INTRODUCTION

Orexins A and B, also known as hypocretin 1 and 2, are neuropeptides derived from a common 130-amino acid precursor peptide, prepro-orexin (PPO), by proteolytic cleavage. They were simultaneously discovered by two independent research groups (1, 2). Orexin A is a 33-amino acid residue peptide with two intramolecular disulphide bonds in the N-terminal region, whereas orexin B is a linear 28-amino acid residue peptide. The discussed peptides have a 46% sequence identity (1). In the investigated vertebrates, the main source of orexins is the lateral hypothalamic area (1, 2), the brain structure that controls energy homeostasis (3). Both orexins act via two closely related G-protein coupled receptors, orexin type 1 receptor (OX1R) and orexin type 2 receptor (OX2R). OX1R appears to be selective for orexin A, while OX2R binds both orexin A and orexin B (2). Neurons containing orexins project to multiple neuronal systems (4). Those hypothalamic peptides and their receptors are widely distributed in the central nervous system and peripheral organs, which suggests their pleiotropic effects. Those proteins are important factors in the regulation of food intake, metabolic rate, cardiovascular function, body temperature, locomotor activity, sleep-wake cycle and stress response (2, 5-11).

Orexins have also been found to affect the reproductive neuroendocrine system, mainly in rats (12-15). Considerable body of evidence has implicated the existence of a common endocrine system that controls metabolism and reproductive functions. The effect of leptin and ghrelin on the organism's metabolic status and reproductive function has been observed (16, 17). Orexins seem to belong to the above group of hormones which regulate metabolic status and reproduction but our knowledge of orexins’ involvement in the control of the female reproductive system is fragmentary and focused mainly on their direct effects on the reproductive system, including the ovaries (18, 19) and the uterus (20). Several studies discussed orexins’ influence on pituitary function (21-23). Further work is needed to investigate the presence of orexins in the highest branch of hypothalamus-pituitary-gonadal axis in sexually mature pigs. To our best knowledge, there is a general scarcity of data, that focus on hypothalamic structures involved in GnRH production and secretion: the mediobasal hypothalamus (MBH), with the...
arcuate nucleus, which is considered to be GnRH pulse generator (26), preoptic area (POA), with the anterior medial preoptic nucleus, which participates in the control of the oestrous cycle in rats (27), and stalk median eminence (SME), where about 1000 neurons in mammals release GnRH peptide from neuroterminals in this structure (28). Additionally, there are limited data concerning the possible influence of the hormonal status on the orexin expression pattern in porcine hypothalamic structures responsible for GnRH production and secretion. Thus, the present study was designed to compare the expression levels of porcine PPO mRNAs by quantitative real-time PCR and the expression levels of orexin A and orexin B proteins by fluorescence immunohistochemistry in the indicated areas of the porcine hypothalamus during the oestrous cycle.

MATERIALS AND METHODS

Animals and tissue collection

The studies were carried out in accordance with ethical standards of the Animal Ethics Committee at the University of Warmia and Mazury in Olsztyn. Mature gilts (Large White x Polish Landrace), 7–8 months old, weighing 120–130 kg, descended from private breeding were used. All gilts were slaughtered during the autumn, therefore day length had no effect on mRNA expression. Diets were balanced (crude protein, metabolisable energy, exogenous amino acids and minerals) in accordance with the nutrient requirements of domestic pigs. Individuals were given free access to water. Twenty gilts were assigned to one of four experimental groups (n=5 per group) as follows: days 2–3, 10–12, 14–16, and 17–19 of the oestrous cycle. The analyzed days are representative of the stages of the oestrous cycle characterized by major hormonal and physiological changes observed throughout the cycle.emales were monitored daily for oestrus behaviour in the presence of an intact boar. The day of the second oestrus onset was designated as day 0 of the oestrous cycle. The phase of the oestrous cycle was also confirmed on the basis of ovarian morphology, highly characteristic ovarian morphology was noted in each of the analysed periods with the presence of corpora haemorrhagica on days 2–3, fully active corpora lutea on days 10–12, corpora lutea with clear signs of luteolysis on days 14–16, and only corpora albicantia with preovulatory follicles on days 17–19 (29). Within a few minutes after the slaughter the hypothalamus has been dissected. Each hypothalamic block was divided into the preoptic area, medial basal hypothalamus and stalk median eminence. The medial basal hypothalamus was defined as a block of tissue bounded rostrally by the optic chiasma, caudally by the mammillary body, laterally by the hypothalamic sulci and dorsally by the cut 5 mm deep. The preoptic area was limited rostrally approximately 5 mm anterior to the optic chiasma and caudally by the rostral border of the medial basal hypothalamic area. The stalk median eminence was easily detached from the basal hypothalamus without cutting and was the cut away at its junction with the pituitary gland (30). All the samples were frozen in liquid nitrogen and stored at −80°C until processing for RNA and protein analysis.

Quantitative real-time PCR

Quantitative real-time PCR analysis was carried out using a PCR System 7300 (Applied Biosystems, USA). The PCR reaction included 100 ng cDNA, 0.5 µM primers, 25 µl SYBR Green PCR Master Mix (Applied Biosystems, USA), and RNase free water in a final volume of 50 µl. The primers for PPO gene were cited: 5'-AAG ACA GCA CCC TTC CTG GAG AC-3' as a sense primer, and 5'-TGA TTG CCA GCG CCG TGT AGC A-3' as an antisense primer. GAPDH gene, sense: 5'-CCT TCA TTG ACC TTC ACT ACA TGG T3-3', and antisense: 5'-CCA CAT ACG TAG CAC CAT C-3'. Preproorexin primers (access no: EF434655) were complementary to positions 41–63 (F) and 240–261 (R) of the pig PPO gene sequence and GAPDH primers (access no: U48832) encompassed positions 61–85 (F) and 219–243 (R) of porcine GAPDH gene sequence. A constitutively expressed gene, GAPDH, was used as the internal control to verify the quantitative real-time PCR. Real-time PCR cycling conditions were as follows: enzyme activation and initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing at 59°C for 1 min. Negative controls were performed in which water was substituted for cDNA, or reverse transcription was not performed prior to PCR. All samples were amplified in duplicate. The specificity of amplification was tested at the end of the PCR by melting-curve analysis. Product purity was confirmed by electrophoresis. Calculation of relative expression level of PPO gene was conducted based on the comparative cycle threshold method (ΔΔCt) (31). Expression of PPO mRNA was calculated by the equation 2−ΔΔCt, where ΔCt was obtained by subtracting the corresponding GAPDH Ct value from the specific Ct of PPO, and ΔΔCt was determined by subtracting the ΔCt of each experimental sample from ΔCt of the reference sample, called the calibrator (the tissue with the lowest expression).

Sequence analysis

PCR-amplified DNA was electrophoresed on 1.5% agarose in Tris-acetate buffer. After isolation from gel, DNA was sequenced (ABI Prism® Big DyeTM Terminator Cycle Sequencing Kit, ABI Prism 3777 DNA sequencer, USA) in both directions to confirm the accuracy of amplification. Comparison of the PCR-amplified DNA sequences to those in the database indicated 100% homology at the nucleotide level.

Fluorescent immunohistochemistry

MBH, POA and SME were cut into 10 µm thick sections using cryostat CM3050 (Leica, USA). Frozen sections of the examined tissues after reaching room temperature were fixed in 4% paraformaldehyde. To decrease nonspecific binding, sections were blocked in 10% normal goat serum (Sigma-Aldrich, USA) diluted in 0.01 M PBS with 0.1% bovine serum albumin (BSA, Sigma-Aldrich, USA) and 1% triton X-100 (Sigma-Aldrich, USA) for 1 hour at room temperature (RT). Then tissues were left in a moist chamber overnight at 4°C with primary antibodies: rabbit anti-orexin A or rabbit anti-orexin B at 1:50 dilution (Phoenix Pharmaceuticals, Inc., USA). The following day, the sections were incubated with biotinylated anti-rabbit IgG (Vector Laboratories, USA) at 1:100 dilution for 1 hour at RT. Next, the slides were incubated with fluorescein streptavidin complex (Vector Laboratories, USA) at 1:50 dilution in 0.01 M PBS for 1 hour at RT. Sections were also stained with propidium iodide for visualization of cell nucleus. For negative control the primary antibody was omitted and the tissues were incubated in 0.01 M PBS or rabbit universal negative control (Daco Cytomation, Denmark). The slides were mounted in fluorescent medium (Sigma-Aldrich, USA).

The intensity of fluorescence immunoreaction was analysed on each section. Images were captured using an fluorescence microscope, using a dual filter cube for FITC and TRITC (Olympus BX 51, Japan) attached to the digital camera, with a high-sensitivity charge coupled device (CCD) to provide a sufficient rate of the quantitative linearity (Olympus DP 72, Japan). The measurement analysis was conducted with corresponding software, as previously described by Khan et al.
(32), and then analysed using cellF Imaging Software for Life Science Microscopy, which is highly recommended by Olympus. Phase analysis was a quantitative evaluation of area with regard to each separate gray value. The image acquiring to analysis was defined by converting images to gray value ranges for the separate phases, the analysis was conducted on a gray-value images. The thresholds were automatically calculated via the image histogram. The spectral data were automatically converted to optical density (OD) units by taking the negative log of the transmitted image divided by the illumination. Ten pictures of the stained tissue were taken randomly from each coverslip. Each measuring area was a square of side 20 µm. Data from each coverslip were archived, analyzed and expressed as the intensity staining (arbitrary units; range: 0–255).

**Data analysis**

All data were analyzed by two-way ANOVA and least significant difference (LSD) post hoc test and are reported as the means ±S.E.M. from five independent observations. Two-way ANOVA indicated that, for both orexin A and B, the differences due to phase of the oestrous cycle and kind of the examined tissues, and the interaction between the phase and the tissue, were significant (p<0.05). Statistical analyses were performed with regard to each separate gray value. The image acquiring was defined by converting images to gray value ranges with the exception of the porcine hypothalamus (Fig. 4C). In POA, the highest signal intensity was observed in SME and lowest in MBH (Fig. 5A-D).

**RESULTS**

**Quantitative real-time PCR**

In the three examined hypothalamic structures, namely MBH, POA and SME, changes in PPO mRNA expression were observed in all structures throughout the oestrous cycle. The lowest PPO expression levels in MBH were noted on days 2–3 of the oestrous cycle. Higher (p<0.05) transcript content was observed in the remaining three stages of the cycle, with no significant differences between the phases (Fig. 1A). In POA, the expression of PPO mRNA was highest on days 17–19 (p<0.05 compared to days 14–16) (Fig. 1B). The highest levels of PPO expression in SME were reported on days 2–3 of the oestrous cycle, and the lowest on days 14–16 (p<0.05) (Fig. 1C).

A comparison of PPO mRNA levels in MBH, POA and SME during the four examined stages of the cycle revealed the highest transcript concentrations in MBH (days 10–12, 14–16, 17–19) (p<0.05). On days 2–3, PPO mRNA expression peaks were observed in both MBH and SME (p<0.05) (Fig. 2A-2D).

**Fluorescent Immunohistochemistry**

Orexin A protein was detected in all examined structures of the porcine hypothalamus (Fig. 3). OXA immunoreactivity changed during the oestrous cycle: the highest signal intensity in MBH was noted on days 14–16 and the lowest on days 17–19 (p<0.05) (Fig. 4A). The highest immunoreactivity levels in POA were observed on days 2–3 and 10–12 (Fig. 4B). In SME, OXA immunoreactivity was lower on days 14–16 (p<0.05), in comparison with the remaining phases of the cycle (Fig. 4C).

A comparison of OXA immunoreactivity in the three examined structures of the hypothalamus points to the highest signal intensity (p<0.05) in POA and SME on days 2–3 and 10–12 of the cycle. During luteolysis (days 14–16), the highest signal intensity was observed in POA and MBH (p<0.05), whereas in the follicular phase (days 17–19), immunoreactivity was highest in SME (p<0.05) and lowest in MBH (Fig. 5A-5D).

Similarly to OXA, the presence of orexin B protein was observed in all examined structures of the porcine hypothalamus (Fig. 6). In MBH, OXB immunoreactivity was highest on days 10–12 of the oestrous cycle and lowest during the follicular phase (p<0.05) (Fig. 7A). In POA, the highest signal intensity was noted on days 14–16, and the lowest on days 10–12 (p<0.05) (Fig. 7B). In SME, immunoreactivity increased gradually during the oestrous cycle, and the highest values were observed in the remaining three stages of the cycle, with no significant differences between the phases (Fig. 1A). In POA, the expression of PPO mRNA was highest on days 17–19 (p<0.05 compared to days 14–16) (Fig. 1B). The highest levels of PPO expression in SME were reported on days 2–3 of the oestrous cycle, and the lowest on days 14–16 (p<0.05) (Fig. 1C).

A comparison of PPO mRNA levels in MBH, POA and SME during the four examined stages of the cycle revealed the highest transcript concentrations in MBH (days 10–12, 14–16, 17–19) (p<0.05). On days 2–3, PPO mRNA expression peaks were observed in both MBH and SME (p<0.05) (Fig. 2A-2D).

**Fig. 1.** Prepro-orexin (PPO) mRNA content, determined by quantitative real-time PCR, in porcine mediobasal hypothalamus (A), preoptic area (B) and stalk median eminence (C) at four different stages of the oestrous cycle. Results are means ±S.E.M. (n=5). Bars with different superscripts are significantly different. Small letters indicate p<0.05.
Fig. 2. Comparison of prepro-orexin (PPO) gene expression between mediobasal hypothalamus (MBH), preoptic area (POA) and stalk median eminence (SME) at different stages of the cycle - on days 2–3 (A), 10–12 (B), 14–16 (C), 17–19 (D) of the oestrous cycle. Results are means ±S.E.M. (n=5). Bars with different superscripts are significantly different. Small letters indicate p<0.05.

Fig. 3. Immunofluorescence localization of orexin A (green) in mediobasal hypothalamus (MBH), preoptic area (POA) and stalk median eminence (SME) of the porcine hypothalamus during the oestrous cycle. Shown in red: cell nuclei stained with propidium iodide. Right upper corners: negative controls with rabbit non-immune immunoglobulins (rabbit universal negative control). Left upper corners: enlarged image of neuron cell bodies (magnification: ×4000) with a positive orexin A immunoreactivity. Scale bar =100 µm; images are representative of n=5.
noted in the follicular phase (p<0.05 in comparison with days 2–3 and 10–12) (Fig. 7C).

A comparison of OXB immunoreactivity in three structures of the porcine hypothalamus acquired during four stages of the oestrous cycle revealed the highest (p<0.05) signal intensity in MBH, whereas the lowest intensity was noted in SME (days 2–3, 14–16 and 17–19) or in POA and SME (days 10–12) (Fig. 8A-8D).

DISCUSSION

The results obtained on the basis of PPO mRNA as well ir-OXA and ir-OXB presence in the porcine hypothalamic structures involved in reproductive processes - MBH, POA, SME may suggest that these structures can be a source of orexin A and orexin B. We also observed variations in prepro-orexin gene expression and the immunoreactivity pattern of both hormones subject to the phase of the oestrous cycle. The expression levels of the prepro-orexin gene are higher in POA and MBH during the follicular phase, whereas the highest PPO mRNA expression levels were reported in SME in the early luteal phase. Differences were noted in the expression patterns of the prepro-orexin gene and the analyzed proteins. Those variations could be attributed to the distinct half-life of orexin A and orexin B proteins derived by proteolytic processing of their common precursor - PPO (2). Our results concerning PPO mRNA expression are partially corroborate of Ning et al. (33)
who studied Suzhong sows and noted the highest mRNA expression of PPO in the hypothalamus (investigated as a whole structure, without dividing into MBH, POA, SME) during proestrus.

Similarly to our studies, orexin A immunoreactive fibers were localized in SME and MBH of the rat hypothalamus (34). These findings were not fully validated by the work of Cutler et al. (35) who did not observe the discussed protein in the median eminence of rodents. In two studies, porcine orexin A has been localized within tissues, using immunohistochemical staining. Li et al. (36) observed orexin-like immunoreactive cells in the testes, adrenal gland, pancreas, small intestine, thyroid gland and thymus. The same research team also investigated orexin A protein in the pig hypothalamus. The authors noted the presence of orexin-like immunoreactive neurons in a number of hypothalamic nuclei (25). Orexin B protein was immunohistochemically localized by Su et al. (37, 38) within the hypothalamus, mainly in the perifornical nucleus, the lateral hypothalamus, the dorsomedial nucleus and the posterior hypothalamus, but with a sparser distribution in the preoptic area, which confirmed our findings with regard to orexin B localization in POA. The results of our earlier work indicate that type 1 and type 2 receptors are also expressed in the porcine MBH, POA and SME at both gene and protein levels (39). The above suggests that OXA and OXB could directly influence hypothalamic structures.

Type 1 and 2 orexin receptors were found in the porcine hypothalamus, pituitary and ovaries, and differences in their expression patterns were noted during the oestrous cycle (39, 21, 19). The expression of OX1R and OX2R genes in selected regions of the porcine hypothalamus was enhanced during the follicular phase (39). Those findings suggest that the hormonal milieu affects the orexinergic system. Other observations further support the above hypothesis. Gender-dependent differences pointed to the presence of significant correlations between orexin expression and hormonal status. Sexual

Fig. 6. Immunofluorescence localization of orexin B (green) in mediobasal hypothalamus (MBH), preoptic area (POA) and stalk median eminence (SME) of the porcine hypothalamus during the oestrous cycle. Shown in red: cell nuclei stained with propidium iodide. Right upper corners: negative controls with rabbit non-immune immunoglobulins (rabbit universal negative control). Left upper corners: enlarged image of neuron cell bodies (magnification: ×4000) with a positive orexin B immunoreactivity. Scale bar =100 µm; images are representative of n=5.
dimorphism is manifested by the expression levels of prepro-orexin and type 1 orexin receptor in rats. The mRNAs expression levels are significantly higher in females than in males (40, 41). Pregnancy can also affect the orexinergic system, but the existing body of research provides contradictory data regarding orexin expression in pregnant and nonpregnant rats. Kanenishi et al. (42) observed higher levels of PPO mRNA in the brains of pregnant than nonpregnant rats, whereas contradictory results were reported by Garcia et al. (43). The expression and concentrations of orexins and their receptors was also investigated in studies of hypothalamic and pituitary tissues during the oestrous cycle, mostly in rats. In the work of Porkka-Heiskanen et al. (44), the concentrations of hypothalamic OXA in rats were higher during proestrus than diestrus and oestrus in young cycling animals. Contrary to the above, Russell et al. (45) have shown that the levels of hypothalamic orexin A were lowest on the day of proestrus and that hyperestrogenization of female rats reduces orexin A concentrations in the hypothalamus. The above findings imply that estrogens have the most potent effect on the central orexinergic system among all ovarian steroids.

The functions of orexins in the hypothalamus has been investigated mainly in the lateral hypothalamus (1, 2).
Orexins’ role in hypothalamic areas involved in reproduction is still poorly understood. The study by Campbell et al. (46) indicated that approximately 80% of GnRH neurons are connected with orexin neuronal fibers and 85% of GnRH neurons express both types of orexin receptors. In the ovine hypothalamus, 30% of the examined GnRH cells had orexin immunoreactive terminals with no regional or sex differences (47). Orexins’ direct influence on reproductive functions is not widely described, but they seem to have an evident effect on LH and GnRH secretion, even though the studies investigating orexins as regulators of GnRH and LH release produced discrepant results. An in vitro study by Su et al. (38) on prepubertal Suzhong pigs demonstrated that orexin B significantly increased GnRH and LH secretion. Similar results were obtained by Barb & Matteri (22), in their experiment orexin B stimulated the secretion of basal and GnRH-induced LH from the pituitary cells of prepubertal gilts. In another study on ovariectomized Suzhong gilts, orexin A transiently promoted LH secretion, also upregulated GnRH expression in the hypothalamus and upregulated LHβ and FSHβ mRNAs expression in the pituitary (48). In the aforementioned earlier study by Porkka-Heiskanen et al. (44), high OXA concentrations in the rat hypothalamus were correlated with luteinizing hormone and prolactin surge from the pituitary. Similar findings were reported by Russell et al. (45), in whose study the release of orexin A at proestrus enhanced GnRH secretion from hypothalamic explants of female rats cultured in vitro. Additionally, GnRH mRNA expression in hypothalamic GT1-7 cells was enhanced by orexin A (49). On the other hand, it should also be noted that orexin A suppressed the pulsatile secretion of LH via β-endorphin in ovariectomized rats (50). The orexin-stimulated production of GnRH and LH in the hypothalamus could have a bimodal effect - orexin A stimulated the release of GnRH and LH in the rostral POA and limited their secretion levels in the medial POA and SME (12). It has been suggested that orexins inhibited LH release through the CRH system (51) apart from mentioned β-endorphin LH suppression. When administered intracerebroventricularly, orexins A and B stimulated LH release in ovariectomized rats pretreated with progesterone and oestradiol benzoate and suppressed LH secretion in unprimed ovariectomized rats (52). Thus, orexin’s ability to affect GnRH secretion and, consequently, LH release seems to be evident.

CONCLUSION

The results of our study imply that orexins A and B are produced in the porcine hypothalamus and that their concentrations vary subject to hormonal status of the pig. The present study confirmed PPO mRNA expression in MBH, POA and SME. Furthermore, our findings supported the localization of orexins A and B in the above porcine hypothalamic structures. However, to fully confirm those structures as areas of orexin synthesis, the peptides should be indicated in the cell bodies in more precise future studies. The new data suggest that orexins may act directly on GnRH neurons in the investigated structures. Our findings imply that orexins may affect reproductive functions at the highest level of the hypothalamic-pituitary-gonadal axis.

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