ACCEPTED AUTHOR VERSION OF THE MANUSCRIPT:

Brain-derived neurotrophic factor affects mRNA and miRNA expression of the appetite regulating centre in the sheep arcuate nucleus
DOI: 10.2478/aoas-2020-0015

Bartosz Jarosław Przybył, Michał Szlis*, Anna Wójcik-Gładysz

Department of Animal Physiology, The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, 05-110 Jabłonna, Poland

*Corresponding author: m.szlis@ifzz.pl

Received date: 7 August 2019
Accepted date: 9 January 2020

To cite this article: (2020). Przybył B.J., Szlis M., Wójcik-Gładysz A. (2020). Brain-derived neurotrophic factor affects mRNA and miRNA expression of the appetite regulating centre in the sheep arcuate nucleus, Annals of Animal Science, DOI: 10.2478/aoas-2020-0015

This is unedited PDF of peer-reviewed and accepted manuscript. Copyediting, typesetting, and review of the manuscript may affect the content, so this provisional version can differ from the final version.
Brain-derived neurotrophic factor affects mRNA and miRNA expression of the appetite regulating centre in the sheep arcuate nucleus

Bartosz Jarosław Przybył, Michał Szlis*, Anna Wójcik-Gładysz

Department of Animal Physiology, The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, 05-110 Jabłonna, Poland

*Corresponding author: m.szlis@ifzz.pl

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.

Funding

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.

Conflict of interest

The authors have declared that no competing interest exists.
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 x 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

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., 2018b; Wójcik-Gładysz et al., 2019; Wójcik-Gladysz 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 (Hormon et al., 2014; Lopaschuk et al., 2010; Wójcik-Gładysz and Szlis, 2016).

Brain-derived neurotrophic factor (BDNF) 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), dorsomedial (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 Deptula 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 alph-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 Agriculture 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; 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 postmortem 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, Holland) 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 (Hasieć et al., 2017; Tillet et al., 2010; A. Wójcik-Gładysz et al., 2018, 2016; Wójcik-Gładysz et al., 2014), as well as preliminary studies (Szlis et al. 2018; Przybył et al. 2018; abstract from V Congress of Polish Society of Neuroendocrinology are available on website of The Polish Society of Endocrinology Journal – Endokrynologia Polska: https://journals.viamedica.pl/endokrynologia_polska/issue/view/4394).

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 x 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 Systems 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) (Fig. 1).

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 (Fig. 2). All tissues were frozen in liquid nitrogen immediately after collection and then stored at −80°C until real-time RT-qPCR analysis.

**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) and synthesized by Genomed (Warsaw, Poland). The respective primer sequences are shown in Table 1.

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\(_2\)O, 2 × 0.5 µL primers (0.5 mM), and 2 µL of cDNA template. 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 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), 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.

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 H2O, 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 in above point 2.2. of 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≤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 exceeded 0.5 ng mL⁻¹. Post-mortem ovary inspection revealed the presence of corpa 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; Fig. 3A). 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 (Fig. 3B). 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; Fig. 3C). 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 (Fig. 4A, B). No statistically significant differences (P<0.05) in miRNA-647 expression in any experimental groups have been observed (Fig. 4C).

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 (Fig. 5A). 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 (Fig. 5B). 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; Fig. 5D). 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 (Fig. 5C).
Discussion

In the present study, an in vivo effect of BDNF on the expression of npy, agrp, cart and pome 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 principle area regulating the maintenance of organism energy balance. Two sub-populations of neurons, NPY/AgRP and CART/α-MSH, are the most important elements of the hypothalamic appetite regulating network (Szlis et al. 2018; 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 depend 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). 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 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; 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/\(\alpha\)-MSH neuron activity requires further investigation concerning the changes in protein level, especially immunochistological co-localization of NPY, AgRP, CART and \(\alpha\)-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 \textit{in vivo} conditions can alter the expression of miRNA molecules in the hypothalamus region associated with body energy regulation.

In the literature, there is a 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), dorsalmedial 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 alph-amidating monooxygenase (pam), third ventricle (IIIv), mediobasal hypothalamus (MBH), glyceraldehyde- 3-phosphate dehydrogenase (GAPDH), β-actin (ACTB), peptidylprolyl isomerase C (PPIC)
References

Balthasar N., Dalgaard L.T., Lee C.E., Yu J., Funahashi H., Williams T., Ferreira M., Tang V., Mcgovern R.A., Kenny C.D., Christiansen L.M., Edelstein E., Choi B., Boss O., Aschkenasi C., Zhang C., Mountjoy K., Kishi T., Elmquist J.K., Lowell B.B. (2005). Divergence of Melanocortin Pathways in the Control of Food Intake and Energy Expenditure. Cell, 123: 493–505.

Barde Y.A., Edgar D., Thoenen H. (1982). Purification of a new neurotrophic factor from mammalian brain. EMBO J., 1: 549–553.

Barnea A., Roberts J. (2001): Induction of functional and morphological expression of neuropeptide Y (NPY) in cortical cultures by brain-derived neurotrophic factor (BDNF). Evidence for a requirement for extracellular-regulated kinase (ERK)-dependent and ERK-independent mechanisms. Brain Res., 919: 57–69.

Benes V., Castoldi M. (2010). Expression profiling of microRNA using real-time quantitative PCR, how to use it and what is available. Methods, 50: 244–249.

Byerly M.S., Simon J., Lebihan-Duval E., Duclos M.J., Cogburn L.A., Porter T.E. (2009). Effects of BDNF, T3, and corticosterone on expression of the hypothalamic obesity gene network in vivo and in vitro. AJP Regul. Integr. Comp. Physiol. 296: R1180–R1189.

Cordeira J.W., Frank L., Sena-Esteves M., Pothos E.N., Rios, M. (2010). Brain-Derived Neurotrophic Factor Regulates Hedonic Feeding by Acting on the Mesolimbic Dopamine System. J. Neurosci., 30: 2533–2541.

Croll S.D., Chesnutt C.R., Rudge J.S., Acheson A., Ryan T.E., Siuciak J.A., DiStefano P.S., Wiegand S.J., Lindsay R.M. (1998). Co-infusion with a TrkB-Fc receptor body carrier enhances BDNF distribution in the adult rat brain. Exp. Neurol., 152: 20–33.

Croll S.D., Wiegand S.J., Anderson K.D., Lindsay R.M., Nawa H. (1994). Regulation of Neuropeptides in Adult Rat Forebrain by the Neurotrophins BDNF and NGF. Eur. J.
De Souza M.J., Leidy H.J., O’Donnell E., Lasley B., Williams N.I. (2004). Fasting ghrelin levels in physically active women: Relationship with menstrual disturbances and metabolic hormones. J. Clin. Endocrinol. Metab., 89: 3536–3542.

Dittrich F., Ochs G., Große-Wilde A., Berweiler U., Yan Q., Miller J.A., Toyka K. V., Sendtner M. (1996). Pharmacokinetics of intrathecally applied BDNF and effects on spinal motoneurons. Exp. Neurol., 141: 225–239.

Evans J.J., Anderson G.M. (2012). Balancing ovulation and anovulation: Integration of the reproductive and energy balance axes by neuropeptides. Hum. Reprod. Update, 18: 313–332.

Fenichel R.M., Dominguez J.E., Mayer L., Walsh B.T., Boozer C., Warren M.P. (2008). Leptin levels and luteinizing hormone pulsatility in normal cycling women and their relationship to daily changes in metabolic rate. Fertil. Steril. 90: 1161–1168.

Grenda A., Budzyński M., Filip A.A. (2013). Biogeneza cząsteczek mikroRNA oraz ich znaczenie w powstawaniu i przebiegu wybranych zaburzeń hematologicznych. Postępy Hig. Med. Dosw., 67: 174–185.

Grill H.J., Ginsberg A.B., Seeley R.J., Kaplan J.M. (1998). Brainstem Application of Melanocortin Receptor Ligands Produces Long-Lasting Effects on Feeding and Body Weight. J. Neurosci., 18: 10128–10135.

Hukowska-Szematowicz B., Deptuła W. (2010). Biologiczna rola mikroRNA (miRNA) nowe dane. Postępy Biol. Komórki, 37: 585–597.

Kawashima H., Numakawa T., Kumamaru E., Adachi N., Mizuno H., Ninomiya M., Kunugi H., Hashido K., (2010). Glucocorticoid attenuates brain-derived neurotrophic factor-dependent upregulation of glutamate receptors via the suppression of microRNA-132 expression. Neuroscience, 165: 1301–1311.
Kishi T., Aschkenasi C.J., Lee C.E., Mountjoy K.G., Saper C.B., Elmquist J.K. (2003). Expression of Melanocortin 4 Receptor mRNA in the Central Nervous System of the Rat. J. Comp. Neurol., 457: 213–235.

Lunstra D.D., Christenson R. K. (1981). Synchronization of Ewes during Anestrus: Influence of Time of Year and Interval to Onset of Estrus on Conception Rate. Journal of Animal Science, 53: 448-457.

Liu H., Kishi T., Roseberry A.G., Cai X., Lee C.E., Montez J.M., Friedman J.M., Elmquist J.K. (2003). Transgenic Mice Expressing Green Fluorescent Protein under the Control of the Melanocortin-4 Receptor Promoter. J. Neurosci., 23: 7143–7154.

Lopaschuk G.D., Ussher J.R., Jaswal J.S. (2010). Targeting Intermediary Metabolism in the hypothalamus as a mechanism to regulate appetite. Pharmacol Rev., 62: 237–264.

Mellios N., Huang H.S., Grigorenko A., Rogaev E., Akbarian S. (2008). A set of differentially expressed miRNAs, including miR-30a-5p, act as post-transcriptional inhibitors of BDNF in prefrontal cortex. Hum. Mol. Genet., 17: 3030–3042.

Navarro V.M., Fernández-Fernández R., Nogueiras R., Vigo E., Tovar S., Chartrel N., Le Marec O., Leprince J., Aguilar E., Pinilla L., Dieguez C., Vaudry H., Tena-Sempere M., (2006). Novel role of 26RFa, a hypothalamic RFamide orexigenic peptide, as putative regulator of the gonadotropic axis. J. Physiol. 573: 237–249.

Nicholson J.R., Peter J.C., Lecourt A.C., Barde Y.A., Hofbauer K.G. (2007). Melanocortin-4 receptor activation stimulates hypothalamic brain-derived neurotrophic factor release to regulate food intake, body temperature and cardiovascular function. J. Neuroendocrinol., 19: 974–982.

Peiris T. S., Machaalani R., Waters K.A. (2004). Brain-derived neurotrophic factor mRNA and protein in the piglet brainstem and effects of Intermittent Hypercapnic Hypoxia. Brain Res., 1029: 11–23.
Pfaffl M.W., Horgan G.W., Dempfle L. (2002). Relative expression software tool ( REST © )
for group-wise comparison and statistical analysis of relative expression results in real-
time PCR. Nucleic Acids Res., 30: e36.

Pfaffl M.W., Tichopad A., Prgomet C., Neuvians T.P. (2004). Determination of stable
housekeeping genes , differentially regulated target genes and sample integrity:
BestKeeper – Excel-based tool using pair-wise correlations. Biotechnol. Lett., 26: 509–
515.

Polkowska J., Wójcik-Gładysz A., Wańkowska M., Bruneau G., Tillet Y. (2008). Prepubertal
changes in the synthesis, storage and release of the somatostatin in the hypothalamus of
female lambs: A morphofunctional study. J. Chem. Neuroanat., 36: 53–58.

Schneeberger M., Gomis R., Claret M. (2014). Hypothalamic and brainstem neuronal circuits
controlling homeostatic energy balance. J. Endocrinol., 220: T25-T46.

Strzetelski J.A., Brzóska F., Kowalski Z.M., Osięgłowski S. (2014). Zalecenia Żywnieniowe dla
Przeżuwaczy i Tabele wartości pokarmowej pasz. Inst. Zootech. PIB, Kraków.

Sullivan S.D., DeFazio R.A., Moenter S.M. (2003). Metabolic Regulation of Fertility through
Presynaptic and Postsynaptic Signaling to Gonadotropin-Releasing Hormone Neurons. J.
Neurosci., 23: 8578–8585.

Szlis M., Krawczyńska A., Polkowska J., Wójcik-Gładysz A. (2015). Neuromodulatory
influence of obestatin on GnRH/LH axis activ- ity and NPY gene expression in
peripubertal sheep. Reproduction in Domestic Animals, 50 (Suppl 3), 79.
https://doi.org/10.1111/rda.12579

Szlis M., Polkowska J., Skrzeczyńska E., Przybył B.J., Wójcik-Gładysz A., (2018); Does
obestatin modulate the hypothalamic appetite-regulating network in peripubertal sheep? J.
Anim. Physiol. Anim. Nutr., 102: 690–700.

Takei N., Furukawa K., Hanyu O., Sone H., Nawa H. (2014). A possible link between BDNF
and mTOR in control of food intake. Front. Psychol., 5: 1–6.

Tillet Y., Picard S., Bruneau G., Ciofi P., Wańkowska M., Wójcik-Gładysz A., Polkowska J. (2010). Hypothalamic arcuate neuropeptide Y-neurons decrease periventricular somatostatin-neuronal activity before puberty in the female lamb: Morphological arguments. J. Chem. Neuroanat., 40: 265–271.

Traczyk W. and Przekop F. (1963). A method for testing the function of the hypothalamus and pituitary in the sheep in chronic experiments. Acta. Physiol. Pol., 14: 217 – 226.

Unger T.J., Calde ron G.A., Bradley L.C., Sena-Esteves M., Rios M. (2007). Selective Deletion of Bdnf in the Ventromedial and Dorsomedial Hypothalamus of Adult Mice Results in Hyperphagic Behavior and Obesity. J. Neurosci., 27: 14265–14274.

Van Wynsberghe P.M., Chan S.P., Slack F.J., Pasquinelli A.E. (2011). Analysis of microRNA Expression and Function. Methods in Cell Biology. 106: 219-252.

Wan S., Browning, K.N., Coleman F.H., Sutton G., Zheng H., Butler A., Berthoud H., Travagli R.A. (2008). Presynaptic Melanocortin-4 Receptors on Vagal Afferent Fibers Modulate the Excitability of Rat Nucleus Tractus Solitarius Neurons. J. Neurosci., 28: 4957–4966.

Wang C., Bomberg E., Billington C., Levine A., Kotz C.M. (2007). Brain-derived neurotrophic factor in the hypothalamic paraventricular nucleus increases energy expenditure by elevating metabolic rate 55417: 992–1002.

Welento J., Szteyn S., Milart Z. (1969). Observations on the stereotaxic configuration of the hypothalamus nuclei in the sheep. Anatom. Anz., 124: 1–27.

Williams D.L., Kaplan J.M., Grill H.J. (2000). The Role of the Dorsal Vagal Complex and the Vagus Nerve in Feeding Effects of Melanocortin-3/4 Receptor Stimulation. Endocrinology, 141: 1332–1337.

Wójcik-Gładysz A., Szlis M., (2016). Hypothalamo-gastrointestinal axis - role in food intake regulation. J. Anim. Feed Sci., 25: 97-108
Wójcik-Gładysz A., Szlis M., Misztal A., Przybył B.J., Polkowska, J. (2018). Obestatin stimulates the somatotrophic axis activity in sheep. Brain Res., 1678: 278–287.

Wójcik-Gładysz A., Wańkowska M., Gajewska A., Misztal T., Szlis M., Polkowska J. (2014). The effect of intracerebroventricular infusions of ghrelin on the secretory activity of the GnRH / LH system in peripubertal ewes. J. Anim. Feed Sci., 23: 299–308.

Wójcik-Gładysz A., Wańkowska M., Gajewska A., Misztal T., Zielińska-Górska M., Szlis M., Polkowska J. (2016). Effects of intracerebroventricular infusions of ghrelin on secretion of follicle-stimulating hormone in peripubertal female sheep. Reprod. Fertil. Dev., 28: 2065–2074.

Xapelli S., Bernardino L., Ferreira R., Grade S., Silva A.P., Salgado J.R., Cavadas C., Grouzmann E., Poulsen F.R., Jakobsen B., Oliveira C.R., Zimmer J., Malva J.O. (2008). Interaction between neuropeptide Y (NPY) and brain-derived neurotrophic factor in NPY-mediated neuroprotection against excitotoxicity: A role for microglia. Eur. J. Neurosci., 27: 2089–2102.

Xu B., Goulding E.H., Zang K., Cepoi D., Cone R.D., Jones K.R., Tecott L.H., Reichardt L.F. (2003). Brain-derived neurotrophic factor regulates energy balance downstream of melanocortin-4 receptor. Nat. Neurosci., 6: 736–742.

Zheng H., Patterson L.M., Phifer C.B., Berthoud H., Patterson L.M., Phifer C.B. (2005). Brain stem melanocortinergic modulation of meal size and identification of hypothalamic POMC projections. Am. J. Physiol. - Regul. Integr. Comp. Physiol., 289: R247–R258.

Abstract from V Congress of Polish Society of Neuroendocrinology, 21-22 September, 2018, Kraków; https://journals.viamedica.pl/endokrynologia_polska/issue/view/4394 (access 22 October, 2019)
Table 1. Sheep-specific primer sequences used in the experiment

| Gene | Primer | Sequence (5'-3') | Product size (nt) | GenBank access no. |
|------|--------|------------------|-------------------|-------------------|
| GAPDH Forward | AGAAGGCTGGGCTCACCT | 134 | NM_001034034 |
| GAPDH Reverse | GCATTGCTGACAATCTTGA | | |
| PPIC Forward | TGGAAAAGTCGTGCCCAAGA | 158 | XM_004008676.1 |
| PPIC Reverse | TGCTTATACCCACCACTGCCA | | |
| ACTB Forward | TGGGCATGGAATCCTG | 194 | NM_001009784 |
| ACTB Reverse | GCCCGCAGATGATCTTGAT | | |
| NPY Forward | ATCACCAGGCAGAGATACGG | 100 | NM_001009452.1 |
| NPY Reverse | CCACGCTAGGCTGGGAATG | | |
| CART Forward | CCCATGAGAAGGAGCTGATTGA | 108 | XM_012097209.1 |
| CART Reverse | TGGGGACTTGGCCATACTTC | | |
| AgRP Forward | TCCTAGAGCTCCAAGGCCTA | 124 | XM_015100491.1 |
| AgRP Reverse | CTTCCGGGATCTAGCACCTC | | |
| POMC Forward | GAAAGTAACCTGCTGGCGTG | 129 | NM_001009266.1 |
| POMC Reverse | GAAATGGCCCATGACGTACTTC | | |
| PAM Forward | GTACTCCAGGTACCATATGGGA | 216 | XM_012179006.2 |
| PAM Reverse | GTGGCAAAAGACATGCTTGA | | |
### Table 2. Comparison of the sequences of tested sheep and human genes

| Gene | GenBank access no. | Product size (nt) | Identities | Gaps |
|------|-------------------|------------------|------------|------|
|      | Homo sapiens:     |                  |            |      |
| NPY  | NM_000905         | 576              | 345/391 (88%) | 3/391 (0%) |
|      | Ovis aries:       | 452              |            |      |
|      | NM_001009452      |                  |            |      |
| AgRP | Homo sapiens:     | 783              | 361/439 (82%) | 8/439 (1%) |
|      | NM_001138         |                  |            |      |
|      | Ovis aries:       | 724              |            |      |
|      | XM_015100491      |                  |            |      |
| CART | Homo sapiens:     | 933              | 607/711 (85%) | 32/711 (4%) |
|      | NM_004291         |                  |            |      |
|      | Ovis aries:       | 708              |            |      |
|      | XM_015101190      |                  |            |      |
| POMC | Homo sapiens:     | 1406             | 687/819 (84%) | 42/819 (5%) |
|      | NM_000939         |                  |            |      |
|      | Ovis aries:       | 792              |            |      |
|      | NM_001009266      |                  |            |      |
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   | GUGCAUUGUAGUUGCAUU GCA | GTGCATTGTCAGTTGCATT GCA |                   |
|             | miRNA-33b-5p   | GUGCAUUGCUGUUGCAUU GC   | GTGCATTGCTGTTGCATTG C   |                   |
|             | miRNA-647      | GUGGCUGCACUCACUCC UUC | GTGGCTGCACTCACTTCCT TC |                   |
| **CART**    | miRNA-377-3p   | AUCACACAAAGGCAACUU UUGU | ATCACACAAAGGCAACTT TTGT |                   |
|             | miRNA-214-3p   | ACAGCAGGACAGACAGG CAGU | AGCAGGACAGACAGG GACG |                   |
| **POMC**    | miRNA-488-3p   | UUGAAAGGCUAUCUUUCUUG GUC | GGGAAAGGCTATTTCTTG GTC |                   |
|             | miRNA-485-5p   | AGAGGCUGGCGGAUGA AUUC | GAAAGGCTATTTCTTG GTC |                   |
Figure Legends

**Figure 1.** Scheme of the experiment.

**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: antero-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.

**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.

**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.
**ESTRUS CYCLE**

**Surgery**
- Beginning of September
- 5 weeks

**Convalescence**
- 21 days before
- icv infusions
- icv infusions

**Estrous synchronization**
- Day 0
- 4th day of estrus

**Day 0**
- 08:00 a.m. – 02:00 p.m.

**Day 1**

**Day 2**

**Day 3**
- Tissue collection

**icv infusions**
- 08:40 a.m. – 09:30 p.m.
- 10:00 a.m. – 10:50 p.m.
- 11:20 a.m. – 12:10 p.m.
- 12:40 p.m. – 01:30 p.m.
