In contrast, cells synthesizing GHRH are exclusively located in the MBH, where neurons transport this hormone to ME terminals (Feng et al., 2011; Hassouna et al., 2012; Wójcik-Gładysz et al., 2018). The direct effect of SRIF and GHRH on somatotrophic pituitary cells occurs through receptors specific to these peptides (Renaville et al., 2002). Both of these opposing acting peptides are responsible for controlling the secretion of growth hormone (GH) by somatotrophic cells located in the anterior pituitary. GH is the primary factor regulating peripheral growth processes, and its secretion depends on the alternating release of SRIF and GHRH from ME nerve terminals. This mechanism results in a pulsatile release of GH from pituitary somatotrophic cells into the peripheral blood (Chen et al., 1989). The main effect of GH is the stimulation of body weight gain by promoting chondrogenesis and osteogenesis in bone cartilage cells. Additionally, growth hormones directly affect the regulation

of carbohydrate metabolism by inducing glycogenolysis and increasing glucose release from the liver (Jamil Sami, 2007).

RFamide (RFa) peptides are a large family of neuropeptides characterized by the presence of an Arg-Phe-NH2 motif at their C-terminus, and they are present in all vertebrates. Members of this family have been shown to play numerous important physiological roles in neuroendocrine, behaviour, sensory and autonomic functions (Findeisen et al., 2011). RFa peptides are expressed in many areas of the vertebrate brain, where they mainly regulate appetite and reproduction (Sandvik et al., 2014). The rising number of RFa peptides identified in mammals can be divided into five groups: the gonadotropin-inhibitory (GnIH) group, the neuropeptide FF (NPFF) group, the kisspeptin group, the prolactin-releasing peptide (PrRP) group, and the pyroglutamylated RFa peptide group (26RFa/QRFP43) (Chartrel et al., 2011; Przybył et al., 2024; Szlis et al., 2024).

QRFP43 is the most recently identified member of this peptide family. A preprotein is encoded by a precursor gene whose post-translational cleavage can generate two peptide forms – 26RFa and 43RFa – both present in many regions of the vertebrate brain (Leprince et al., 2017). The C-terminal sequence of those peptides (KGGFXFRF-NH2), responsible for the biological activity, is highly evolutionarily conserved (Chartrel et al., 2011). Moreover, the biological activity of these peptides is very similar, but QRFP43 shows more potent effects (Jiang et al., 2003). Numerous studies have implicated that the expression of both the QRFP43 gene and peptide occur in hypothalamic nuclei involved in critical survival processes, including the arcuate nucleus (ARC), ventromedial hypothalamic nucleus (VMN), lateral hypothalamic area (LHA), PVN and AHA (Bruzzone et al., 2007; Chartrel et al., 2011). Furthermore, the presence of GPR103, a specific receptor for QRFP43, has been demonstrated in all these hypothalamic structures (Kampe et al., 2006; Bruzzone et al., 2007). These findings and previous data suggest that QRFP43 may be another neuropeptide modulating the hormonal activity of the somatotrophic axis.

In fact, QRFP43 and 26RFa have been shown to influence hormone secretion and behaviour, especially in rodents. Navarro et al. (2006) demonstrated that 26RFa stimulated gonadotrophin release in female rats. In addition, this peptide has been shown to exert an orexigenic effect in mice (Lectez et al., 2009). QRFP43 administration in rodents stimulated aldosterone release, induced feeding behaviour, and increased the rate of metabolism (Jiang et al., 2003; Takayasu et al., 2006). Our studies in sheep model also revealed that QRFP43 could modulate the activity of the hypothalamic appetite-regulating centre, gonadotrophic axis, and hypothalamo-pituitary-thyroid axis (Przybył et al., 2024; Szlis et al., 2024)among others by the peptides belonging to the RFamide peptide family. However, the knowledge concerning on the impact of recently identified member of this family (QRFP43). Other studies performed in different animal models also demonstrated that QRFP43 and other RFa peptides might influence somatotrophic axis activity, primarily through changes in blood GH concentrations (Iijima et al., 2001; Sakamoto et al., 2003; Moriyama et al., 2007; Luque et al., 2011; Qaiser et al., 2012). Nevertheless, current literature data shows that the effect of RF-amide peptides is different for each family member and this effect may also be distinct in various species as we have already noted in our other work relating to QRFP43 (Przybył et al., 2024, 2025). The results obtained in the presented project fulfil the criteria of basic research, as well as provide new unique data on the effects of anorexigenic/orexigenic neuropeptides on the growth processes modulation.

The objective of the study was to investigate the role of QRFP43 in modulating the activity of key somatotrophic axis hormones. The research hypothesis assumed that QRFP43 could modulate gene expression, storage and release of somatotrophic axis hormones both at the hypothalamic and pituitary levels. To verify this hypothesis, we determined the expression of *SRIF*, *GHRH* mRNA and peptides in the hypothalamus, mRNA expression of the *SRIF* receptors 2 and 5 (*SSTR2*, *SSTR5*), and *GHRHR* genes, as well as *GH* mRNA and peptide expression in the ovine pituitary. Furthermore, we investigated the changes of GH concentration in blood and pituitaries homogenates.

Material and methods

Animals and experimental design

All procedures were conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and the EU Directive 2010/63/EU on animal experimentation. Approval for the study was obtained from the Local Ethics Committee affiliated with the Warsaw University of Life Sciences (Resolution no. WAW2/193/2019),

in compliance with the Polish Law for the Animal Care and Use of January 21, 2005, and the Polish Law for Animal Protection of September 16, 2011.

Forty-eight female Polish Merino sheep (42-week-old, average weight 38.6 ± 3.5 kg) were used in the study. The animals were housed indoors under natural lighting conditions (52°N, 21°E) and fed a standard hay diet with commercial concentrates twice daily, according to the Polish Recommendations for Growing Sheep (Strzetelski et al., 2014). The feed was balanced in terms of energy and appropriate nutrients to ensure optimal development of the growing animals. Water and salt licks were available *ad libitum*.

Stainless steel cannulas were implanted directly into the third ventricle (IIIv) of the brain under anesthesia with atropinum sulfuricum (0.44 mg kg^{-1}; Polfa, Warsaw, Poland), ketamine (400 mg per sheep; Vetoquinol Biowet, Gorzów, Poland), and dexmedetomidine (0.05 mg kg^{-1}, Dexdomitor®; Orion Pharma, Turku, Finland). Permanent stainless-steel guide cannulas (ø = 0.8 mm) were inserted into the IIIv at specific coordinates: anteroposterior position – 31 mm, horizontal position – 0.5 mm, and mid-sagittal position – 0.10 mm (Przybył et al., 2021a). The correct placement of the guide cannula was verified by the outflow of cerebrospinal fluid (CSF) upon removal of the guide tube stiletto, as well as by post-mortem examination. After surgery, penicillin-streptomycin (0.1 ml kg^{-1}; ScanVet, Gniezno, Poland) and tolfenamic acid (0.05 ml kg^{-1}, Tolfine®; Vetoquinol Biowet, Gorzów, Poland) were administered by intramuscular injection for four consecutive days, followed by a five-week recovery period. In all experimental animals, oestrus synchronization was performed 21 days before intracerebroventricular (ICV) infusion using Chronogest CR sponges (MSD Animal Health, UK), as described by Przybył et al. (2021a). Animals entered the experiment on day 4 to 5 after ovulation, coinciding with a decrease in peripheral blood oestrogen levels in sheep (Kennedy, 2012).

The experiments were conducted from the end of October till the first week of December. Animals were randomly divided into three experimental groups: a control group receiving an ICV infusion of Ringer-Locke solution (artificial cerebrospinal fluid; 480 µl per day; n = 16), group I receiving an ICV infusion of QRFP43 (Phoenix Pharmaceuticals Inc., USA) at a dose of 10 µg per day (RFa10 group; n = 16), and group II receiving QRFP43 at a dose of 50 µg per day (RFa50 group; n = 16). Both doses of QRFP43 were diluted in 480 µl of Ringer-Locke solution, and infused at a rate of 120 µl h^{-1}. QRFP43 dosing in this experiment were selected based on information from the literature, as well as our preliminary results and previous neuroendocrine research (Wójcik-Gładysz et al., 2019; Przybył et al., 2021a; Szlis et al., 2024; Przybył et al., 2024, 2025).

During the experiment, one hour prior to infusion, cannulas were introduced through the guides and locked in position with the tips positioned approximately 2.0 to 2.5 mm above the base of the brain; CSF flowed into the infused cannulas when their tips reached the IIIv. Subsequently, all sheep received four 50-min ICV infusions with 30-min intervals between infusion, carried out from 08:40 to 13:30 on three consecutive days; the flow rate of the microinjection pump was set to 2 µl min^{-1}. Blood samples were collected on the third day of infusion from 08:00 to 13:50 at 10-min intervals (a total of 36 samples were taken from each sheep).

Directly after the last blood sample collection, animals were weighed, anaesthetized intravenously using dexmedetomidine (0.05 ml kg^{-1}) and pentobarbital (80 ng kg^{-1}, Morbital®; Vetoquinol Biowet, Poland), and subsequently decapitated. For molecular biological analysis, isolation of the selected hypothalamic structures from eight sheep from each group (n = 8/group) was performed according to the coordinates (area and depth of cuts) defining the location of individual hypothalamic nuclei described in the stereotaxic atlas of the sheep hypothalamus (Welento et al., 1969). Hypothalamic structures were prepared as described by Przybył et al. (2020). For immunohistochemistry hypothalamic structures from eight sheep from each group (n = 8/group) were prepared as described below.

Real TimeRT-qPCR

Total RNA from the AHA, MBH and anterior pituitary was extracted using the NucleoSpin RNA/Protein kit (Macherey-Nagel GmbH & Co., Düren, Germany) according to the manufacturer's instructions. The yield of the isolated RNA was estimated spectrophotometrically (Nanodrop, NanoDrop Technologies, DE USA), and its integrity was evaluated electrophoretically by separation on a 1.5% agarose gel containing ethidium bromide. For complementary DNA (cDNA) synthesis, 1500 ng ml^{-1} mRNA from the selected hypothalamic regions in a total volume of 20 µl was reverse transcribed using a TranScriba Kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's protocol. Specific primers for sheep species (*Ovis aries*) that determined the expression of housekeeping genes and genes of interest

Table 1. Primer sequences used in the experiment

Gene	Primer	Sequence (5'-3')	Product size (nt)	References
GAPDH	Forward	AGAAGGCTGGGGCTCACT	134	Wojtulewicz et al., 2023
	Reverse	GGCATTGCTGACAATCTTGA		
PPIC	Forward	TGGAAAAGTCGTGCCCAAGA	158	Wójcik et al., 2023
	Reverse	TGCTTATACCACCAGTGCCA		
ACTB	Forward	TGGGCATGGAATCCTG	194	Szczepkowska et al., 2023
	Reverse	GGCGCGATGATCTTGAT		
SRIF	Forward	CTCCATCGTCCTGGCTCTT	113	Przybył et al., 2021
	Reverse	ACTTGGCCAGTTCCTGTTTG		
GHRH	Forward	CCTCTCAGGATTCCACGGTA	146	
	Reverse	CGTACCTTTGCTCCTTGCTC		
GH	Forward	CAGGTTGCCTTCTGCTTCTC	164	
	Reverse	ACCAGGCTGTTGGTGAAGAC		
GHRHR	Forward	CTTCTGGTGGCTGGTTCTCG	173	
	Reverse	GCCCAAAGTTCACCCCAACA		
SSTR2	Forward	GGAGCGGAGTGACAGTAAGC	178	
	Reverse	GGTCTCCATTGAGGAGGGTC		
SSTR5	Forward	GTCATGAGCGTGGATCGCTA	71	
	Reverse	AGGTGAGGTTGCAGGTGTTC		

GAPDH – glyceraldehyde-3-phosphate dehydrogenase, ACTB – β-actin, PPIC – peptidylprolyl isomerase C, SRIF – somatostatin, GHRH – growth hormone releasing hormone, GHRHR – growth hormone releasing hormone receptor, GH – growth hormone, SSTR2 – somatostatin receptor type 2, SSTR5 – somatostatin receptor type 5

were designed using Primer 3 software (The Whitehead Institute, Cambridge, MA) and synthesised by Genomed (Warsaw, Poland). The primer sequences are shown in Table 1 (Przybył et al., 2021b)

Real Time qPCR was conducted using 5× FIREPol EvaGreen qPCR Mix Plus (noROX; Solis BioDyne, Tartu, Estonia) in a total volume of 15 µl containing 3 µl Master Mix, 9 µl RNase free H2O, 2 × 0.5 µl primers (0.5 mM), and 2 µl cDNA template was used. Amplification was performed using a Rotor Gene 6000 thermocycler (Corbett Research, Mortlake, Australia) according to the following protocol: one cycle at 95 °C for 15 min (enzyme activation) followed by 35 cycles at 94 °C for 5 s (denaturation), 59 °C for 20 s (annealing), and 72 °C for 15 s (elongation) and one cycle at 72 °C for 7 min (product stabilisation). The melting curve was performed using the range of 70–95 °C at 0.5 °C intervals. Negative controls without the cDNA template were included for each reaction. Real Time qPCR for each cDNA sample was performed twice in triplicate. The identities of the PCR products were confirmed by direct sequencing (Genomed, Warsaw, Poland) (Przybył et al., 2021b).

The relative gene expression was quantified using the comparative quantitation option of the Rotor Gene 6000 software 1.7 (Qiagen GmbH, Hilden, Germany) 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) (Pfaffl et al., 2002, 2004). The expression of each investigated gene was normalized to the geometrical mean expression of three reference genes, i.e., glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), β-actin (*ACTB*) and peptidylprolyl isomerase C (*PPIC*). The results are presented in arbitrary units as the ratio of target gene expression to the mean expression of housekeeping genes with the relative gene expression for the group of sheep that received only Ringer-Locke solution infusion set to 1.0 ± SEM (Szlis et al., 2018).

Immunohistochemical procedures

Immediately after decapitation, brains underwent carotid artery perfusion with 1500 ml 0.1 M phosphate-buffered saline (PBS; Sigma, Missouri, USA), followed by 2000 ml 0.1 M PBS containing 4% (w/v) paraformaldehyde (Sigma-Aldrich, Missouri, USA), pH 7.4. The hypothalami and pituitaries were dissected 20 min after the beginning of perfusion and postfixed for 72 h (hypothalami) or 48 h (pituitaries) by immersion in the same fixative and next washed with 0.01 M PBS. Hypothalami jointly

with the ME and pituitary stalk were cryoprotected in a 20% sucrose solution in 0.1 M PBS at 4 °C for at least two days. The hypothalamic blocks were frozen at −20 °C and cut on a cryostat (Jung CM 1500, Leica Instruments GmBH, Nussloch, Germany) in coronal planes at 30 μm thickness between the septum and the mamillary body, including the AHA, the MBH, the ME and the pituitary stalk (Wójcik-Gładysz et al., 2018).

Postfixed pituitaries were dehydrated in graded alcohol, embedded in paraplast and cut through at 4 μm thickness in the sagittal plane. Brain sections were washed in 0.1 M PBS and then incubated for 4 h in 1% hydrogen peroxide in 0.1 M PBS, 24 h in 3% pre-immune lamb serum in 0.1 MPBS and in 0.5% Triton X-100 in 0.01 M PBS for 30 min. The pituitary sections were deparafined, rehydrated, washed in 0.01 M PBS, incubated for 30 min in 2% pre-immune lamb serum in 0.01 M PBS and 30 min in 0.1% hydrogen peroxide in 0.01 M PBS (Wójcik-Gładysz et al., 2018).

The hypothalamic sections were incubated for 14 days at 4 °C with the primary antiserum (rabbit anti-somatostatin ref. T-4103; BMA Biomedicals, Augst, Switzerland; rabbit polyclonal GHRH antibody Ref. ab187512; Abcam, Amsterdam, Netherlands), diluted 1:1500 and 1:800, respectively, in accordance with the procedure used in our laboratory(Wójcik-Gładysz et al., 2018). The pituitary sections were incubated for 48 h at 4 °C with the primary antiserum anti-hGH ref. 19538 (methodological details and the specificity of the antibody as described by Dubois et al. (1971); Polkowska et al. (2011) diluted 1:1000. After the incubation with primary antibodies, the sections were incubated for 2 h at room temperature (~20 °C) with secondary antibody (sheep anti-rabbit Ig [H+L] labeled with peroxidase; ABcam, Cambridge, UK) at a dilution of 1:400 (hypothalamic sections), or 1:800 (pituitary sections), in 0.1% normal lamb serum in 0.01 M PBS. The color reaction was developed by incubating sections with 0.05% 3′3-diaminobenzidine tetrahydrochloride chromogen (Sigma, St. Louis, MO, USA) and 0.001% hydrogen peroxide in 0.05 M Tris buffer. Selected hypothalamic material was also stained using the silver intensification method, as described by Liposits et al. (1984).

The specificity of the primary antibodies used in the staining was checked using control reactions. For this purpose, a solution of diluted antibodies was incubated with the respective antigen for 24 h at +4 °C (used: synthetic SRIF (Sigma, Missouri, USA), synthetic GHRH (UCB, Brussels, Belgium) and GH (Sigma, Missouri, USA). The mixture was then applied to control slides prepared according to the protocol described earlier and stained according to the instructions described above. The specificity of the secondary antibodies was checked by incubating the control preparations with rabbit serum (instead of the primary antibodies). A stained background control was also performed, in which slides were incubated with bovine serum (instead of primary and secondary antibodies). None of the above control stains showed specific cell staining (data not shown).

A Nikon type 104 projection microscope (Nikon Corporation, Tokyo, Japan) was used for histological analyses of hypothalamic and pituitary sections. Staining was analysed using a Lucia version 3.51ab image analysis computer system (Laboratory Imaging Ltd, Prague, Czech Republic). Immunostained sections were captured by a camera (Panasonic KR222, Matsushita Electric Industrial Co, Osaka, Japan) and transferred to a colour monitor. Pictures were adjusted for optimal contrast, converted to greyscale, fixed at the same brightness levels, and saved in a buffering system. Analyses were performed using a 20× objective lens for SRIF and GHRH perikarya, an objective of 4× for the nerve terminals in the ME, and an objective of 40× for the pituitary GH cells. The parameter of area fraction, which indicates the percentage of stained elements in a delineated area, was used for immunoreactive (IR) nerve terminals in the ME, and IR GH- cells in the adenohypophysis (Wójcik-Gładysz et al., 2018).

Quantitative analysis was performed within the sub-areas of interest in each hypothalamus and anterior pituitary gland. This was performed by applying a threshold function to select a range of grey values that were optically identified as positively stained. All other values were referred to as unstained. Before taking measurements, the images were processed to subtract the background and remove artefacts. The threshold values used were different for the ME, adenohypophysis, and individual hormones, but constant for all measured images of one histochemical product. The frame size was kept constant for the duration of the image analysis. The area fractions of the immunoproduct in somatostatin neurons were estimated in (i) every 4th section through the hypothalamus on both sides of the third ventricle of the brain (~24 fields of 0.1643 mm^2 measured in each section), and (ii) every 2nd section in the MBH in the delineated area containing the middle part of the ME (total area of 100 mm^2 for each hypothalamus).

Using every 40th mounted and stained section, we analysed the IR GH-cells in the four sections of each adenohypophysis (24 fields of 0.0837 mm^2 were measured in each section). The quantitative measurements taken from each section of the ME, or adenohypophysis were averaged to obtain a mean from each area, for each animal (Wójcik-Gładysz et al., 2018).

Preparation of pituitary homogenates

For homogenization, the 30 mg of anterior part of pituitary were placed in 1 ml phosphate-buffer (pH 7.0) containing 1.5 µl of aprotinin (SigmaAldrich, St. Louis, MO, USA) to protect proteins from degradation. Homogenization was performed using steel balls (50 Hz, 5 min). Next samples were centrifuged (3 000 rpm, 3 min) and the obtained supernatant was collected and frozen at −20 °C for radioimmunoassay (RIA) analysis.

Radioimmunology of GH

The concentration of GH in pituitary and plasma was estimated according to the protocol described previously by Przybył et al. (2021b). The GH concentration of the plasma samples was determined using a double-antibody RIA with anti-ovine GH (rabbit) and anti-rabbit gamma-globulin antisera, as described in detail by Slaba et al. (1994). The reference standard for GH and anti-ovine GH antiserum were provided by Dr A.F. Parlow (National Institutes of Health, Bethesda, MD, USA). The assay sensitivity was 0.68 ng/ml, and the intra- and inter-assay coefficients of variability were 5.9 and 10.2%, respectively.

Statistical analysis

GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA) was used for all statistical calculations. All data presented in the graphs are means ± SEM for each group. Normality of data distribution was tested using the Shapiro-Wilk test. Statistical analyses were performed using the Kruskal-Wallis test with the post-hoc Dunn multiple comparisons test. Differences resulting in $P \leq 0.05$ were considered statistically significant.

Results

SRIF and GHRH mRNA expression

Real Time qPCR analyses revealed the presence of *SRIF* mRNA in AHA and *GHRH* transcripts in the MBH in all experimental groups of sheep. A significant increase in *SRIF* mRNA expression was observed in the RFa10 ($P \leq 0.001$, 2.28 ± 0.19) and RFa50 ($P \leq 0.05$, 1.88 ± 0.21) groups compared to control animals (1.00 ± 0.20). However, no significant differences in *SRIF* mRNA expression were recorded between the RFa10 and RFa50 groups (Figure 1A). Conversely, mRNA expression of *GHRH* was significantly ($P \leq 0.0001$) lower in the RFa10 group compared to the control group of animals (0.56 ± 0.05 and 1.00 ± 0.11 respectively). In parallel, a significant ($P \leq 0.05$) increase in *GHRH* transcript was observed in the RFa50 group compared to the RFa10 group (0.75 ± 0.05 and 0.56 ± 0.05, respectively). No significant differences were observed between the RFa50 and control groups (Figure 1B).

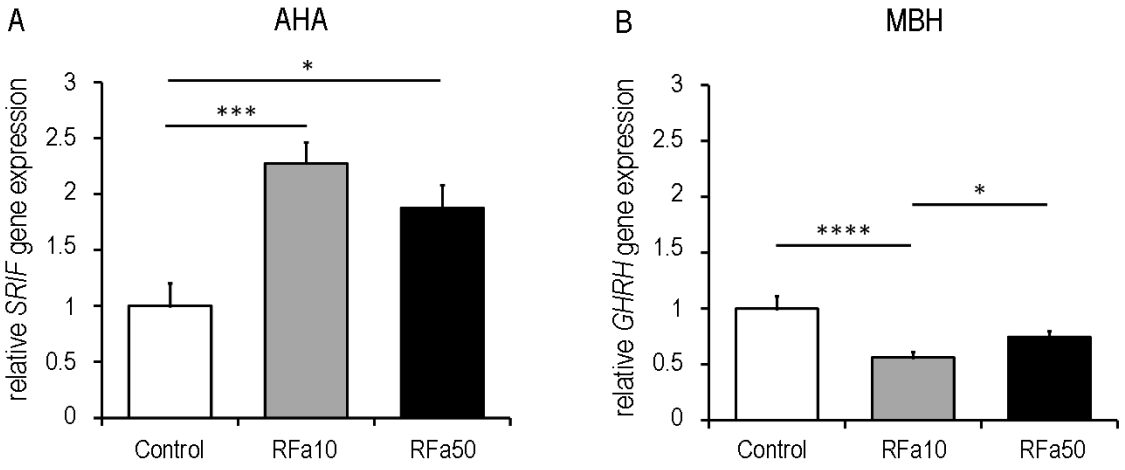

Figure 1. Expression of somatostatin (*SRIF*) mRNA in the anterior hypothalamus area (A) and growth hormone releasing hormone (*GHRH*) mRNA in the medio-basal hypothalamus (B). Data are means ± standard error of the measurement; treatment groups: control group – animals administered Ringer-Locke infusion at a dose of 480 µl per day (n = 8), QRFP43 RFa10 – animals administered QRFP43 infusion at a dose 10 µg/480 µl (n = 8), QRFP43 RFa50 – animals administered QRFP43 infusion at a dose 50 µg/480 µl (n = 8); AHA – anterior hypothalamus, MBH – medio-basal hypothalamus); *,***,**** – indicate significantly different values at $P \leq 0.05$, $P \leq 0.001$ and $P \leq 0.0001$, respectively

SRIF and GHRH immunoreactivity

Microscopic observations revealed the presence of IR SRIF and IR GHRH nerve terminals in the external zone of the ME, spanning its entire width from the postero-anterior part to the pituitary stalk, with the highest accumulation in the medial part. In addition, infusion of QRFP43 induced marked changes in IR SRIF localized in nerve terminals of the ME, leading to a reduction in its abundance in the ME compared to animals from the control group (Figure 2A,B,C). At the same time, an increase in the amount of IR GHRH was observed in the ME of sheep receiving ORFP43 infusions compared to control animals (Figure 2D,E,F).

SSTR2, SSTR5, GHRHR and GH mRNA expression

At the pituitary level, Real Time qPCR analyses revealed that *SSTR2*, *SSTR5*, *GHRHR* and *GH* mRNA transcripts were present in sheep pituitary cells from all experimental groups. A significant increase in *SSTR2* mRNA expression was observed in the RFa10 group (1.46 ± 0.08, $P \leq 0.01$) compared to the control group (1.00 ± 0.07). Similarly, a significant difference ($P \leq 0.01$) was observed between the RFa10 group in comparison to the RFa50 group (1.46 ± 0.08 and 0.89 ± 0.08, respectively), while no significant changes were recorded between the control and RFa50 groups of

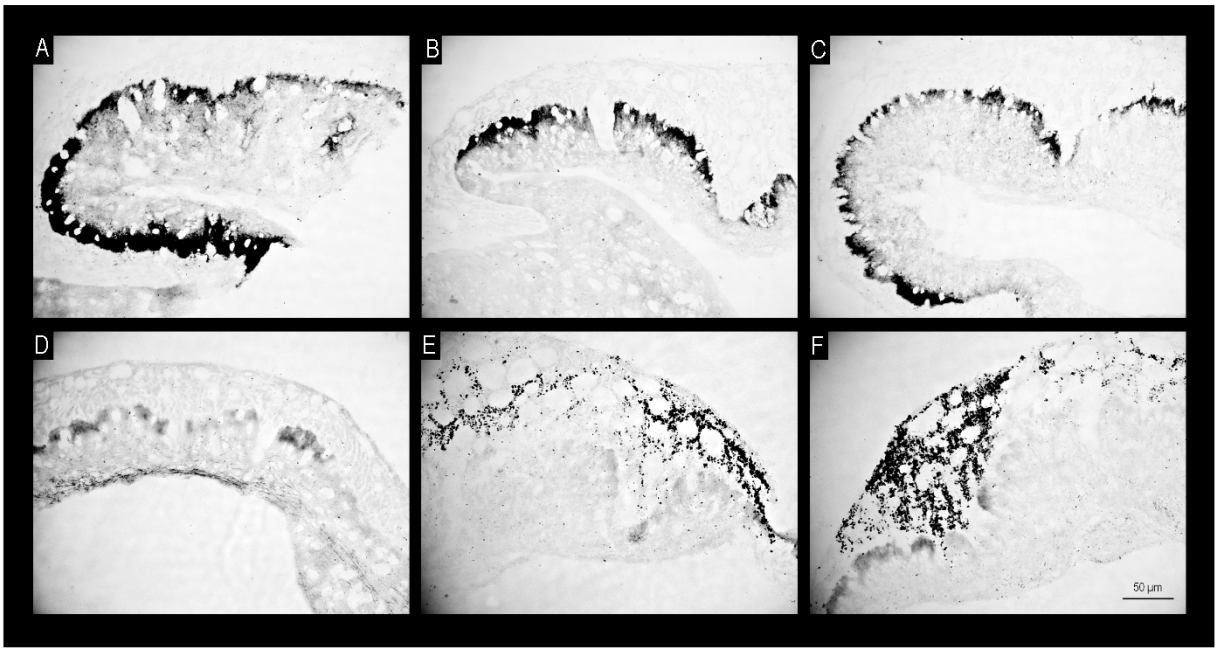

Figure 2. Immunoreactive (IR) somatostatin (SRIF; A, B, C) and IR growth hormone releasing hormone (GHRH; D, E, F) nerve terminals in the median eminence (ME) of representative sheep from the control group (A, D), RFa10 group (B, E) and RFa50 group (C, F). Treatment groups: control – animals administered Ringer-Locke infusion at a dose of 480 µl per day (n = 8), QRFP43 RFa10 – animals administered QRFP43 infusion at a dose 10 µg/480 µl (n = 8), QRFP43 RFa50 – animals administered QRFP43 infusion at a dose 50 µg/480 µl (n = 8). Scale bars: 50 µm

Microscopic observations were confirmed by quantitative image analysis, demonstrating that the percentage of area showing positive IR SRIF staining decreased in the ME ($P \leq 0.001$ and $P \leq 0.005$) in both groups infused with QRFP43 compared to the control group. Differences have also been observed between the RFa10 and RFa50 groups of animals ($P \leq 0.001$; Figures 3A and 2E,F). Moreover, the percentage of area with positive IR GHRH staining in both QRFP43-infused groups of sheep was increased ($P \leq 0.001$) compared to the control group (Figure 3B).

sheep (Figure 4A). *SSTR5* mRNA expression, compared to control animals, was statistically higher ($P \leq 0.05$) in the RFa10 group (1.00 ± 0.06 and 1.88 ± 0.14, respectively). The abundance of *SSTR5* transcript was significantly ($P \leq 0.0001$) lower in the RFa50 group compared to the RFa10 group of animals (1.88 ± 0.14 and 0.70 ± 0.04, respectively). However, no significant differences were noted between the control and RFa50 groups (Figure 4B). Additionally, *GHRH* mRNA expression was significantly ($P \leq 0.01$) lower in the RFa50 group than in the control group of sheep (0.50 ± 0.03 and

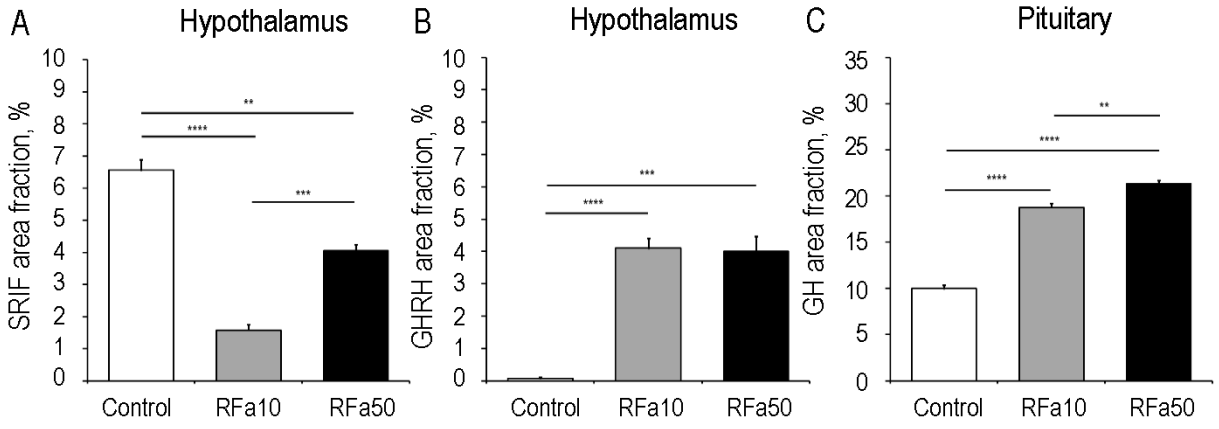

Figure 3. Mean percentage of area fraction for immunoreactive (IR) somatostatin (SRIF; A), growth hormone releasing hormone (GHRH; B) in nerve terminals of median eminence (ME), and IR growth hormone (GH; C) in ovine pituitary cells. Data are means ± standard error of the measurement. Treatment groups: control – animals that received Ringer-Locke infusion at a dose of 480 µl per day (n = 8), QRFP43 RFa10 – animals administered QRFP43 infusion at a dose of 10 µg/480 µl (n = 8), QRFP43 RFa50 – animals administered QRFP43 infusion at a dose 50 µg/480 µl (n = 8). *,**,***,**** – indicate significantly different values at $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$ and $P \leq 0.0001$, respectively

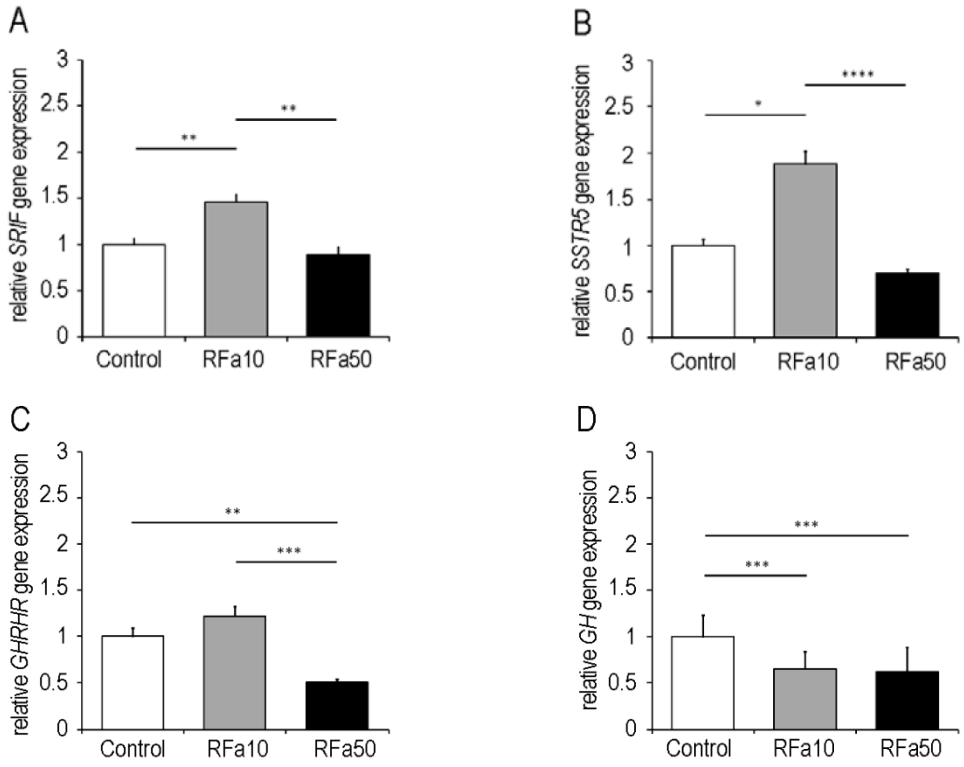

Figure 4. Expression of somatostatin receptor type 2 (SSTR2; A), somatostatin receptor type 5 (SSTR5; B), growth hormone releasing hormone receptor (GHRHR; C) and growth hormone (GH; D) mRNA in the pituitary. Data are means ± standard error of the measurement; treatment groups: control – animals administered Ringer-Locke infusion at a dose of 480 µl per day (n = 8), QRFP43 RFa10 – animals administered QRFP43 infusion at a dose 10 µg/480 µl (n = 8), QRFP43 RFa50 – animals administered QRFP43 infusion at a dose 50 µg/480 µl (n = 8); *,**,***,**** – indicate significantly different values at $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$ and $P \leq 0.0001$, respectively

1.00 ± 0.09, respectively). A significant decrease in *GHRH* mRNA ($P \leq 0.001$) was also observed between the RFa10 and RFa50 groups (1.22 ± 0.10 and 0.50 ± 0.03, respectively), while there were no significant differences between the RFa10 and the control groups (Figure 4C). Regarding *GH* mRNA expression, a significant decrease ($P \leq 0.001$) was observed in both the RFa10 and RFa50 group (0.65 ± 0.20 and 0.62 ± 0.26, respectively) compared to control animals (1.00 ± 0.23), whereas no differences were recorded between the RFa10 and RFa50 groups (Figure 4D).

GH immunoreactivity

Visible changes in IR GH pituitary cells were observed between control sheep and those administered RFa10 and RFa50. The number of GH-stained cells and the intensity of immunoreactions were higher in both QRFP43-infused sheep than in the control sheep (Figure 5A,B,C). This was reflected in the increased ($P \leq 0.0001$) percentage of IR GH cells in the adenohypophysis in the RFa10 and RFa50 groups compared to the control group, with a similar increase observed between RFa10 and RFa50 groups of sheep ($P \leq 0.05$; Figure 3C).

GH concentration in the pituitary gland

The mean concentration of GH hormone in pituitary in control, RFa10 and RFa50 animals was 116.8 ± 10.53, 240.1 ± 22.62 and 247.3 ± 18.63 µg/pituitary, respectively. The GH concentrations were higher ($P \leq 0.01$) in both QRFP43-treated groups in comparison to control group (Figure 6).

GH plasma concentration

The mean plasma GH concentration in the RFa10 group (6.9 ± 0.19 ng/ml) was higher ($P \leq 0.0001$) than in control (5.2 ± 0.10 ng/ml). Furthermore, a similar increase ($P \leq 0.0001$) was observed in the RFa50 group (6.9 ± 0.14 ng/ml) in comparison to control animals (5.2 ± 0.10 ng/ml). No statistically significant differences were observed between both groups receiving QRFP43 infusion (Figure 7).

Discussion

As noted earlier, RFa peptides are a large family of peptides renowned for their pivotal

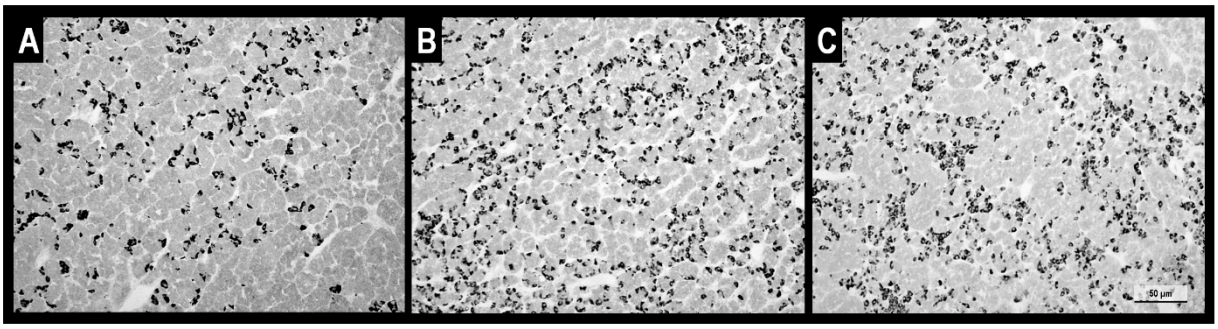

Figure 5. Immunoreactive GH pituitary cells from representative sheep: control (A), RFa10 (B) and RFa50 (C) groups of animals. Treatment groups: control – animals administered Ringer-Locke infusion at a dose of 480 µl per day (n = 8), QRFP43 RFa10 – animals administered QRFP43 infusion at a dose of 10 µg/480 µl (n = 8), QRFP43 RFa50 – animals administered QRFP43 infusion at a dose of 50 µg/480 µl (n = 8). Scale bars: 50 µm

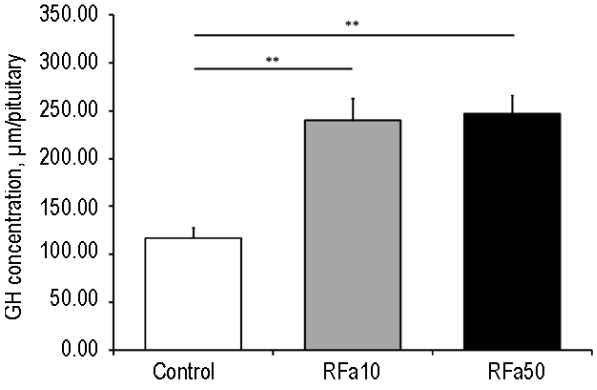

Figure 6. The concentration of growth hormone (GH) in pituitary homogenate. Data are means ± standard error of measurement; treatment groups: control group of animals which received Ringer-Locke infusion at dose of 480 µl per day (n = 8), RFa10 – group of animals which received QRFP43 infusion at dose 10 µg/480 µl (n = 8), RFa50 – group of animals which received QRFP43 infusion at dose 50 µg/480 µl (n = 8); ** – indicates values significantly different at $P \leq 0.01$

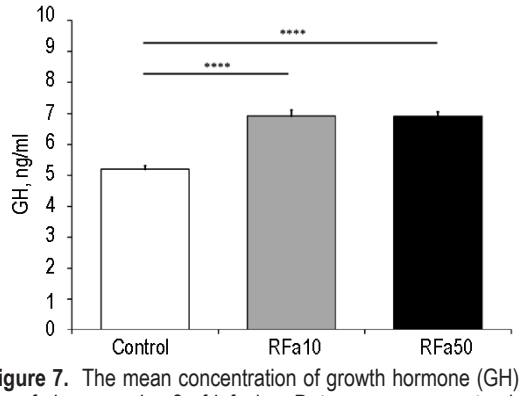

Figure 7. The mean concentration of growth hormone (GH) in plasma of sheep on day 3 of infusion. Data are means ± standard error of measurement; treatment groups: control group of animals which received Ringer-Locke infusion at dose of 480 µl per day (n = 16), RFa10 – group of animals which received QRFP43 infusion at dose 10 µg/480 µl (n = 16), RFa50 – group of animals which received QRFP43 infusion at dose 50 µg/480 µl (n = 16); **** – indicates values significantly different at $P \leq 0.0001$

physiological functions in the body. However, there is limited information in the currently available literature on how QRFP43 affects the function of the somatotrophic axis. Therefore, in this study, we investigated the effects of ICV QRFP43 infusion into the IIIv of the sheep brain on the secretory activity of key somatotrophic axis hormones that regulate growth processes.

The result obtained in the present study showed that centrally infused QRFP43 affected the activity of the somatotrophic axis in female sheep at both the hypothalamic and pituitary levels. Exogenous QRFP43 caused an increase in *SRIF* mRNA expression in the AHA of sheep. However, despite this enhanced *SRIF* mRNA expression, there was a decrease in IR SRIF material in ME nerve terminals. The consequence of these changes could be the suppression of the inhibitory effect of SRIF on the activity of pituitary somatotrophic cells. This interpretation was further supported by the observed increase in IR GHRH following QRFP43 administration in ME. According to our knowledge, those are first observation of *in vivo* effects of QRFP43 at the hypothalamus-pituitary level.

At the pituitary level, QRFP43 appeared to trigger specific alterations in *SSTR* mRNA expression. We observed an increase in mRNA expression for both *SSTR2* and *SSTR5* in the RFa10 group, as well as decrease in the expression of both mRNA receptors in the RFa50 group. Moreover, QRFP43 exhibited an inhibitory effect on *GHRHR* mRNA expression, but only at the higher dose of the peptide. Furthermore, a decrease in *GH* mRNA expression in pituitary was observed however, the immunohistochemistry and RIA from pituitary homogenates results suggested the opposite outcome. Microscopic observation showed an increase in IR GHRH in the ME and IR GH in pituitary cells following QRFP43 infusion. Also the same outcome have been noted in GH concentration in pituitary homogenates. In addition to changes involving post-transcriptional mRNA regulation, it is possible that these alterations between mRNA and protein expression levels, occurred due to auto-regulation *via* short or long feedback loops (Katoh et al., 2007; Bartke, 2022). The results may suggest that QRFP43 over-stimulated the activity of the somatotrophic axis at the time of tissue collection. This could be confirmed by the excessive production of GH peptide in somatotrophic pituitary cells, leading to increased expression of *SRIF* mRNA and subsequent inhibition of pituitary somatotrophic cell activity by SRIF. This theory seems to be partially confirmed by the results regarding GH concentration in the peripheral blood of animals. In the presented experiment, a significant increase in GH concentration was observed in the blood of sheep receiving QRFP43 infusion. However, to fully explain the observed differences in the expression of mRNA and proteins of the hormones studied, further research and analysis is required.

In study of Qaiser et al. (2012) effect of administration of QRFP43 on GH secretion has been studied. The latter study was conducted on adult male rhesus monkeys that were administered different QRFP43 doses by intravenous injection. Analysis of changes in mean plasma GH levels showed the inhibitory effect of QRFP43 on GH secretion, but only at the highest doses of the peptide. Interestingly, these authors observed a dose-dependent trend characterized by a minor and transient increase in mean GH concentrations followed by a progressive decrease after intravenous injection RFa administration (Qaiser et al., 2012). To the best of our knowledge, this is the only article reporting the effect of QRFP43 on somatotrophic axis neuronal activity. Interestingly, the latter authors also investigated the effect of 26RFa on GH concentrations in the blood and showed that 26RFa, in contrast to QRFP43, stimulated GH secretion and increased GH levels. Moreover, similarly as in the case of QRFP43, this effect was dose dependent (Qaiser et al., 2012). These findings indicate that peptides derived from the same prepropeptide, which generally exhibit the same biological activities, may modulate *in vivo* the activity of hormonal axes in a different manner.

The available literature provides some other reports concerning peptides belonging to the RFa family and their influence on GH (Sari et al., 2009; Luque et al., 2011). For instance, an *in vitro* study in sockey salmon pituitary cell cultures has demonstrated that LPXRF-amide peptides were able to stimulate GH release (Amano et al., 2006). In relation to our results, the latter study also established that the stimulatory effect of this RFa was dose-dependent. This effect has also been observed for other RFa in a study involving cultured frog pituitary cells (Koda et al., 2002). Further, a study involving rats receiving ICV injections of RFRP-3 suggested that yet another peptide from this family could influence somatotrophic axis activity in a dose-dependent manner (Johnson et al., 2007). In the cited work, adult male Sprague Dawley rats were administered various doses of RFRP-3 (10, 100 and 500 ng) through steel guide cannulas to the lateral ventricle of the brain, followed by blood collection to determine alterations in plasma GH levels. Radioimmunoassay results indicated that RFRP-3 at a dose of 100 ng increased plasma GH levels, whereas lower

and higher doses showed no effect or even decreased plasma levels of this hormone (Johnson et al., 2007). Considering the aforementioned studies, the effect of RFa on the activity of the somatotrophic axis appears to vary depending on the specific type of peptide administered, its dosage, duration and pattern of administration, as well as the model organism utilized in the experiment. Therefore, these findings seems to support our hypothesis that QRFP43 stimulates the activity of the somatotrophic axis in sheep, although prolonged administration may also activate processes suppressing GH release due to overstimulation of the axis. In order to better understand the mechanism underlying these changes, further studies are needed, especially to determine the effect of long-term QRFP43 administration on the secretory activity of the somatotrophic axis.

Conclusions

In summary, it was shown that QRFP43 may be involved in the growth processes regulation at the level of the central nervous system in sheep. In the hypothalamus QRFP43 shows to effect on somatostatin expression, as well as increase growth hormone-releasing hormone levels in the median eminence, thereby stimulating growth hormone synthesis in the pituitary and growth hormone concentration in sheep blood. Furthermore, long-term administration of exogenous QRFP43 may activate physiological processes at the mRNA level that prepare the attenuation of somatotrophic axis activity and reduce growth hormone release into the peripheral blood. However, noting that changes in mRNA levels do not always accurately reflect the respective cellular translational activity, further studies are necessary to fully understand the action of the QRFP43 in relation to the somatotrophic axis. To better understand its mechanism of action further research should focus to investigate changes in cellular transmission (both in nerve cells and pituitary cells) that occur after administration of QRFP43.

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Conflict of interest

The Authors declare that there is no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors did not use any artificial intelligence-assisted technologies in the writing process.

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