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CROSS-TALK BETWEEN LEPTIN, GHRELIN AND OREXINS IN THE CENTRAL NERVOUS SYSTEM OF SEASONAL ANIMALS – A REVIEW*

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Abstract

The maintenance of energy homeostasis is achieved with 'detectors' that receive signals from the external and internal environment and with multidirectional 'communication routes' including neuronal networks and body fluids, such as blood and cerebrospinal fluid. Changes in the energy demands of organisms are caused by current physiological status and environmental conditions, including season and food availability. Little is known about the interactions between the metabolic indicators involved in the maintenance of energy homeostasis, e.g., leptin, orexins and ghrelin. Sheep and other seasonal animals are highly adaptable to their environments because of the plasticity of their neural and endocrine systems. Sheep exhibit leptin resistance and are thus an extremely interesting model for research on the relationship between hormonal indicators of energy metabolism. The paper is focused mainly on the anatomical and functional communication between leptin, ghrelin and orexins, which play principal roles in the adaptation of energetic demands to environmental fluctuations.

Key words: leptin, ghrelin, orexins, energy homeostasis, sheep, seasonality

In the past several years, intensive research has shown that food is an important light-independent synchronizer of mammalian biological clocks (Challet et al., 2003). These effects of food are based on complex bi-directional relationships between nerve centers located in the hypothalamus and in peripheral organs, such as the stomach and adipose tissue. Energy resources are sensed by hormonal metabolic indicators such as leptin and ghrelin. Receptors for these hormones are located on the suprachiasmatic nuclei neurons (the overriding part of the mammalian biological clock; SCN) (Guan et al., 1997; Lamont et al., 2014) as well as in the arcuate nucleus (ARC) (Ferrini et al., 2009; Li et al., 2012) (Figure 3). The ARC is directly wired

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to the SCN and the dorsomedial hypothalamus (DMH), a region considered a key hypothalamic element in the non-light synchronization of the mammalian biological clock (Landry et al., 2007). These brain structures remain in functional contact with the paraventricular nucleus (PVN) and the lateral hypothalamic area (LHA), which are important for orexin biosynthesis (Smart and Jerman, 2002) (Figure 3). The neural communication controlling the circadian rhythms of food intake is generally regulated as described above. However, in seasonal animals such as sheep or the Siberian hamster, food intake depends not only on the availability of food during the year but also on annual fluctuations in the secretion of metabolic hormones such as leptin, orexins and ghrelin, which are involved in maintaining energy homeostasis. Food intake and the annual rhythms of the production of these hormones are strictly interdependent (Clarke, 2014).

Regulation of the activity of orexin and ghrelin in the central nervous system by leptin

The role of leptin in the food intake process

Leptin, which is produced mainly in adipocytes but also in the central nervous system (CNS) and secreted into the bloodstream, is a signal describing the long-term energy stocks in an organism and is mainly active in the hypothalamus (Ahima and Flier, 2000). Leptin in the ARC stimulates a group of anorexigenic neurons, the proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated (CART) transcript neurons, to increase the secretion of α-melanocyte stimulating hormone (α-MSH) and stimulate a feeling of satiety. Leptin also inhibits the synthesis and secretion of appetite stimulants, such as neuropeptide Y (NPY) and agouti-related peptide (AgRP) (Morgan and Mercer, 2001). The signal generated in the ARC by leptin is further modulated and amplified in the PVN, DMH, and LHA, which are anatomically and functionally related to the ARC (Figure 3). Leptin inhibits the pathway responsible for hunger by decreasing orexin and melanin-concentrating hormone (MCH) synthesis in the perifornical area (pfA), LHA and DMH and increasing the activity of corticotropin-releasing hormone and melanocortin type 4 receptor expression in the PVN (Henry et al., 2000; Iqbal et al., 2001 a, b). When the energy stocks stored in adipose tissue become smaller and the concentration of leptin decreases, the NPY/AgRP neurons are activated, and POMC neurons are hampered, stimulating the acquisition and storage of energy by the animal. These anorectic actions create a sense of satiety leading to a reduction of food intake and an increase in energy expenditure caused by peripherally secreted hormones, e.g. islet amyloid polypeptide or peptide YY, or centrally secreted hormones, e.g. CART or α-MSH.

Anatomical and functional connections between leptin and orexins

Orexins (orexin A and B) belong to a group of orexigenic hormones. The effects of orexins on ingestive behavior appear to depend upon interactions with other food-related signaling systems, such as NPY, leptin, MCH, ghrelin, galanin, and agouti-related protein (Anukulkitch et al., 2010). Orexins are derived from the same precursor protein, prepro-orexin (pORX), which is expressed in a small number of perikaryons in the lateral and anterior hypothalamus (Smart and Jerman, 2002) and

increases during hypoglycemia, fasting and negative energy balance (Diano et al., 2003). Although orexin perikaryons are a relatively small group of cells, their projections are scattered in many parts of CNS, including the brain stem, hippocampus, cerebral cortex and spinal cord (Cutler et al., 1999). In sheep, orexins are expressed in the ventromedial hypothalamus (VMH), the ARC (Qi et al., 2008), the PVN (Qi et al., 2009), and the intergeniculate leaflet, which plays a key role transmitting non-photic signals to the SCN (Marston et al., 2008).

Orexin synthesis is dependent on the degree of fat cover; however, pORX expression levels and MCH gene expression in the LHA, pfA and DMH are negatively correlated with the amount of accumulated adipose tissue. Anukulkitch et al. (2009) indicated that fluctuations in the circannual availability of food and the degree of fat deposition are accompanied by changes in pORX and MCH gene expression in the LHA, DMH and pfA in ovariectomized ewes maintained under natural photoperiod conditions. Specifically, in the months when sheep have a low body fat percentage, determined by the double-beam X-ray technique, increased MCH and pORX production was observed in the hypothalamus. In contrast to these results, Iqbal et al. (2003) observed no relationship between the expression level of pORX in the hypothalamus of sheep and the quantity of amassed adipose tissue, body weight (BW) or nutritional status of the animals.

It is difficult to determine the mechanism of action of leptin on orexin neurons in small ruminants. Anukulkitch et al. (2009, 2010) reported that changes in the expression of NPY and POMC genes in the ARC, as detected by *in situ* hybridization, did not correspond with leptin receptor (LRb) gene expression or the plasma leptin concentration. There were no significant differences in POMC mRNA synthesis between groups of obese, lean and normal weight ewes, and NPY gene expression did not change significantly between the obese and control sheep. Therefore, the effect of leptin on pORX-synthesizing neurons might be independent from its influence on primary neurons located in the ARC, where NPY and POMC expression occurs. Using neuroanatomical brain tracking methods, it was observed that the nerve connections between the ARC and LHA in the hypothalamus of sheep are not as strong as they are in humans and rodents (Clarke, 2014; Elias et al., 1998; Qi et al., 2010). However, the LHA of sheep receives numerous nerve projections from other areas of the hypothalamus, including the VMH, PVN and DMH (Figure 1), which in turn remain in close functional and anatomical contact with the ARC (Qi et al., 2010). There is also evidence of a direct leptin effect on DMH, VMH and PVN activity (Figure 1) in sheep (Irani et al., 2007; Qi et al., 2009; Williams et al., 1999). Therefore, it can be presumed that the effect of leptin on neurons synthesizing orexins can occur either indirectly through the VMH, PVN and DMH or directly in the LHA beyond the ARC (Figure 3).

The possibility of a direct effect of leptin on orexin neurons in the LHA is supported by the expression of LRb and signal transducer and activator of transcription 3 (STAT-3) proteins in pORX-synthesizing cells in that area of the hypothalamus (Håkansson et al., 1999). Currently, the mechanism of orexin release by leptin in the LHA involves neurons synthesizing neurotensin (Leinninger et al., 2011). However, these results come from a study conducted on rodents. Therefore, it is necessary to

perform similar tests on sheep to obtain a reliable picture of the interactions between leptin and orexins in the LHA.

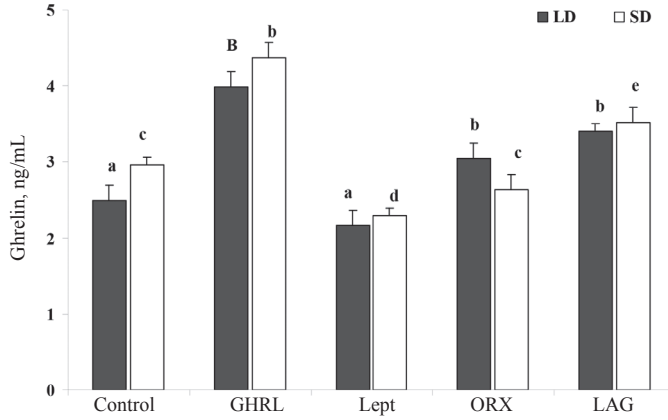

Figure 1. Mean concentrations (±SEM) of ghrelin in control and roleptin-(Lept), ghrelin- (GHRL), orexin- (ORX), and leptin antagonist and ghrelin- (LAG) treated ewes (n = 8/treatment) during short-day (ShD) and long-day (LD) photoperiods. Means without a common letter differ; lowercase letter ($P<0.05$), uppercase letter ($P<0.01$). Adapted from: Kirsz et al., 2012

"Ghrelin-leptin tango"

Ghrelin is one of the hormones that maintains energy homeostasis, and the secretion of ghrelin is regulated in part by leptin. To emphasize the close relationship between leptin and ghrelin in the regulation of energy homeostasis, appetite and body weight, Cummings and Foster (2003) coined the term "ghrelin-leptin tango", which accurately describes the nature of the interaction between these hormones.

Secretion of ghrelin mobilizes mechanisms counteracting energy shortages, stimulates the search and intake of food and influences appetitive behavior (Bagińska and Stokowska, 2006). The main sources of endogenous ghrelin are peripheral tissues and, above all, the oxyntic (parietal) cells of the stomach (Sakata et al., 2002) and the abomasum of ruminants (Hayashida et al., 2001). The mechanism of ghrelin secretion in the abomasum of ruminants has not been fully elucidated. Sugino et al. (2002, 2004) showed that the ghrelin secretion profile of sheep varies depending on the frequency of feeding and arises from sheep learning of the feeding time. Stockwell-Goering et al. (2015) reported that in the plasma of sheep subjected to short-term nutritional stress, the concentration of ghrelin increased in response to the smell, sound and sight of food alone. The above results indicate that ghrelin secretion by the autonomic nervous system is strongly affected by the CNS and is therefore not induced solely by a lack of nutrients in the intestinal tract. Ghrelin synthesis and target locations are usually distant from each other. The orexigenic and lipogenic properties of this hormone are associated mainly with NPY/AgRP neuronal activation with simultaneous, GABAergic-reversible inhibition of POMC neurons in the ARC (Kojima and Kangawa, 2005). The amount of ghrelin transported from the blood into the cerebrospinal fluid (CSF) is very low. In sheep, after 40–50 minutes

of intravenous ghrelin administration, the concentration in the blood and in the CSF increases tenfold and twofold, respectively. Grouselle et al. (2008) showed that the physiological concentration of endogenous ghrelin in the CSF in sheep is one thousand times smaller than its concentration in the peripheral circulation.

Initial reports describing the antagonistic relationship between leptin and ghrelin concerned their peripheral interactions. Experiments performed on rodents have proved that changes in the plasma concentration of ghrelin are correlated with changes to the endogenous concentration of leptin. During fasting, the ghrelin plasma concentration increases and the concentration of leptin decreases, whereas during feeding the situation is reversed (Bagnasco et al., 2002). In animals with strongly marked seasonality, this type of adjustment also translates into the storage of energy stocks in the form of adipose tissue. A good example of this phenomenon occurs in animals that hibernate, such as the golden-backed squirrel. During the months preceding hibernation, leptin secretion decreases, and ghrelin secretion, lipogenesis and food intake increase (Healy et al., 2010). Other studies confirm the existence of circadian differences in the influence of leptin on the activity of the ghrelinergic system (Finger et al., 2011).

There are only a few studies on the interaction between leptin and ghrelin in sheep. Kirsz et al. (2012) demonstrated the inhibitory effect of intracerebroventricular (ICV)-infused leptin on the plasma concentration of ghrelin in sheep during the fall-winter period. A higher dose of leptin lowered the secretion of ghrelin in sheep during both short and long photoperiods (Kirsz, unpublished data). The inhibitory effect of leptin on ghrelin secretion was apparent from experiments that administered an ovine pegylated leptin antagonist (mutant D23L/L39A/D40A/F41A; SOLA) in conjunction with exogenous ghrelin (Kirsz et al., 2012). Central infusions of SOLA significantly increased the concentration of plasma ghrelin in sheep during both seasons and resulted in increased ghrelin secretion during the LD photoperiod compared with the SD photoperiod (Figure 1). These results indicate that circannual differences in ghrelin secretion can be revealed using SOLA and emphasized the role of leptin in ghrelin secretion. Increased secretion of ghrelin after central infusions of SOLA and exogenous ghrelin might result from an increase in the expression of LRb in the ARC during the LD season (Clarke et al., 2003; Mercer et al., 2000).

Ghrelin and orexin relationship at CNS

Based on the available literature, it is difficult to determine the manner in which orexins affect ghrelin secretion in sheep. However, experiments performed on rodents indicate that a two-way exchange of information might occur between ghrelin and the orexins. Ghrelin affects orexin neurons through the activation of the NPY in the ARC and the LHA. Orexins can, by the same means, stimulate neurons in which ghrelin gene expression occurs. Experiments performed on hibernating rodents showed that exogenous orexin A and orexin B can stimulate or inhibit the activity of NPY and POMC neurons isolated from the ARC. Stimulation of NPY cells mainly involves the orexin 1 receptor, whereas inhibition of the POMC cells is mediated by the orexin 2 receptor (Muroya et al., 2004). Another potential interaction between ghrelin and orexins likely occurs in the dopaminergic system in the

midbrain. Interactions between ghrelin and orexins in that area of the brain primarily control locomotor activity (Kaur et al., 2008), waiting for feeding time (Lamont et al., 2012) and the feeling of pleasure (Perello et al., 2010). Protrusions of orexin neurons also contact other brain structures, including the DMH, PVN and the nucleus tractus solitarius. In these areas of the CNS, Fos protein expression increases as a result of central injections of ghrelin (Olszewski et al., 2003). Therefore, it should be assumed that interactions between ghrelin and orexins occur in many centers of the CNS. However, research confirming these observations in larger mammals, such as sheep, is needed. The data on sheep highlight the possibility of a two-way dependency between ghrelin and orexins that intensifies during the LD season. The weakened impact of both peptides on the regulation of their mutual secretion during the SD season probably stems from the adapted physiology of seasonal animals to environmental conditions. This is particularly important in the winter when animals expend significant energy in search of food. Therefore, a reduced CNS response to orexigenic hormones and a hypothalamus that is more sensitive to anorexigenic hormones, such as leptin, allows animals to survive periods with limited access to natural food. Such regulation leads to a reduction in the metabolic rate, which in sheep drops by approximately 25% during the SD season, a time when the environment offers limited possibilities of obtaining energy (Argo et al., 1999).

Effects of photoperiod on leptin, ghrelin and orexin interactions

Circadian and circannual rhythms of orexin secretion

Archer et al. (2002) reported that in small ruminants, the photoperiod is the main factor that interferes with orexin synthesis, and they noted an increase in pORX expression levels in the LHA during a short day (SD; 8 h of light: 16 h of dark) and a decline in pORX expression in the LHA during a long day (LD; 16 h of light: 8 h of dark). This was confirmed for sheep fed *ad libitum* and those subjected to nutritional restrictions. However, as demonstrated on Figure 2 leptin administered to sheep three times ICV inhibited the secretion of orexin B during both the fall and the spring (Kirsz et al., 2012). Although the aforementioned experimental data are ambiguous, they indicate that the photoperiod and leptin are factors affecting orexin synthesis in sheep.

Effect of melatonin on the interaction between ghrelin and leptin

Experiments performed on rams, wethers (Anukulkitch et al., 2007) and lean ovariectomized ewes (Kurose et al., 2005) during the LD season showed increased LRb expression together with an accompanying increase in the expression of NPY, ghrelin and the ghrelin receptor (GHSR; Growth Hormone Secretagogues Receptor) in the ARC. This was a surprising result due to the well-described antagonistic relationship between leptin, NPY and ghrelin and suggests that leptin has a limited impact on the activity of these proteins during an LD season. This is likely caused by a weakened leptin signal resulting from increased activity of suppressor of cytokine signaling 3 (SOCS-3) in the hypothalamus of sheep at that time. During LD, sheep experience natural leptin resistance because of autosuppression that is caused by SOCS-3 expression, which is stimulated by leptin (Zieba et al., 2008).

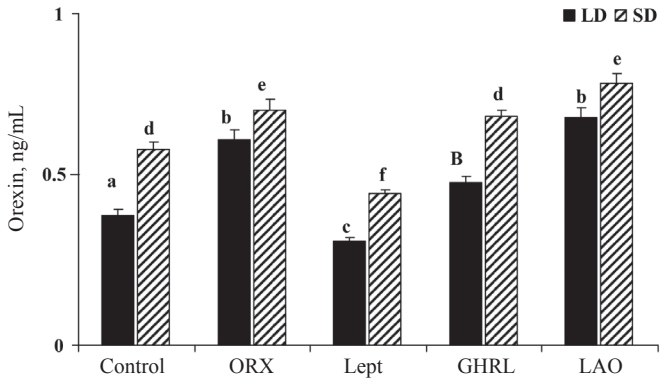

Figure 2. Mean (±SEM) concentrations of orexin in control and roleptin-(Lept), orexin- (ORX), ghrelin- (GHR), and leptin antagonist and orexin- (LAO) treated ewes during short- (SD) and long-day (LD) photoperiods. Denotes a difference * ($P<0.05$) within groups. Different letters indicate statistical differences between groups. Adapted from Kirsz et al., 2012

Another factor that might interfere with leptin and ghrelin interactions in sheep is melatonin, a biochemical signal of day length changes (Zieba et al., 2011). Studies performed on the Siberian hamster indicated that exogenous melatonin decreases the concentration of leptin (Korhonen et al., 2008). The stimulatory effects of melatonin on the concentration of leptin in the bloodstream have been reported in seasonally reproducing mink (*Mustela vision*) with melatonin implants during the fall season (Mustonen et al., 2000). In sheep, the daily relationship between melatonin and leptin has the features of a positive dependency. The concentrations of both melatonin and leptin increase at night and decrease throughout the day (Bertolucci et al., 2005). An experiment performed on sheep perirenal adipose tissue explants incubated in culture medium maintained in additional melatonin confirmed a tendency to stimulate leptin secretion by exogenous hormone during a shortening day. In one of the test months (July), melatonin appeared to have an inhibitory effect on leptin secretion (Szczesna et al., 2011). Thus, it can be assumed that melatonin in sheep has a stimulating effect on the secretion and activity of leptin during the SD season, whereas during the LD season, the weaker melatonin signal might cause a reduction in leptin activity.

Ghrelin signaling mediates appetite through lateral hypothalamic orexin pathways: the role of photoperiod

After leptin, ghrelin is the second factor that affects the level of orexin secretion in the plasma of sheep. Triple intraventricular infusions of ghrelin (Figure 2) significantly stimulated the secretion of orexin B during an extended day season (Kirsz et al., 2012). The nature of ghrelin interaction might be associated with circannual changes in the neuroendocrine activity of the hormone in sheep, which to date are mainly considered to regulate the somato-, lacto- and gonado-tropic axes (Harrison et al., 2008; Zieba et al., 2015). Harrison et al. (2008) observed for the first time

that depending on the photoperiod, ghrelin administered centrally to wethers stimulates food intake, modulates growth hormone (GH) secretion and inhibits the release of reproductive axis. Ghrelin injections to the ventricle (IIIV) of the brain caused a temporary increase in food intake during the LD season, which was not observed during the SD season. Interestingly, Kirsz et al. (2012) confirmed that sheep showed an increase in the concentration of endogenous ghrelin after central administration of orexin B and that this concentration was higher during the LD season than the SD season (Figure 2). The reciprocal control of the secretion of ghrelin and orexin B might have metabolic consequences enhancing the individual biological functions of both hormones. It is possible that the interaction between ghrelin and orexin B during the spring and summer seasons is associated with their orexigenic activity and affects seasonal food intake in sheep during the spring and summer, which are months that are characterized by a large abundance of natural food. Existing data in the literature confirm the effects of photoperiod on ghrelin-induced stimulation of appetite in seasonal animals (Bradley et al., 2010; Harrison et al., 2008). For instance, ICV infusions of exogenous ghrelin in sheep increased their *ad libitum* food intake during an LD (16 h light: 8 h darkness) but did not affect appetite during an SD (8 h light: 16 h darkness) (Harrison et al., 2008). Similar observations were made in Siberian hamsters, in which the temporary effects of exogenous ghrelin were manifested during an LD (15 h light: 9 h darkness) (Bradley et al., 2010). Iqbal et al. (2006) applied either chronic or disposable central infusions of ghrelin in sheep during the SD photoperiod and noted no changes in food intake among these animals. Lack of a noticeable ghrelin effect on the appetite of sheep during a shortening day might be associated with increased leptin activity because studies on small ruminants have demonstrated that leptin reduces appetite during the SD season and stimulates it during the LD season (Adam et al., 2006; Miller et al., 2002).

Experimental data on the relationship between ghrelin and orexins relate primarily to their central control of food intake and the stimulation of behavior connected with searching for food (Lamont et al., 2012; Perello et al., 2010). It was found that axons of neurons synthesizing orexins and ghrelin come into synaptic contact in the ARC and LHA (Olszewski et al., 2003; Toshinai et al., 2003). Under the influence of exogenous ghrelin, there was a 23% increase in the expression of Fos protein on orexin neurons in the LHA, but a similar effect was not observed in MCH neurons, which constitute the second important population of cells in the LHA (Toshinai et al., 2003). Similar results were reported in a study using ICV infusions of ghrelin receptor agonist (GHRP-6) (Lawrence et al., 2002). In addition, *in vivo* experiments showed that central infusions of anti-orexin A and anti-orexin B antibodies into the IIIV inhibited ghrelin-stimulated food intake (Toshinai et al., 2003) in mice. Injections of either the agonist of the Y1 NPY receptor and ghrelin or anti-orexin A and B antibodies reduced the exogenous ghrelin-induced appetite by 41% and 87%, respectively (Toshinai et al., 2003). Thus, it can be concluded that orexins and ghrelin have interdependent anatomical functions and NPY might mediate communication between them. Observations performed under *in vitro* conditions revealed that exogenous ghrelin also directly affected orexin neurons isolated from the LHA, triggering their depolarization and increasing their excitability (Yamanaka et al., 2003).

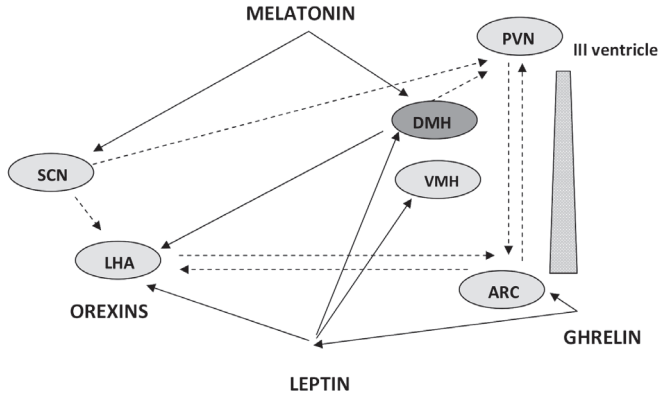

Figure 3. Schematic representation of potential hypothalamic sites of interaction between photoperiod (melatonin) and leptin, ghrelin and orexins (A and B) signaling. Melatonin receptors are localized in the suprachiasmatic nuclei (SCN) and dorsomedial hypothalamus (DMH), and leptin and ghrelin receptors are localized in the arcuate nucleus (ARC), DMH and lateral hypothalamic area (LHA) together with orexins receptors. Connections exist, as shown, between these hypothalamic nuclei, and it is speculated that feedback from melatonin and the other three hormones may ultimately be integrated within the DMN. Potential primary sites of action of the hormones melatonin and leptin, ghrelin and orexins; potential secondary communications between the hypothalamic nuclei

Crossroads

Studies of the roles of leptin, ghrelin and orexins in metabolic control have demonstrated that all of these hormones react differently to adapt to challenging food availability. However, these hormones are not isolated from one another, and many studies have noted crosstalk between these hormones. In sheep, the photoperiod is involved in the interactions between ghrelin, leptin and orexins, although there are little data in the literature confirming these observations. However, in both Siberian hamsters (Bradley et al., 2010) and sheep (Clarke et al., 2003; Polkowska et al., 2012), a day length- and nutritional status-dependent influence of ghrelin on the activity of the NPY neurons was observed. Accounting for a role of NPY in leptin regulation and as a connector between ghrelin and orexins, it can be presumed that the circannual changes in NPY activity might, to some extent, be an expression of changes in the relationships between these hormones. Experiments on Siberian hamsters fed normally revealed that after exogenous ghrelin treatment, the immune reactivity of the Fos protein on NPY neurons in the ARC increased, and this effect was significant only during the LD season (15 h light: 9 h darkness) (Bradley et al., 2010). In a study of Soya rams, a fourfold increase in food intake was accompanied by increased expression of NPY mRNA in the ARC during the LD season (Clarke et al., 2003). In ICV ghrelin-infused lambs, NPY mRNA expression in the VMH did not change after 72 hours of food restriction (Polkowska et al., 2012).

Conclusions

Intensiveness of research provided to explain the relationships between hormones engaged in regulation of energy homeostasis and metabolism, due to the im-

portance of these interactions and processes controlled by them. Those studies are conducted not only for cognitive purposes, but also in practical terms, for treatment of pathological phenomena associated with endocrine dysfunction in humans and animals or relating to the economic viability of farming. Due to the strict adaptation to environmental conditions related to the plasticity of the endocrine system, as well as the presence of many physiological phenomena, seasonal animals are a very interesting model in such studies. Observations mentioned above emphasize close relationship that exists between photoperiod, a powerful factor which influences the course of many processes in them, its main biochemical indicator – melatonin and peptides involved in the regulation of energy homeostasis such as leptin, ghrelin and orexins A and B.

Declaration of interest

The authors have no conflicts of interest to declare.

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