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Short communication

QRFP43 modulates the activity of the hypothalamic appetite regulatory centre in sheep

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ABSTRACT. The neuromodulatory effect of pyroglutamylated RFamide peptide 43 (QRFP43) on the hypothalamic appetite regulation centre in sheep has not yet been investigated. The present work focuses on the role of QRFP43 in modulating mRNA expression of pyroglutamylated RFamide peptide receptor (*QRFPR*), neuropeptide Y (*NPY*), agouti related neuropeptide (*AGRP*), cocaine- and amphetamine-regulated transcript prepropeptide (*CARTPT*), proopiomelanocortin (*POMC*), peptidyglycine alpha-amidating monooxygenase (*PAM*) and NPY protein expression in the hypothalamic arcuate nucleus in sheep. The aim of this study was to investigate whether QRFP43 could affect gene expression of the appetite-regulating centre in the hypothalamus. The experiment was conducted from September to December and included forty-eight female Polish Merino sheep randomly assigned to three groups. The control group received an intracerebrovenctricular infusion of Ringer-Locke solution (480 µl/day), whereas the experimental groups were administered QRFP43 in two doses: 10 or 50 µg/480 µl/day (referred to as the RFa10 and RFa50 groups). Selected brain structures were collected from animals for immunohistochemical and real-time PCR analyses. Central infusions of QRFP43 induced changes in mRNA expression of the *NPY*, *CARTPT*, *POMC* and *QRFPR* genes. A decrease in *NPY* and *QRFPR* mRNA expression, and an increase in *CARTPT* mRNA expression were detected. Furthermore, infusion of QRFP43 in the RFa50 group resulted in decreased *POMC* mRNA expression. Our findings suggest that QRFP43 may exert an inhibitory effect on the hypothalamic neuronal network responsible for appetite regulation in sheep. Moreover, it appears that the effect of QRFP43 may vary in different animal species, thus further research is required, especially involving hormonal proteomic analyses.

Introduction

Recent studies have revealed numerous connections between appetite regulation and other regulatory systems within the body, explaining the profound impact of eating disorders on overall physiological functioning (Wójcik-Gładysz and Szlis, 2016). The main site of integration of these regulatory processes in the central nervous system (CNS) is located in the hypothalamus. Neurons present in the arcuate nucleus (ARC) of the hypothalamus, which are involved in regulating metabolic processes and maintaining body's energy homeostasis, constitute one of the most important appetite regulating centres in the CNS. This centre comprises two distinct subpopulations of neurons, responsible for controlling energy expenditure and food intake. The first one consists of neurons co-expressing neuropeptide Y (NPY) and agouti-related peptide, collectively known as NPY/AGRP neurons, associated with generation

of orexigenic signals that stimulate appetite. The second subpopulation is formed by neurons showing co-expression of cocaine- and amphetamine-regulated transcript protein (CARTP), and α-melanocortin, referred to as CARTP/α-MSH neurons, which generate anorexic signals suppressing appetite (Wójcik-Gładysz and Szlis, 2016).

RF-amide peptides (RFa) are a group of regulatory peptides with diverse biological functions, distinguished by a characteristic sequence at the C-terminus (Leprince et al., 2017). Pyroglutamylated RFamide peptide 26 (26RFa) and 43 (QRFP43) are among well-described proteins belonging to this group. In mammals, cleavage of the 26RFa precursor can generate two forms of this peptide: 26RFa and QRFP43, the latter being an N-terminal elongated form. Studies have demonstrated that both of them possess the same biological activity. The C-terminal region of this neuropeptide (KGGFXFRF-NH_2), responsible for its biological activity, shows high evolutionary conservatism, a hallmark of peptides playing crucial roles in the functioning of organisms (Takayasu et al., 2006; Chartrel et al., 2011).

The results of the current study have revealed expression of the *QRFP* gene and its receptor – pyroglutamylated RFamide peptide receptor (*QRFPR*) – in the ventromedial hypothalamic nucleus (VMN), lateral hypothalamic area (LHA), and ARC (Chartrel et al., 2011). Considering that the distribution of QRFP43 neurons and QRFPR-containing neurons overlaps with the location of the hypothalamic appetite regulating centre, investigating the potential impact of this neuropeptide on appetite regulation seems highly relevant. Studies performed in rodent models have demonstrated that 26RFa/QRFP43 administration can, under certain conditions, stimulate appetite or the expression of main orexigenic neuropeptides. However, the magnitude of this effect appears to be influenced by various factors such as diet type, animal hormonal status, sex or circadian rhythm (Primeaux, 2011; Primeaux et al., 2013). This emphasises the need for further research, particularly on non-rodent animal models, to better understand the role of this neuropeptide.

The present study aimed to verify the hypothesis that QRFP43 might act as a modulator that affects the appetite regulation centre in the hypothalamus in sheep. Therefore, we investigated the impact of QRFP43 administrated intracerebroventricularly (ICV) into the third ventricle (IIIv) of the sheep brain on mRNA expression of *NPY*, agouti related neuropeptide (*AGRP*), CART prepropeptide (*CARTPT*), proopiomelanocortin (*POMC)*, peptidylglycine alpha-amidating monooxygenase (*PAM*) and *QRFPR*, as well as on NPY protein expression.

Material and methods

Animals and experimental design

All procedures were approved by the Local Ethics Committee affiliated with the Warsaw University of Life Sciences (Resolution no. WAW2/193/2019).

Forty-eight female Polish Merino sheep, aged 42 weeks, with an average body weight of 38.6 ± 3.5 kg, were used in the present study. The animals were housed indoors under natural lighting conditions (52°N, 21°E) and were fed a standard hay diet with commercial concentrates twice daily. Both water and salt licks were available *ad libitum*.

Stainless steel cannulas were surgically implanted directly into the third ventricle (IIIv) of the brain to each animal under anaesthesia induced by atropinum sulfuricum (0.44 mg/kg; Polfa, Warsaw, Poland), ketamine (400 mg per sheep; Vetoquinol Biowet, Gorzów, Poland), and dexmedetomidine (0.05 mg/kg, Dexdomitor®; Orion Pharma, Turku, Finland). Prior to ICV infusion, all experimental animals underwent oestrus synchronization 21 days earlier using Chronogest CR sponges (MSD Animal Health, UK). This process has been described in detail in Przybył et al. (2021). Sheep entered the experiment on day 4–5 after ovulation.

The experiments were conducted from the last week of October until the first week of December. Each animal was randomly assigned to one of three experimental groups, each consisting of 16 animals: a control group receiving an ICV infusion of Ringer-Locke solution (480 µl/day), and two experimental groups treated with QRFP43 in two doses: 10 or 50 µg/480 µl/day (RFa10 and RFa50 groups). QRFP43 doses were diluted using 480 µl of Ringer-Locke solution, and administered at an infusion rate of 120 µl/h; dosing was established based on our unpublished, preliminary research. Four 50-min ICV infusions were administered at 30-min intervals from 8:40 to 13:30 on each of three consecutive days. Directly after the last infusion, animals were weighed and anaesthetised intravenously with dexmedetomidine (0.05 ml/kg) and pentobarbital (80 ng/kg, Morbital®, Vetoquinol Biowet, Poland), then euthanised by decapitation. Isolation of the selected hypothalamic structures: ARC, median eminence (ME), as well as their preservation, was conducted according to the method described in Szlis et al. (2018).

Real-time PCR

Total RNA extraction from the ARC nucleus was performed using the NucleoSpinRNA/Protein kit (Macherey-Nagel GmbH & Co., Düren, Germany), according to the manufacturer's protocol. For complementary DNA (cDNA) synthesis, 1500 ng/ml RNA from the ARC nucleus in a total volume of 20 µl was used for reverse transcription using a TranScriba Kit (A&A Biotechnology, Gdynia, Poland), according to the manufacturer's instructions. Species-specific primers for sheep (*Ovis aries*) were designed using Primer 3 software (The Whitehead Institute, Cambridge, MA, USA) and synthesised by Genomed (Warsaw, Poland) to determine the expression of housekeeping genes and genes of interest. The corresponding primer sequences are presented in Table 1.

Real-time PCR was performed using 5 × FIREPol EvaGreen qPCR Mix Plus (no ROX; Solis BioDyne, Tartu, Estonia). Relative gene expression was calculated using the comparative quantitation option implemented in the Rotor Gene 6000 software 1.7 (Qiagen GmbH, Hilden, Germany) and determined using the Relative Expression Software Tool, following the protocol described by Przybył et al. (2021). Housekeeping genes used in the experiment included glyceraldehyde-3-phosphate dehydrogenase (*GAPDH,* average expression stability = 0.936), actin beta (*ACTB,* average expression stability = 0.758) and peptidylprolyl isomerase C (*PPIC,* average expression stability = 0.609). *GAPDH* was identified using the BestKeeper software (https://www.gene-quantification.de/bestkeeper.html) as the most suitable endogenous control gene for normalising gene expression in this study. The results are presented as relative gene expression of the target gene normalised to the housekeeping gene (*GAPDH*) with the relative gene expression for the group of sheep that received Ringer-Locke solution infusion set to 1.0.

Immunohistochemical procedure and image analyses

The hypothalamic sections were incubated for 14 days at 4 °C with a primary NPY antiserum (rabbit anti-NPY [ref. 9528]; Sigma, Saint Louis, MO, USA), diluted at a ratio of 1:3000, following the procedure used in our laboratory (Szlis et al., 2018) with minor modifications. After incubation with primary antibodies, the sections were incubated for 2 h

Table 1. Primer sequences used in the experiment

Gene	Primer	Sequence (5'→3')	Product size, bp	References
GAPDH	Forward	AGAAGGCTGGGGCTCACT	134	
	Reverse	GGCATTGCTGACAATCTTGA		
PPIC	Forward	TGGAAAAGTCGTGCCCAAGA	158	
	Reverse	TGCTTATACCACCAGTGCCA		
ACTB	Forward	TGGGCATGGAATCCTG	194	
	Reverse	GGCGCGATGATCTTGAT		
NPY	Forward	ATCACCAGGCAGAGATACGG	100	
	Reverse	CCAGCCTAGTTCTGGGAATG		
CARTPT	Forward	CCCATGAGAAGGAGCTGATTGA	108	Przybył et al., 2000
	Reverse	TGGGGACTTGGCCATACTTC		
AGRP	Forward	TCCTAGAGCTCCAAGGCCTA	124	
	Reverse	CCTTCCGGATCTAGCACCTC		
POMC	Forward	GAAAGTAACCTGCTGGCGTG	129	
	Reverse	GAAATGGCCCATGACGTACTTC		
PAM	Forward	GTACTCCAGGTACACTATGGGGA	216	
	Reverse	GTAGGCAAAGACATGCATTGGA		
QRFPR	Forward	GCATCCCGGTCACTATGCTC	128	NM_001192681.1
	Reverse	TGGACTGGACAAATGGCACC		

GAPDH – glyceraldehyde-3-phosphate dehydrogenase, *PPIC* – peptidylprolyl isomerase C, *ACTB* – actin beta, *NPY* – neuropeptide Y, *CARTPT* – CART prepropeptide, *AGRP* – agouti related neuropeptide, *POMC* – proopiomelanocortin, *PAM* – peptidylglycine alpha-amidating monooxygenase, *QRFPR* – pyroglutamylated RFamide peptide receptor

at room temperature (~20 °C) with a secondary antibody (sheep anti-rabbit IgG [H + L] labelled with peroxidase; ABcam, Cambridge, UK) at a dilution of 1:400 in 0.1% normal lamb serum in 0.01 M PBS. The colour reaction was induced by incubating sections with 0.05% 3/3-diaminobenzidine tetrahydrochloride chromogen (Sigma, Saint Louis, MO, USA) and 0.001% hydrogen peroxide in 0.05 M Tris buffer.

Histological analyses of hypothalamic sections were performed using a Nikon type 104 projection microscope (Nikon Corporation, Tokyo, Japan). Images of immunostained sections were captured using a Panasonic KR222 camera (Matsushita Electric Industrial Co., Osaka, Japan) and transferred to a colour monitor for adjustments of optimal contrast and brightness. Subsequently, the images were converted to grayscale and saved in a buffering system. Analysis was performed using a × 40 objective lens.

Statistical analysis

Statistical calculations were conducted using GraphPad software (GraphPad Sofware Inc., La Jolla, CA). Data shown in the graphs are expressed as means ± SEM for each groups. The Shapiro-Wilk test was used to verify the normality of data distribution. For mRNA expression analysis, the Kruskal-Wallis test, followed by *post hoc* Dunn multiple comparisons test, were utilised. Statistical significance was defined as differences resulting in $P \leq 0.05$.

Results and discussion

The presented study was designed to investigate the influence of QRFP43 on mRNA expression of *QRFPR* and key neuropeptides co-forming the hypothalamic appetite regulatory centre in the sheep ARC. The analyses revealed the presence of *QRFPR* mRNA transcripts in the sheep ARC in all experimental groups. Interestingly, the expression of *QRFPR* mRNA was significantly ($P < 0.0001$) reduced in the RFa50 group compared to the control group of animals (0.65-fold ± 0.05 and 1.00-fold ± 0.03; respectively; Figure 1A). Additionally, a dose-dependent decrease in *NPY* mRNA expression was observed in the RFa10 and RFa50 groups (0.82-fold ± 0.04, $P = 0.0493$; and 0.65-fold ± 0.03, $P = 0.0163$; respectively) compared to the control group (1.00-fold ± 0.06) (Figure 1B). No significant differences in *AGRP* mRNA expression were observed between sheep from either experimental group (Figure 1C). Microscopic observation of NPY peptide distribution and location in the sheep hypothalamus revealed only a small amount of stained immunoreactive NPY material in each group of animals tested; however, no stained parikarya were recorded in any of the hypothalamic fragments examined (Figure 2).

The present results differ from those obtained in experiments on rodents. In a study carried out on male rats with food restriction, Lectez et al. (2009) showed that a single ICV injection of 26RFa increased *NPY* mRNA expression in the VMN. The same authors also demonstrated increased secretion of NPY peptide by rat VMN explants in an *in vitro* experiment. The observed differences between our experimental model and that mentioned above may result from different feeding strategies (monogastric animals – rodents, ruminants – sheep), the time of day when the animals eat, and the season.

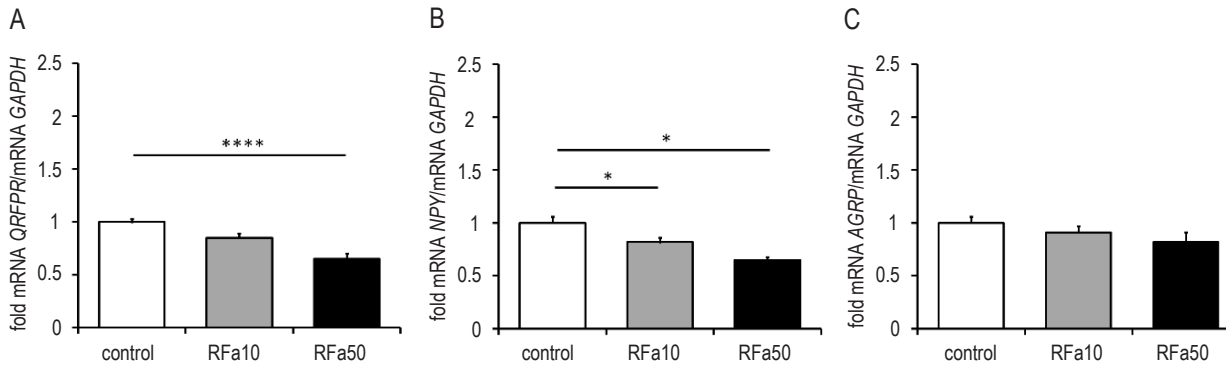

Figure 1. Expression of mRNA in the arcuate nucleus: pyroglutamylated RFamide peptide receptor (*QRFPR*; A) neuropeptide Y (*NPY*; B); agouti related neuropeptide (*AGRP*; C). All values are relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Data are presented as means ± standard error of the measurement; treatment groups: control – animals infused Ringer-Locke solution at a dose of 480 µl per day (n = 16), RFa10 – animals that received QRFP43 at a dose of 10 µg/480 µl (n = 16), RFa50 – animals that received QRFP43 infusion at a dose of 50 µg/480 µl (n = 8). Statistically significant differences: * $P < 0.05$, **** $P < 0.0001$

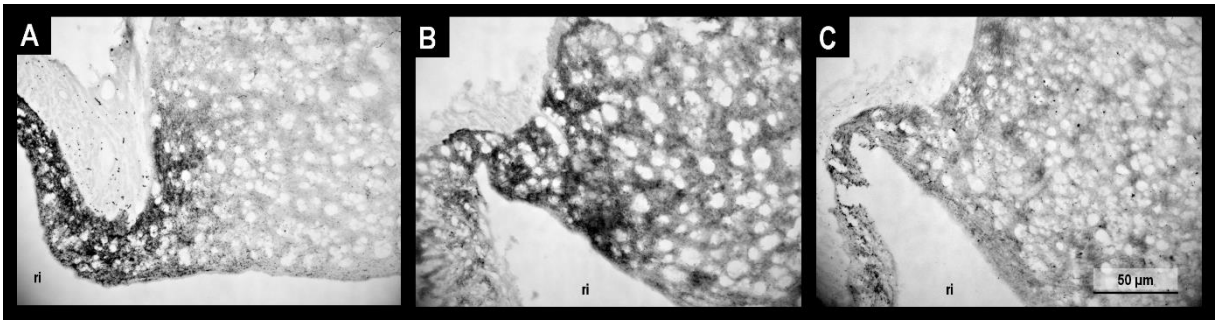

Figure 2. Population of immunoreactive NPY material in the arcuate nucleus of representative sheep from the control group (A), RFa10 group (B), and RFa50 group (C). Treatment groups: control animals that received Ringer-Locke infusion at a dose of 480 µl per day (n = 16), RFa10 – animals that received QRFP43 at a dose of 10 µg/480 µl (n = 16), RFa50 – animals that received QRFP43 infusion at a dose of 50 µg/480 µl (n = 8). Scale bars: 50 µm (A–C)

Analyses of *CARTPT* mRNA expression in the sheep ARC showed a significant ($P < 0.0001$) increase of this transcript in the RFa50 group in comparison to the control group (2.05-fold ± 0.06 and 1.00-fold ± 0.10; respectively). In addition, an increase in *CARTPT* mRNA expression was observed in the RFa50 group compared to the RFa10 group of sheep ($P = 0.0045$; 2.05-fold ± 0.06 vs. 1.19-fold ± 0.12, respectively; Figure 3A). *POMC* mRNA expression was increased in the RFa10 group compared to the control group ($P = 0.0180$; 1.52-fold ± 0.05 and 1.00-fold ± 0.10, respectively). On the other hand, there was a significant ($P < 0.0001$) decrease in *POMC* mRNA expression in the RFa50 group compared to the RFa10 group (0.63-fold ± 0.07; Figure 3B). Nevertheless, no statistically significant differences in *PAM* (an enzyme that converts POMC to α-MSH) mRNA expression were observed between sheep from any experimental groups (Figure 3C). These results contradict findings reported by Lectez et al. (2009), who showed that a single injection of 26RFa decreased *POMC* mRNA expression in male rats. Additionally, an *in vitro* study of the latter authors demonstrated a decrease in α-MSH concentration in media after incubation of rat hypothalamus explants with 26RFa.

It seems that in rodents, RFamide may exert an orexigenic effect under certain conditions. Studies investigating the effect of food restriction in male mice have shown a stimulating effect of ICV injected 26RFa/QRFP43 on food intake and body weight (Chartrel et al., 2003; do Rego et al., 2006; Moriya et al., 2006). However, the results of do Rego et al. (2006) have demonstrated that the influence of QRFP43 on the regulation of energy homeostasis may depend on the type of diet administered to the experimental animals. Similar observations have been reported in other studies, where centrally administered 26RFa selectively increased food intake in female rats on a high-fat diet, but had no effect in animals fed a low-fat diet (Primeaux, 2011). Nevertheless, it cannot be excluded that the influence of 26RFa/QRFP43 on the body may depend not only on the type of diet or duration of peptide administrating, but also on the time of day at which it is administered. Results from experiments on rats indicate that

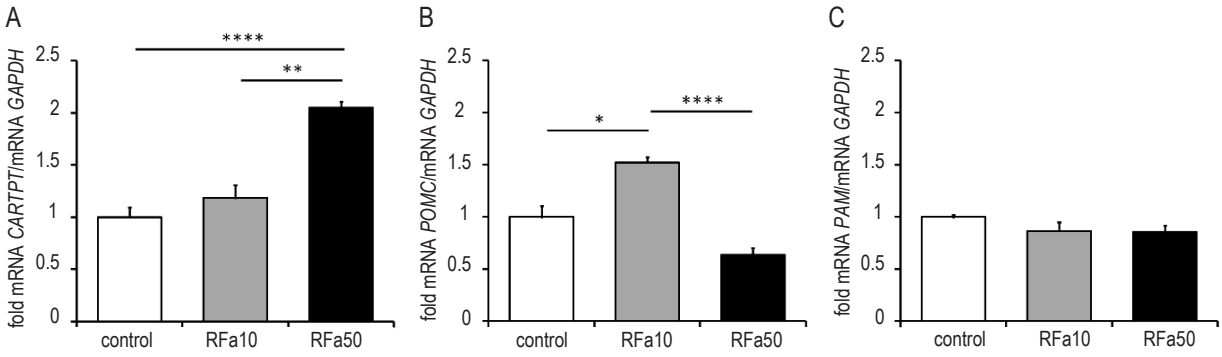

Figure 3. Expression of mRNA in the arcuate nucleus: CART prepropeptide (*CARTPT*; A), proopiomelanocortin (*POMC*; B), peptidylglycine alpha-amidating monooxygenase (*PAM*; C). Data are presented as means ± standard error of the measurement. Treatment groups: control – animals that received Ringer-Locke infusion at a dose of 480 µl per day (n = 16), RFa10 – animals that received QRFP43 at a dose 10 µg/480 µl (n = 16), RFa50 – animals that received QRFP43 infusion at a dose 50 µg/480 µl (n = 8). Statistically significant differences: * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$

ICV injection of 26RFa/QRFP43 before the dark phase does not affect the amount of food consumed (Kampe et al., 2006).

Conclusions

In summary, the data presented demonstrate that pyroglutamylated RFamide peptide 43 (QRFP43) modulates pyroglutamylated RFamide peptide receptor (*QRFPR*), neuropeptide Y (*NPY*), cocaine- and amphetamine-regulated transcript prepropeptide (*CARTPT*) and proopiomelanocortin (*POMC*), peptidyglycine alpha-amidating monooxygenase (*PAM*) mRNA expression in the sheep ARC. These results suggest that QRFP43 may exert an inhibitory effect on the hypothalamic neuronal network responsible for appetite regulation in sheep. Additionally, it appears that the effect of QRFP43 may vary between individual animal species, highlighting the need for further research, particularly involving hormonal proteomic analyses.

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Conflict of interest

The Authors declare that there is no conflict of interest.

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