Ann. Anim. Sci., Vol. 20, No. 3 (2020) 853–869 *DOI: 10.2478/aoas-2020-0015*

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BRAIN-DERIVED NEUROTROPHIC FACTOR AFFECTS mRNA AND miRNA EXPRESSION OF THE APPETITE REGULATING CENTRE IN THE SHEEP ARCUATE NUCLEUS*

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Abstract

The neuromodulatory effects of brain-derived neurotrophic factor (BDNF) on appetite regulation centre peptide gene activity in the sheep hypothalamus have not been examined yet. The aim of this study was to determine whether BDNF participates in modulation of neuropeptide Y (*npy),* agouti-related peptide (*agrp*), cocaine and amphetamine regulated transcript (*cart*), and proopiomelanocortin (*pomc*) mRNA expression and selected microRNAs in the sheep hypothalamic arcuate (ARC) nucleus. Animals (Polish Merino sheep, n=24) were divided into three groups. The control group received a central infusion of Ringer-Locke solution (480 µl/day) whereas the experimental groups were treated with BDNF in two doses: 10 or 60 µg/480 µl/day. All sheep received four intracerebroventricular infusions (performed from 08:40 a.m. to 01:30 p.m.; infusion scheme: 4 × 50 min infusions with 30 min intervals between them) on each of three consecutive days. Immediately after the last infusion, the sheep were slaughtered, and selected structures of the hypothalamus were frozen for further real-time qPCR analysis. Central infusion of BDNF evoked dose-dependent changes in *npy, agrp, cart, pomc* and peptidylglycine alpha-amidating monooxygenase (*pam*) mRNA expression in the sheep ARC nucleus. An increase in *npy, agrp* and *pomc* mRNA expression but also a decrease in *cart* mRNA expression in the ARC nucleus were detected. Moreover, a decrease in *pam* (gene encoding an enzyme that converts POMC into α-MSH) mRNA expression, was also noted. Furthermore, after central BDNF administration, changes in miRNA-33a-5p, miRNA-33b-5p, miRNA-377-3p, miRNA-214-3p, miRNA-485 and miRNA-488 expression were observed. Based on the presented results, it can be concluded that BDNF may affect the appetite regulating centre activity through modulation of *npy, agrp, cart, pomc* and *pam* mRNA expression in the ARC nucleus. It was also revealed that BDNF modulates miRNA expression in the sheep ARC nucleus.

Key words: BDNF, appetite regulating centre, miRNAs, arcuate nucleus, sheep

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*This research was partially supported by funds provided by the National Science Centre, Poland, PRELUDIUM 9 grant no. 2015/17/N/NZ9/01110 and 'Start Grant' funding by The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences.

A serious problem concerning obesity and obesity-related diseases has recently emerged in civilized societies. Eating disorders and addiction symptoms are often related to disturbances in neuroendocrine control of metabolic processes and brain neurohormone activity and may generate similar neurobiological pathologies resulting from neuroadaptive changes at the central nervous system (CNS) level (De Souza et al., 2004; Evans and Anderson, 2012; Lopaschuk et al., 2010). Recent studies focused on central and peripheral mediators of both energy homeostasis and behavioural addictions (i.e., neuropeptide Y (NPY)), brain-derived neurotrophic factor (BDNF), ghrelin, obestatin or leptin) action on the hypothalamic appetite regulating network (Fenichel et al., 2008; Sullivan et al., 2003; Szlis et al., 2018 a; Wójcik-Gładysz et al., 2019; Wójcik-Gładysz and Szlis, 2016).

The 'appetite-regulating centre' localized in the arcuate (ARC) nucleus of the hypothalamus is co-created mainly by two sub-populations of neurons, referred to as 'first-order neurons'. The first neuronal sub-population is characterized by co-expression of NPY and agouti-related peptide (AgRP; NPY/AgRP neurons), the second, by co-expression of cocaine and amphetamine regulated transcript (CART) and α-melanocortin (α-MSH; CART/α-MSH neurons). These neurons are essential for detection and integration of central and peripheral signals, allowing precise control of appetite and energy expenditure at the hypothalamic level (Lopaschuk et al., 2010; Wójcik-Gładysz and Szlis, 2016).

Brain-derived neurotrophic factor belongs to the neurotrophin family and participates in processes related to neuroprotection and neuroplasticity of neurons, such as stimulation of neuronal growth and formation of synaptic connections (Peiris et al., 2004). BDNF mRNA as well as its receptor (tyrosine kinase isoform B receptor, TrkB) were found in immunoreactive neurons in rodents: ventromedial (VMN), dorsalmedial (DMN), paraventricular (PVN), ARC nuclei and median eminence (ME) (Barde et al., 1982; Unger et al., 2007). It has been shown that BDNF expression and activity at the CNS level can be changed during the state of nutritional addiction. Simultaneously, there are data showing that BDNF is also a mediator or neuromodulator of food intake control (Cordeira et al., 2010; Xu et al., 2003).

Studies suggest that BDNF could be involved in central regulation of appetite modulation processes, acting through NPY/AgRP and CART/α-MSH neurons. It was found that intraventricular administration of exogenous BDNF in mice resulted in activation of hypothalamic neurons in areas responsible for regulation of metabolism and increased c-Fos expression in the VMN, PVN nuclei and lateral hypothalamic area (Unger et al., 2007). Additionally, it has been shown that the main points of BDNF expression and its action in the hypothalamus overlap with the presence of NPY and the area of NPY action. Moreover, *in vivo* and *in vitro* studies revealed that BDNF may affect NPY mRNA and protein expression in the ARC nucleus (Barnea and Roberts, 2001; Xapelli et al., 2008). BDNF may also affect proopiomelanocortin (POMC, a precursor of α-MSH), AgRP mRNA and α-MSH protein and receptor (MC4R) expression (Byerly et al., 2009; Nicholson et al., 2007; Takei et al., 2014).

The discrepancy between the expression of mRNAs and their proteins in *in vivo* research on physiological changes induced by biologically active substances is a frequently observed phenomenon. One possible explanation is post-transcriptional

mRNA regulation guided by the small RNA molecule fraction called microRNA (miRNA) and alterations of numerous essential physiological processes that can be caused by deregulation of miRNA expression (Benes and Castoldi, 2010; Grenda et al., 2013; Hukowska-Szematowicz and Deptuła, 2010; Van Wynsberghe et al., 2011). There is a lack of real information about miRNA expression and function in the sheep CNS, especially from research conducted *in vivo*.

The present study aimed to test the hypothesis that BDNF in the sheep CNS acts as a hormonal signal affecting mRNA expression of the main neurotransmitters, constituting the hypothalamic network for appetite regulation. Therefore, we have investigated the effect of exogenous BDNF on the mRNA expression of neuropeptides creating the appetite regulating network, i.e., *npy*, *agrp, cart, pomc* and peptidylglycine alpha-amidating monooxygenase (*pam*), the gene encoding the enzyme mediating the conversion of POMC peptide into an active form of α-MSH. Furthermore, we have studied the effect of BDNF on expression of selected miRNAs (which could be related to post-transcriptional regulation of *npy*, *agrp*, *cart* and *pomc* mRNA expression) in the sheep ARC nucleus.

Sheep as a ruminant with a seasonal reproductive strategy and an animal with a daily phase of activity, constitutes a diametrically different research model from rodents, which are nocturnal animals with a seasonal-independent model of reproduction. Moreover, lately the need to perform physiological studies on other animal research models that are longer-lived than rodents has been increasingly emphasized. In recent years, sheep constitute a great, universally approved and recognized animal model to investigate the regulation of many neuroendocrine processes occurring in the CNS.

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 for animal experiments and were approved by the Local Ethics Committee (decision no 22/2015 of 21st May 2015) affiliated with Warsaw Agricultural University, according to the Polish Law for Animal Care and Use (21st January 2005) and the Polish Law for Animal Protection (16th September 2011).

Twenty-four female Polish Merino sheep (42 weeks old, average weight = 38.6 ± 3.5 kg) were used in the study. Animals were housed indoors under natural lighting conditions (52°N, 21°E) and fed a standard hay diet with commercial concentrates twice per day, according to the Polish Recommendation for Growing Sheep (Strzetelski et al., 2014). The fodder was balanced in terms of energy and adequate nutrients to provide optimal development of growing animals. Water and salt licks were available *ad libitum*. The reproductive status of each animal was assessed every week (starting from the beginning of August) by evaluating plasma progesterone concentrations and was verified by post-mortem ovary examination (data not shown).

Stainless-steel guide cannulae were implanted directly into the third ventricle (IIIv) under 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) anaesthesia. Permanent stainless-steel guide cannulae (0.8 mm) were inserted into IIIv (coordinates of guide cannulae implantation: antero-posterior position – 31 mm, horizontal position – 0.5 mm, mid-sagittal position – 0.10 mm) according to Traczyk and Przekop (1963) and Welento et al. (1969). The localization of the guide cannula was verified by the efflux of cerebrospinal fluid upon the removal of the guide tube stylet, as well as by post-mortem inspection. 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 subcutaneous injection over 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). One polyester polyurethane sponge containing 20 mg of flugestone acetate (synthetic analogue of progesterone) was administered intra-vaginally independently of animal body weight and individual day of oestrus cycle. Next, after 14 days, the sponge was gently removed and an intra-muscular injection of pregnant mare's serum gonadotropin (PMSG; 500 i.u. per animal; Folligon, Intervet, the Netherlands) was performed to enhance ovulation. Oestrus occurred within 48–72 h after PMSG administration. The appearance of oestrus was additionally verified using vasectomized rams. Animals entered the experiment on the 4th to 5th day after ovulation, when the oestrogen in the peripheral blood reached its lowest level during the oestrus cycle (Lunstra and Christenson, 1981).

Experiments were conducted from the end of October to the first week of December. Animals were randomly divided into three experimental groups: the control group received an icv infusion of Ringer-Locke solution (artificial cerebrospinal fluid; 480 µL per day; n = 8); group I received an icv infusion of BDNF (Santa Cruz Biotechnology, Inc., Dallas, Texas; cat. no SC-4554) in a dose of 10 µg per day (BDNF 10 group; n = 8), and group II received BDNF in a dose of 60 µg per day (BDNF 60 group; n = 8), both diluted in 480 µL of Ringer-Locke solution, at an infusion rate of 120 µL h^{-1}. The doses of BDNF used in this experiment were chosen on the basis of information from the literature (Dittrich et al., 1996), our previous experience concerning the action of other peptides engaged in modulation of the hypothalamic energy centre such as leptin, ghrelin or obestatin in the sheep hypothalamus (Hasiec et al., 2017; Tillet et al., 2010; Wójcik-Gładysz et al., 2018, 2016, 2014; Szlis et al., 2015), as well as preliminary studies (Szlis et al., 2018 b; Przybył et al., 2018).

During the experiment, 1 h prior to infusion, cannulae were introduced through the guide cannulae and locked in position with tips placed approximately 2.0–2.5 mm above the base of the brain; when the tips of the cannulae were in the IIIv, the cerebrospinal fluid flowed into the infused cannulae. Then, all sheep received four icv infusions (performed from 08:40 a.m. to 01:30 p.m., infusion scheme: 4 × 50 min infusions with 30 min intervals between them) on each of three consecutive days using a BAS Bee MD_1020 904 microinjection pump (Bioanalytica Sys-

tems Inc, West Lafayette, IN, USA). To avoid a rapid increase in pressure through Ringer-Locke or BDNF infusions into the IIIv, the flow rate of the microinjection pump was set to 2 μL min^{-1}. The infusion pattern was designed to mimic the pulsatile release of neurohormones synthetized in the CNS in the sheep model (Polkowska et al., 2008; Wójcik-Gładysz et al., 2018, 2016) (Figure 1).

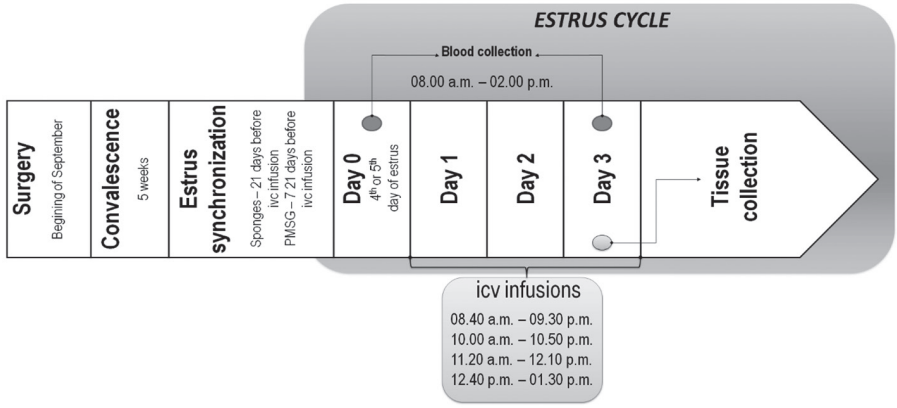

Figure 1. Scheme of the experiment

Immediately after the last infusion, the animals were weighed, anaesthetized intravenously using dexmedetomidine (0.05 mL kg^{-1}) and pentobarbital (80 ng kg^{-1}, Morbital®; Vetoquinol Biowet, Poland), and decapitated. Isolation of selected hypothalamic structures (area and depth of the cut) was performed in accordance with the stereotaxic coordinates defining the individual hypothalamic nuclei described in the anatomic atlas of the sheep hypothalamus (Welento et al., 1969). The brain encompassing the preoptic area and hypothalamus was sectioned sagittally and dissected from both sides of the ARC nucleus (cut coordinates: anterio-posterior 30.82–32.50; sagittal 0.00–1.88; horizontal −0.54–+4.53), the depths of the cuts were 2–2.5 mm (Figure 2). All tissues were frozen in liquid nitrogen immediately after collection and then stored at −80°C until real-time RT-qPCR analysis.

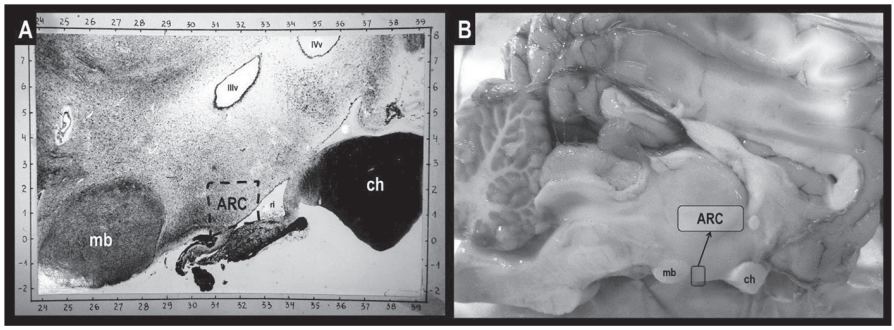

Figure 2. Scheme of dissected structure localization: (A) sagittal cut through the sheep hypothalamus (from Welento et al., 1969), in the black box is the arcuate nucleus (ARC; cut coordinates: anterio--posterior 30.80–32.50; sagittal 0.00–2.88; horizontal −0.54–+4.53); (B) the sagittal cut through the sheep brain, in the black box are the approximate boundaries of dissected structures of the ARC nucleus; ch – chiasma, mb – mammillary bodies, IIIv – third ventricle of the brain

Real-time RT-qPCR

Total RNA from the ARC nucleus was extracted using a NucleoSpin RNA/Protein kit (Macherey-Nagel GmbH & Co., Düren, Germany) according to the manufacturer's instructions. The yield of isolated RNA was estimated spectrophotometrically (Nanodrop, NanoDrop Technologies, Wilmington, DE), 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} of RNA from the ARC nucleus in a total volume of 20 µL was retro-transcribed using a TranScriba Kit (A&A Biotechnology, Gdynia, Poland), according to the manufacturer's protocol. Specific primers for sheep species (*Ovis aries*) determining the expression of housekeeping genes and the genes of interest were designed using Primer 3 software (The Whitehead Institute, Cambridge, MA, USA) and synthesized by Genomed (Warsaw, Poland). The respective primer sequences are shown in Table 1.

Table 1. Sheep-specific primer sequences used in the experiment

Gene	Primer	Sequence (5'-3')	Product size (nt)	GenBank access no.
GAPDH	Forward	AGAAGGCTGGGGCTCACT	134	NM_001034034
	Reverse	GGCATTGCTGACAATCTTGA		
PPIC	Forward	TGGAAAAGTCGTGCCCAAGA	158	XM_004008676.1
	Reverse	TGCTTATACCACCAGTGCCA		
ACTB	Forward	TGGGCATGGAATCCTG	194	NM_001009784
	Reverse	GGCGCGATGATCTTGAT		
NPY	Forward	ATCACCAGGCAGAGATACGG	100	NM_001009452.1
	Reverse	CCAGCCTAGTTCTGGGAATG		
CART	Forward	CCCATGAGAAGGAGCTGATTGA	108	XM_012097209.1
	Reverse	TGGGGACTTGGCCATACTTC		
AgRP	Forward	TCCTAGAGCTCCAAGGCCTA	124	XM_015100491.1
	Reverse	CCTTCCGGATCTAGCACCTC		
POMC	Forward	GAAAGTAACCTGCTGGCGTG	129	NM_001009266.1
	Reverse	GAAATGGCCCATGACGTACTTC		
PAM	Forward	GTACTCCAGGTACACTATGGGGA	216	XM_012179006.2
	Reverse	GTAGGCAAAGACATGCATTGGA		

Real-time qPCR was conducted using a 5× FIREPol EvaGreen qPCR Mix Plus (no ROX; Solis BioDyne, Tartu, Estonia) in a total volume of 15 µL containing 3 µL of Master Mix, 9 µL of RNase-free H_2O, 2 × 0.5 µL primers (0.5 mM), and 2 µL of cDNA template. Amplification was performed using a Rotor Gene 6000 thermocy-

cler (Corbett Research, Mortlake, Australia) according to the following protocol: one cycle at 95°C for 15 min (enzyme activation), followed by PCR including 35 cycles at 94°C for 5 s (denaturation), 59°C for 20 s (annealing), and 72°C for 15 s (elongation), then one cycle at 72°C for 7 min (product stabilization). The melting curve was performed over 70–95°C at 0.5°C intervals. Negative controls without the cDNA template were included in each reaction. Real-time qPCR for each cDNA sample was performed twice in triplicate. The identity of PCR products was confirmed by direct sequencing (Genomed, Warsaw, Poland).

Relative gene expression was calculated 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). In the experiment, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin (ACTB) and peptidylprolyl isomerase C (PPIC) genes were tested as housekeeping genes, and using the Best-Keeper software (http://www.gene-quantification.de/bestkeeper.html), GAPDH was chosen as the best endogenous control gene to normalize mRNA expression in the study. The results are presented as relative expression of the target mRNA vs housekeeping mRNA (GAPDH), with the relative mRNA expression for the group of sheep that received only the Ringer-Locke solution infusion set to 1.0.

Total miRNA isolation and cDNA synthesis

Total miRNA from the ARC nucleus was extracted using a NucleoSpin miRNA kit (Macherey-Nagel GmbH & Co., Düren, Germany) according to the manufacturer's instructions. The yield of isolated RNA was estimated spectrophotometrically (Nanodrop, NanoDrop Technologies, Wilmington, DE, USA), and its integrity was evaluated electrophoretically by separation on a 1.5% agarose gel containing ethidium bromide. For complementary DNA (cDNA) synthesis, 500 ng mL^{-1} of miRNAs in a total volume of 20 µL were retro-transcribed using the MistiCp microRNA cDNA Synthesis Mix (Sigma Aldrich, USA) according to the manufacturer's protocol. The RT-PCR reaction was preceded by a polyadenylation reaction of the 3' end, which allowed us to synthesize a sequence allowing attachment to the cDNA of the universal primer (reverse primer; included in the kit) used in the real time qPCR reaction.

In silico typing of miRNA sequences

The miRNAs investigated in the experiment were typed using bioinformatics tools as well as data from the literature. First, the target sheep mRNA was compared to adequate human mRNA using Basic Local Alignment Search Tool (BLAST; U.S. National Library of Medicine) to check the complementarity between them. The alignment of two sequences was used because they contain a similar sequence to which predicted miRNAs (characterized by non-full complementarity to target mRNA) can be attached. If the sequence complementarity was higher, the probability of selecting an miRNA (based on human sequences deposited in bioinformatics databases miRBase and miRDB) that could regulate the expression of the sheep

mRNA, was also greater. Next, to find miRNAs that can influence our target mRNA, two databases containing miRNA sequences, miRBase and miRDB, were searched. The sequences were used if miRNAs regulating a target mRNA were matching in both databases. In the process of miRNA profiling, no matches for AgRP were found. Therefore, only the expression of predicted miRNAs for NPY (miRNA-33a-5p; miRNA-33b-5p; miRNA-647), CART (miRNA-377-3p; miRNA-214-3p) and POMC (miRNA-488-3p; miRNA-485-5p) were investigated.

Table 2. Comparison of the sequences of tested sheep and human genes

Gene	GenBank access no.	Product size (nt)	Identities	Gaps
NPY	Homo sapiens: NM_000905	576	345/391 (88%)	3/391 (0%)
	Ovis aries: NM_001009452	452		
AgRP	Homo sapiens: NM_001138	783	361/439 (82%)	8/439 (1%)
	Ovis aries: XM_015100491	724		
CART	Homo sapiens: NM_004291	933	607/711 (85%)	32/711 (4%)
	Ovis aries: XM_015101190	708		
POMC	Homo sapiens: NM_000939	1406	687/819 (84%)	42/819 (5%)
	Ovis aries: NM_001009266	792		

Table 3. Forward primer sequences used in the miRNA experiment

Target Gene	miRNA Name	miRNA Sequence	Primer Forward Sequence (5'-3')	Primer Reverse
NPY	miRNA-33a-5p	GUGCAUUGUAGUUG-CAUUGCA	GTGCATTGTAGTTG-CATTGCA	Universal PCR Primer
	miRNA-33b-5p	GUGCAUUGCUGUUG-CAUUGC	GTGCATTGCTGTTG-CATTGC	
	miRNA-647	GUGGCUGCACUCACU-UCCUUC	GTGGCTGCACT-CACTTCCTTC	
CART	miRNA-377-3p	AUCACACAAAGGCAAC-UUUUGU	ATCACACAAA-GGCAACTTTTGT	
	miRNA-214-3p	ACAGCAGGCACAGACA-GGCAGU	AGCAGGCACAGACA-GGCAGT	
POMC	miRNA-488-3p	UUGAAAGGCUAUU-UCUUGGUC	GGGAAAGGC-TATTTCTTGGTC	
	miRNA-485-5p	AGAGGCUGGCCGU-GAUGAAUUC	GAAAGGCTATTTCTTG-GTC	

miRNA real-time qPCR

Real-time qPCR was conducted using 5× FIREPol EvaGreen qPCR Mix Plus (no ROX; Solis BioDyne, Tartu, Estonia) in a total volume of 15 µL containing

3 µL of Master Mix, 9.6 µL of RNase-free H_2O, 0.2 µL forward primer (0.5 mM), 0.2 µL universal primer – reverse primer (0.5 mM), and 2 µL of cDNA template. The 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 PCR including 40 cycles at 95°C for 15 s (denaturation), 60°C for 25 s (annealing), and 72°C for 25 s (elongation), then one cycle at 72°C for 7 min (product stabilization). The melting curve was performed over 70–95°C at 0.5°C intervals. Negative controls without the cDNA template were included in each reaction. Real time qPCR for each cDNA sample was performed twice in triplicates. The identity of PCR products was confirmed by direct sequencing (Genomed, Warsaw, Poland). Relative gene expression was calculated using the method described above in the material and methods section.

Statistical analysis

GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA) was used for all statistical calculations. All data values presented in graphs represent means ± SEM for each group. The normal distribution of data was tested using the Shapiro-Wilk test. Statistical data evaluations for mRNA expression were carried out using one-way analysis of variance (ANOVA) following *post hoc* tests. Differences resulting in $P \leq 0.05$ were considered statistically significant.

Results

Animals

In all sheep, the progesterone concentration in blood plasma before the start of the experiment did not exceed 0.5 ng mL^{-1}. Post-mortem ovary inspection revealed the presence of corpora lutea and no pre-ovulatory follicles were noticed.

Expression of npy, agrp, cart, pomc and pam mRNA

Real-time qPCR analyses revealed the presence of *npy, agrp, cart, pomc,* and *pam* mRNA transcripts in the ARC nucleus in sheep from all experimental groups. The dose-dependent increase of *npy* mRNA expression in both BDNF 10 and 60 groups in comparison to the control group was observed (3.77-fold and 7.38-fold, respectively; Figure 3 A). Similarly, significantly ($P<0.001$) higher *agrp* mRNA expression in BDNF 10 and 60 groups (2.87-fold, 5.99-fold, respectively) in comparison to the control group was observed (Figure 3 B). The *cart* mRNA expression was significantly ($P<0.01$ and $P<0.001$, respectively) lower in the ARC nucleus in both groups receiving BDNF infusion than in the same area in the control group (0.67-fold and 0.21-fold, respectively; Figure 3 C). Moreover, a dose-dependent increase in *pomc* mRNA expression, as well as a dose-dependent decrease in *pam* mRNA expression were observed in both groups receiving BDNF infusions.

Expression of miRNAs in the ARC nucleus

Real-time qPCR analyses revealed the presence of all investigated miRNAs in

the sheep ARC nucleus from all experimental groups. A significant (P<0.01 and P<0.001, respectively) increase of miRNA-33a-5p (P<0.001 and P<0.05, respectively) in BDNF 10 and BDNF 60 (1.92, 1.48-fold, respectively), as well as miRNA-33b-5p (1.40-fold, P<0.01) in BDNF 10 groups in comparison to the control group have been observed (Figure 4 A, B). No statistically significant differences (P<0.05) in miRNA-647 expression in any experimental groups have been observed (Figure 4 C).

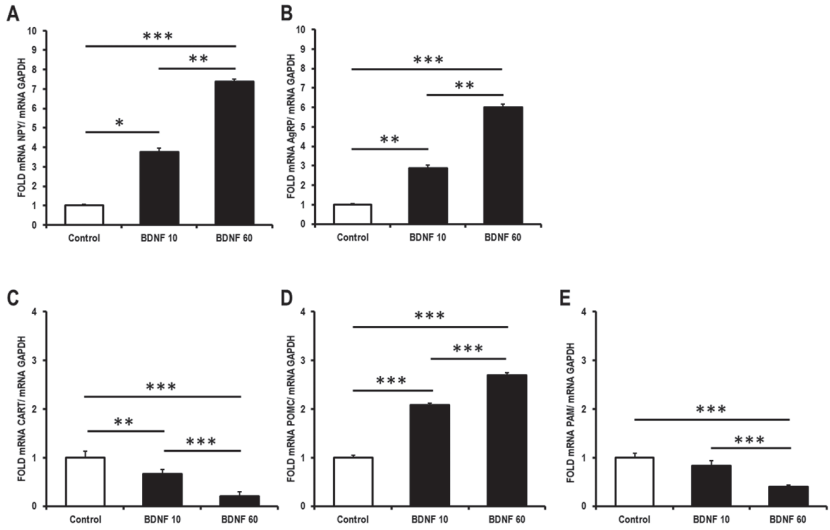

Figure 3. mRNA expression in the arcuate nucleus: neuropeptide Y (A); agouti-related peptide (B); cocaine and amphetamine regulating transcript (C); proopiomelanocortin (D) and peptidylglycine alpha-amidating monooxygenase (E) in control and BDNF infused sheep. Data are means ± SEM; Significant differences: * P<0.05, ** P<0.01, *** P<0.001.

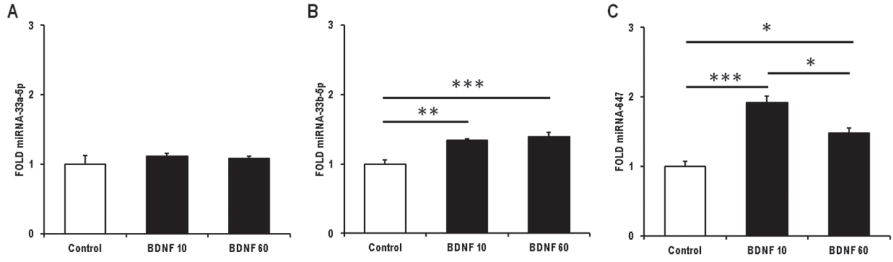

Figure 4. miRNA expression in the arcuate nucleus: miRNA-33a-5p (A); miRNA-33b-5p (B); miRNA-647 (C) in control and BDNF infused sheep. Data are means ± SEM; Significant differences: * P<0.05, ** P<0.01, *** P<0.001

Furthermore, a significant (P<0.01 and P<0.001, respectively) decrease of miRNA-377-3p expression in the BDNF 60 group (0.69-fold) compared to control and BDNF 10 groups has been shown (Figure 5 A). The expression of miRNA-214-3p was also significantly (0.47-fold, P<0.001) lower in the BDNF 60 group than in the control and BDNF 10 groups (Figure 5 B). Moreover, the expression of miRNA-485 was also significantly (P<0.001 and P<0.01, respectively) higher in both BDNF groups than in the control group (1.62 and 1.44-fold, respectively; Figure 5 D). Additionally, significant differences (P<0.001 and P<0.01, respectively) have been observed in miRNA-488 expression between BDNF 10 and control groups as well as between BDNF 10 and BDNF 60 groups (Figure 5 C).

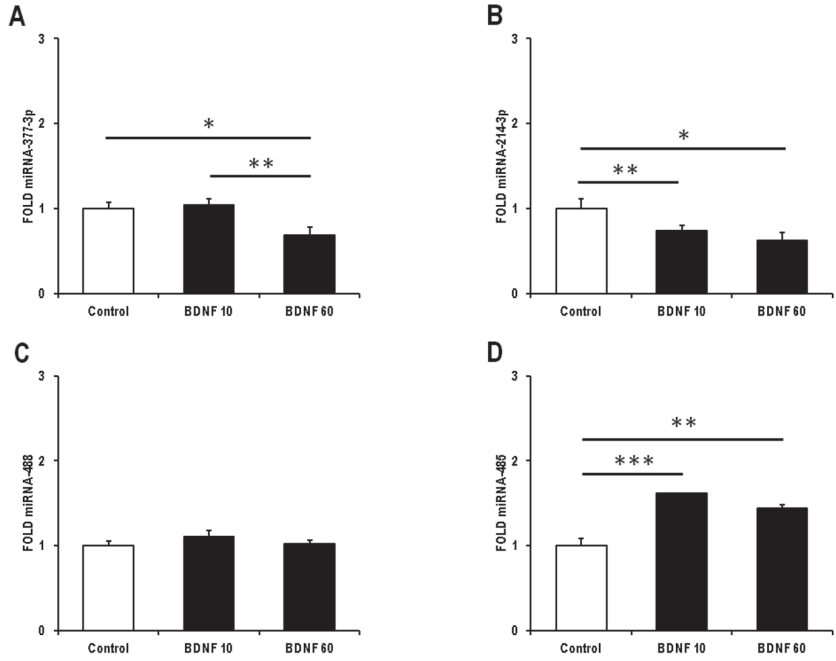

Figure 5. miRNA expression in the arcuate nucleus: miRNA-377-3p (A); miRNA-488 (B); miRNA-214-3p (C); miRNA-485 (D) in control and BDNF infused sheep. Data are means ± SEM; Significant differences: * P<0.05, ** P<0.01, *** P<0.001

Discussion

In the present study, an *in vivo* effect of BDNF on the expression of *npy*, *agrp*, *cart* and *pomc* mRNA in neurons co-creating the hypothalamic appetite-regulating network in domestic animals, namely, ruminants, was demonstrated. Obtained data suggest the participation of BDNF in the central regulation of appetite modulation processes, and its action may occur through NPY/AgRP and CART/α-MSH neurons.

The results of our studies also revealed the expression of selected miRNAs in the sheep ARC nucleus. Moreover, it was demonstrated that BDNF in *in vivo* conditions can alter the expression of these miRNA molecules in the hypothalamus region associated with appetite regulation.

The mediobasal hypothalamus containing the ARC nucleus is known as a principal area regulating the maintenance of the organism's energy balance. Two subpopulations of neurons, NPY/AgRP and CART/α-MSH, are the most important elements of the hypothalamic appetite regulating network (Szlis et al., 2018 a; Wójcik-Gładysz et al., 2016). Our findings revealed that BDNF affects the expression of *npy* and *agrp* mRNA in the ARC nucleus. Moreover, observed changes were BDNF dose-dependent. This result corresponds to data obtained in *in vitro* studies, where supplementation of culture medium with exogenous BDNF peptide affected NPY protein synthesis and this effect was also dose-dependent (Barnea and Roberts, 2001). It has been shown that intraventricular administration of BDNF increased the expression of mRNA and amount of immunoreactive NPY material in neuronal cells of the anterior neocortex of newborn rats (Xapelli et al., 2008). In research conducted on rats, it was also shown that central infusion of BDNF increases NPY mRNA expression in the striatum (Croll et al., 1994). These authors suggest that endogenous BDNF may regulate NPY peptide levels during normal functioning of the adult rat brain, and/or during pathological states. Moreover, the effect of BDNF on neuropeptide expression may depend on the location of NPY neurons within the brain.

The effect of BDNF on NPY and AgRP expression may also be dependent on the organism's energy status. Wang and co-workers (2007) have shown that the inhibition potency of BDNF in normally fed rats is stronger than that observed in rats subjected to deprivation-induced feeding (Wang et al., 2007). In the time of food deprivation, 'first-order neuron' activity is subject to dramatic changes; orexigenic peptides, such as NPY, AgRP, orexin A, or ghrelin are upregulated, while anorectic peptides such as leptin, POMC, α-MSH, and CART are downregulated. Thus, the abovementioned food deprivation-induced neuroendocrine changes may affect the modulation effects of BDNF (Wang et al., 2007; Xapelli et al., 2008).

The remaining neurons localized in the ARC nucleus expressing CART/α-MSH are further important components of the hypothalamic appetite-regulating network (Schneeberger et al., 2014; Zheng et al, 2005). In the present study, a very intensive dose-dependent decrease in *cart* mRNA expression was noted. Moreover, a dose-dependent increase in the expression of *pomc* mRNA, as well as a dose-dependent decrease in *pam* mRNA expression have also been shown. Considering the above observation, we could suspect that changes in *pam* mRNA expression may in consequence affect PAM protein expression, which in turn will cause POMC to not be converted into α-MSH. In assumption, we should observe a decrease in the α-MSH protein level in the ARC nucleus. Moreover, studies indicated that BDNF/TrkB signalling exerts its anorexigenic effects downstream of the melanocortinergic system. It has been shown that the hyperphagic and obese phenotype of agouti yellow mice, in which ectopic expression of the agouti protein antagonizes MC4R, can be partially reversed by infusion of exogenous BDNF (Balthasar et al., 2005; Croll et al., 1998; Grill et al., 1998; Kishi et al., 2003; Liu et al., 2003; Wan et al., 2008; Williams et

al., 2000; Baoji Xu et al., 2003). We could conclude that, in sheep, BDNF can possess the ability to suppress CART and α-MSH protein expression in CART/α-MSH neurons.

Unfortunately, in this study we have investigated only the effect of BDNF on mRNA expression in the sheep ARC nucleus. Considering the fact that changes in mRNA levels do not precisely reflect the respective cellular translational activity, exact determination of the effect of BDNF on NPY/AgRP and CART/α-MSH neuron activity requires further investigation concerning the changes in protein level, especially immunohistological co-localization of NPY, AgRP, CART and α-MSH peptides.

In the performed studies, we observed changes in selected miRNA expression which may be involved in post-transcriptional modulation of NPY, AgRP, CART and POMC mRNA expression. BDNF changed the expression of miRNA-33b-5p, miRNA-647, miRNA-647, miRNA-377-3p, miRNA-214-3p, and miRNA-485, which were selected as being able to participate in the processes of post-transcriptional modulation of NPY, CART and POMC mRNA respectively. To the best of our knowledge, these findings are the first data demonstrating that BDNF in *in vivo* conditions can alter the expression of miRNA molecules in the hypothalamus region associated with body energy regulation.

In the literature, there is little information about the influence of BDNF on miRNA expression. BDNF regulates the expression of miRNA-132, which occurs via cAMP response element binding protein (CREB). In fact, overexpression of miRNA-132 causes enhanced neurite outgrowth and dendritic morphogenesis. BDNF selectively upregulated the expression of miRNA-132 in cultured neurons, whereas no change occurred in the expression of other miRNAs such as miRNA-9, miRNA-124, miRNA-128a, miRNA-128b, miRNA-134, miRNA-138, and miRNA-16 (Kawashima et al., 2010). Moreover, inhibition of the intracellular ERK pathway suppressed the BDNF-induced increase of miRNA-132, suggesting that upregulation of this miRNA by BDNF occurs via the ERK1/2 pathway. It is pertinent that both BDNF and ERK1/2 are implicated in stress and depression. Interestingly, BDNF itself is a target of several miRNAs. For example, miRNA-30a and miRNA-195 directly target the BDNF 3'-UTR, thus reducing its expression, but more studies on this subject need to be performed to better understand the influence of miRNAs on BDNF expression in neuronal cells (Mellios et al., 2008).

The present study shows that BDNF modulates *npy, agrp, cart, pomc,* and *pam* mRNA expression in the sheep ARC nucleus, which suggests that BDNF may be another neurotransmitter with the ability to modulate body energy homeostasis. Moreover, obtained data suggest that BDNF may also change the expression of selected miRNAs, which could be involved in the post-transcriptional regulation of NPY, CART and POMC mRNA expression. The results of this study support the hypothesis that BDNF as neuromodulator may affect the hypothalamic neuronal network responsible for appetite regulation in the sheep ARC nucleus.

Abbreviations

Central nervous system (CNS), brain-derived neurotrophic factor (BDNF), tyrosine kinase family isoform B receptors (TrkB), ventromedial nuclei (VMN), dor-

salmedial nuclei (DMN), paraventricular nuclei (PVN), arcuate nuclei (ARC), median eminence (ME), neuropeptide Y (NPY), agouti-related peptide (AgRP), cocaine and amphetamine regulated transcript (CART), α-melanotrophin (α-MSH), proopiomelanocortin (POMC), microRNA (miRNA), peptidylglycine alpha-amidating monooxygenase (pam), third ventricle (IIIv), mediobasal hypothalamus (MBH), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin (ACTB), peptidylprolyl isomerase C (PPIC).

Acknowledgements

The authors would like to thank veterinary surgeons K. Roszkowicz-Ostrowska and J. Rutkowski for help in brain surgery; E. Skrzeczyńska, A. Misztal for help in performing the experiments; and W. Mrozek and R. Druchniak for animal care. The presented study is part of B.J. Przybył's PhD dissertation.

Conflict of interest

The authors have declared that no competing interest exists.

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Received: 7 VIII 2019
Accepted: 9 I 2020