The effect of the estrous cycle on the expression of prepro-orexin gene and protein and the levels of orexin A and B in the porcine pituitary

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Hypothalamic peptides orexin A (OXA) and orexin B (OXB) are derived from the proteolytic cleavage of a common precursor molecule, prepro-orexin (PPO). They act via two orexin receptors (OX1R and OX2R), which belong to the G-protein coupled receptor superfamily. Orexins are implicated in the regulation of arousal states, energy homeostasis and reproductive neuroendocrine function. The objective of this study was to investigate the presence and changes in orexin expression in the porcine pituitary during the estrous cycle. Adenohypophysis (AP) and neurohypophysis (NP) tissue samples were harvested on days 2 to 3, 10 to 12, 14 to 16, and 17 to 19 of the estrous cycle. The expression of the PPO gene increased in AP and NP during the estrous cycle. The highest PPO protein concentrations in AP were reported on days 2 to 3 (P < 0.05), and in NP – on days 10 to 12 and 17 to 19 (P < 0.05). The expression of PPO mRNA was lower in AP than in NP, but PPO protein levels were higher in AP. In AP, OXA immunoreactivity was higher (P < 0.05) on days 10 to 12 and 14 to 16. In NP, the highest (P < 0.05) content of the analyzed protein was observed on days 10 to 12 and the lowest (P < 0.05) – on days 14 to 16 and 17 to 19. OXB immunoreactivity in AP reached the highest level (P < 0.05) on days 2 to 3, and the lowest level (P < 0.05) was determined on days 10 to 12 and 17 to 19. OXB protein concentrations in NP peaked (P < 0.05) on days 10 to 12 of the cycle. Our study was the first experiment to demonstrate the expression of the orexin gene and orexin proteins in the porcine pituitary and the correlations between expression levels and the phase of the estrous cycle.

Keywords: prepro-orexin, orexin A and B, pituitary, estrous cycle, pig

Implications

The relationship between nutritional status and reproductive success in animals has been studied for many years. Considerable evidence has been accumulated to implicate the existence of a common endocrine system that controls metabolism and the reproductive system. It is suggested that orexins belong to the group of hormones, which regulate metabolic status and reproduction. In this study, we demonstrated differences in orexin gene and orexin proteins in the porcine pituitary during the estrous cycle. The results imply the impact of hormonal milieu on orexins’ expression in the porcine pituitary. A sound knowledge of mechanisms that control energy homeostasis and reproduction is needed in animal breeding practice.

Introduction

Orexin A and B (OXA, OXB), also known as hypocretin 1 and 2, are hypothalamic peptides with a 46% sequence homology. OXA is a 33-amino acid peptide with a molecular mass of ~3.5 kDa, which has an N-terminal pyroglutamyl residue and C-terminal amidation. OXB is a 28-amino acid peptide with a molecular mass of ~2.9 kDa and C-terminal amidation. Orexins are cleaved from a 130-amino acid common precursor molecule, prepro-orexin (PPO), which is synthesized mainly in neurons inside and in the area of the lateral and posterior hypothalamus. Orexin action is mediated by two different receptors, termed orexin receptor 1 (OX1R) and orexin receptor 2 (OX2R), both coupled with protein-G. OXA is a more selective ligand for OX1R, whereas OX2R binds both orexins with similar affinity (De Lecea et al., 1998; Sakurai et al., 1998). The orexins

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were originally identified as regulators of food intake and energy homeostasis (Sakurai et al., 1998) and later as key modulators of the sleep-wake cycle and arousal (Chemelli et al., 1999), but multiple lines of evidence also demonstrate that orexins participate in the control of different endocrine axes, including the hypothalamic–pituitary–adrenal axis (Voisin et al., 2003; Spinazzi et al., 2006) and the hypothalamic–pituitary–ovarian axis (Silveyra et al., 2007a and b; Nitkiewicz et al., 2010; Kaminski et al., 2010a and 2010b).

Orexins regulate the secretion of pituitary hormones by acting on hypothalamic neurons and/or directly on the pituitary. It has been found that ~30% of GnRH neurons in pigs (Su et al., 2008) and 75% to 85% in rats were contacted by orexin fibers and that 85% of GnRH neurons coexpressed OX1R (Campbell et al., 2003). OXA was shown to stimulate the secretion of corticotropin-releasing hormone (CRH) and GnRH from rat hypothalamic explants in vitro (Russell et al., 2000; Small et al., 2003). Moreover, OXB enhanced the release of basal and GnRH-stimulated LH from porcine pituitary cells (Barb and Matteri, 2005), whereas OXA suppressed the secretion of CRH-stimulated ACTH and basal LH release from rat pituitaries (Russell et al., 2001; Samson and Taylor, 2001). Intracerebroventricular (i.c.v.) injection of both orexins stimulated LH release in ovariectomized (ovx) rats pretreated with estradiol and progesterone. However, orexins decreased LH secretion levels in unprimed ovx rats (Pu et al., 1998). The above results suggest that the animals’ hormonal milieu affects the orexin system. The expression of orexins and their receptors in the pituitary of humans (Blanco et al., 2001 and 2003), rats (Date et al., 2000; Jöhren et al., 2003; Silveyra et al., 2007b) and pigs (receptors only) (Kaminski et al., 2010a) also supports the possibility that orexin participates in autocrine and/or paracrine control of the pituitary gland. OXA was found in lactotrophs, thyreotrophs, somatotrophs and gonadotrophs but not in corticotrophs in the human anterior pituitary. By contrast, OXB was observed in all corticotrophs of the adenohypophysis (Blanco et al., 2003). The expression of orexin receptors in the pituitary is also determined by gender, which once again emphasizes the importance of the animals’ hormonal status. The expression of the OX1R gene was higher in the pituitary of male rats than in female rats (Jöhren et al., 2001).

Research advances over the past decade have confirmed that the orexin system participates in the control of rat and human pituitary. In the existing body of research, there are no studies investigating the presence of orexins in the porcine pituitary and possible impact of hormonal status of the animals connected with the phase of the estrous cycle on orexin concentrations. For this reason, the objective of this study was to compare the expression levels of: (1) PPO gene by quantitative real-time PCR, (2) PPO protein by Western blotting and (3) OXA and OXB protein by fluorescent immunohistochemistry in anterior (AP) and posterior pituitary (NP) on days 2 to 3, 10 to 12, 14 to 16, and 17 to 19 of the estrous cycle.

Material and methods

Experimental animals

The studies were carried out in accordance with the principles and procedures of the Animal Ethics Committee at the University of Warmia and Mazury in Olsztyn. Mature gilts (Large White × Polish Landrace), 7 to 8 months old, weighing 120 to 130 kg, were obtained from private breeders. The pigs were last fed the afternoon of the day before their slaughter. Twenty gilts were assigned to one of four experimental groups (n = 5 per group) as follows: (1) days 2 to 3 of the estrous cycle: the early luteal phase, development of new corpora lutea; (2) days 10 to 12: the mid-luteal phase, fully active corpora lutea; (3) days 14 to 16: the late-luteal phase, luteolysis; and (4) days 17 to 19: the follicular phase of the estrous cycle. Females were monitored daily for estrus behaviour in the presence of an intact boar. The day of onset of the second estrus was designated as day 0 of the estrous cycle. The phase of the estrous cycle was also confirmed on the basis of the morphology of the ovaries (Akins and Morrisssette, 1968) and plasma progesterone concentration during the cycle. Within a few minutes after slaughter the pituitary gland was removed and separated into anterior and posterior lobes. All the samples were frozen in liquid nitrogen and maintained at −80°C until RNA and protein analysis were performed. Moreover, blood samples were collected into heparinized tubes, centrifuged (2,500 × g, 15 min, 4°C) and the obtained plasma was stored at −80°C until progesterone measurements.

Measurement of plasma progesterone level

To confirm correctness of the evaluation of the estrous cycle phase, the level of progesterone (P₄) was determined according to the method described by Cierny et al. (1998). Validity of the assay was confirmed by parallelism between the standard curves and a series of dilutions of the samples. The sensitivities of the assays for P₄ was 2 pg per tube. The plasma level of P₄ on days 2 to 3, 10 to 12, 14 to 16 and 17 to 19 was as follows: 4 ± 2 ng/ml, 19 ± 3.4 ng/ml, 8 ± 2.2 ng/ml and 0.2 ± 0.03 ng/ml, respectively, and corresponds with earlier published data pertaining to the steroid concentration in pig plasma during the estrous cycle (Henricks et al., 1972). Intra-assay coefficients of the variations of P₄ were 1.46% and samples were done in one assay.

Total RNA isolation, cDNA synthesis and quantitative real-time PCR

Total RNA was extracted from all collected tissues using the Absolutely RNA Miniprep Kit (Stratagene, La Jolla, CA, USA). RNA concentration and quality were determined spectrophotometrically (NanoDrop ND-1000, NanoDrop Technologies Inc., Wilmington, DE, USA). Approximately 1 μg of RNA was reverse-transcribed into cDNA in a total volume of 20 μl with 0.5 μg oligo(dT)15 primer (Roche, Meylan, France) using the Omniscript RT Kit (Qiagen, Valencia, CA, USA) at 37°C for 1 h and was terminated by incubation at 93°C for 5 min. Quantitative real-time PCR analysis was performed using a PCR
System 7300 (Applied Biosystems, Grand Island, NY, USA) with SYBR Green. Selected forward and reverse primers were: PPO, forward: 5’-AAG AGT ACA CCC TTC CTG GAC AC-3’; reverse: 5’-TGA TTG CCA GGG CCG TGT AGC A-3’; GAPDH, forward: 5’-CTC TCA TTG CCA GGT ACT ACA TGG T-3’; reverse: 5’-CCA CAA CAT ACG TAG CAC CAT C-3’. PPO primers (access No: EF-434655) were complementary to positions 41-63 (F) and 240-261 (R) of the pig PPOX 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. The PCR reaction included 100 ng cDNA, 0.5 µM primers, 25 µL SYBR Green PCR Master Mix (Applied Biosystems), and RNase free water in a final volume of 50 µL. Quantitative real-time PCR cycling conditions were as follows: initial denaturation and enzyme activation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 59°C for 1 min. The hypothalamus was used as a positive control for the gene expression. Negative controls were performed in which water was substituted for cDNA, or reverse transcription was not performed before 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 was conducted based on the comparative cycle threshold method (Livak and Schmittgen, 2001). Expression of PPO mRNA was calculated by the equation 2\(^{-\Delta\Delta CT}\), where \(\Delta CT\) was obtained by subtracting the corresponding GAPDH \(\Delta CT\) value from the specific \(\Delta CT\) of the target (PPO), and \(\Delta\Delta CT\) was determined by subtracting the \(\Delta CT\) of each experimental sample from \(\Delta CT\) of the reference sample, called the calibrator (the tissue with the lowest expression). \(\Delta CT\) was calculated to normalize the differences in the amount of total nucleic acid added to cDNA reaction mixture and the efficiency of reverse transcription reaction. To note, no significant differences \((P < 0.05)\) in \(CT\) values were observed for GAPDH in AP and NP derived from different stages of the estrous cycle. Therefore, GAPDH was an acceptable housekeeping gene for this study. PCR-amplified DNA was electrophoresed on 1.5% agarose in Tris-acetate buffer. After isolation from gel, DNA was sequenced (ABI Prism™ BigDye™ 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.

**Western blotting**

Western blotting analysis was performed as described by Smolinska et al. (2007). Briefly, equal amounts of porcine pituitary (anterior and posterior parts, 10 µg) were resolved by SDS-PAGE (12.5%) for separating PPO and actin and transferred to nitrocellulose membranes (Whatman, Piscataway, NJ, USA). Membranes were blocked for 5 h at 4°C in Tris-buffered saline Tween-20 containing 5% skimmed milk powder, then overnight at 4°C with rabbit polyclonal antibodies to mouse PPO (Merck Millipore, Billerica, MA, USA) at a dilution of 1 : 250 or rabbit polyclonal antibodies to human actin (Sigma-Aldrich, Saint Louis, MO, USA) at a dilution of 1 : 200, which were used as an internal control for equal loading as well as to quantify porcine PPO proteins. To identify immunoreactive bands, membranes were incubated for 1.5 h at room temperature (RT) with mouse anti-rabbit IgG conjugated with alkaline phosphatase (Sigma-Aldrich; diluted 1 : 500 for PPO protein or 1 : 5000 for actin protein). Non-specific fetal calf serum (MP Biomedicals, Santa Ana, CA, USA) was used instead of primary antibodies to produce negative control blots. The immunocomplexes were visualized using 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). The results of Western blotting were quantified by densitometric scanning of immunoblots with GelScan for Windows ver. 1.45 software (Kucharczyk, Warsaw, Poland). Data were expressed as a ratio of PPO protein relative to actin protein in arbitrary optical density units.

**Fluorescent immunohistochemistry**

Fluorescent immunohistochemistry was performed as described by Nitkiewicz et al. (2012). Porcine anterior pituitary and posterior pituitary were sectioned on a cryostat CM3050 (Leica, Wetzlar, Germany) to 10 µm thickness and thaw-mounted onto poly-lysine-coated glass microscope slides (Menzel-Glaser, Braunschweig, Germany). Slides were brought to RT and a hydrophobic marking pen (Dako Cytomation, Glostrup, Denmark) was used to encircle the tissue to prevent reagent run-off. Slides were immersed in a freshly prepared solution of 4% paraformaldehyde (Sigma-Aldrich) in 0.01 M phosphate-buffered saline (PBS, pH 7.4) for 10 min at 4°C then washed three times in 0.01 M PBS. After being fixed and washed endogenous proteins were blocked in 10% normal goat serum (Sigma-Aldrich) diluted in 0.01 M PBS with 0.1% bovine serum albumin (BSA, Sigma-Aldrich) and 0.05% saponin (Sigma-Aldrich). The sections were then incubated with rabbit anti-OXA or rabbit anti-OXB primary antibodies (1 : 50; Phoenix Pharmaceuticals Inc., Burlingame, CA, USA) diluted in antibody diluent (0.01 M PBS containing 0.1% BSA, 0.2% bovine gelatin (Sigma-Aldrich) and 0.05% saponin (Sigma-Aldrich)). The sections were then incubated with rabbit anti-OXA or rabbit anti-OXB secondary antibodies (1 : 200, which were used as an internal control for equal loading, 0.01 M PBS containing 0.1% BSA and 0.3% triton X-100) at 4°C overnight. After primary incubation, the sections were washed three times in wash buffer and incubated with biotinylated anti-rabbit IgG (1 : 100; Vector Laboratories, Burlingame, CA, USA) diluted in antibody diluent (0.01 M PBS containing 0.1% BSA and 0.3% triton X-100) at 4°C overnight. After primary incubation, the sections were washed three times in wash buffer and incubated with fluorescent mounting medium (Sigma-Aldrich) and stored in the dark at 4°C. The labeled tissues were photographed using a DS-5Mc-U2 Camera mounted on an Eclipse 80i fluorescence microscope using a dual filter cube for FITC and TRITC.
(Nikon Corporation, Tokyo, Japan). Fluorescence intensity was analyzed in each section using NIS-Elements BR image analysis software (Nikon Corporation). 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. Data from each coverslip were archived and expressed as the intensity staining (arbitrary units; range: 0 to 255).

**Statistical analysis**
All data were analyzed by two-way ANOVA and least significant difference (LSD) post hoc test and are reported as the mean ± s.e.m. from five independent observations. Statistical analyses were performed using the Statistica program (StatSoft Inc., Tulsa, OK, USA). Values for \( P < 0.05 \) were considered statistically significant.

**Results**

**Quantitative real-time PCR**
In AP, the expression levels of PPO mRNA were 1.6 times higher (\( P < 0.05 \)) on days 17 to 19 of the cycle than on days 2 to 3, and in NP, PPO gene expression was 1.9 and 1.7 times more pronounced (\( P < 0.05 \)) on days 17 to 19 than on days 2 to 3 and 10 to 12, respectively. The differences between the remaining stages of the estrous cycle were not significant in AP and NP (Figures 1a and b). The PPO gene was more highly expressed (\( P < 0.01 \)) in NP than in AP during the entire cycle (Figure 1c).

**Western blotting**
In AP, PPO protein content was from about 1.4 to 1.5 times significantly (\( P < 0.05 \)) higher on days 2 to 3 than in the remaining stages of the estrous cycle (Figure 2a). In NP, PPO protein was observed from about 2.1 to 3.2 times in greater abundance (\( P < 0.05 \)) on days 10 to 12 and 17 to 19 than on days 2 to 3 and 14 to 16 of the cycle (Figure 2b). Contrary to the PPO gene, the expression of protein was higher (\( P < 0.001 \)) in AP than in NP during all phases of the estrous cycle (Figure 2c).

**Fluorescent immunohistochemistry**
OXA and OXB proteins were localized in the porcine AP and NP on days 2 to 3, 10 to 12, 14 to 16 and 17 to 19 of the cycle. No staining was detected in the porcine pituitary when 0.01 M PBS or rabbit universal negative control was used instead of primary antibodies (Supplementary Figure S1). In AP, OXA immunoreactivity was from about 1.2 to 1.3 times higher (\( P < 0.05 \)) on days 10 to 12 and 14 to 16 of the estrous cycle than on days 2 to 3 and 17 to 19. The lowest (\( P < 0.05 \)) OXA protein concentrations were noted during the follicular phase of the estrous cycle (Figure 3a). In NP, from about 1.3 to 2.0 times markedly higher (\( P < 0.05 \)) expression of OXA protein was observed on days 10 to 12, and the lowest (\( P < 0.05 \)) expression levels were reported on days 14 to 16 and 17 to 19 (Figure 3b). Similarly to the PPO protein, the expression of OXA protein was higher (\( P < 0.001 \)) in AP than in NP throughout the entire cycle (Figure 3c).

In AP, OXB immunoreactivity levels were highest (\( P < 0.05 \); from about 1.2 to 1.4 times higher) on days 2 to 3 and lowest (\( P < 0.05 \)) on days 14 to 16 and 17 to 19 of the cycle (Figure 3c).

![Figure 1](image)

**Figure 1** A comparison of prepro-orexin (PPO) mRNA expression determined by quantitative real-time PCR in porcine anterior (a) and posterior (b) pituitary glands between days 2 to 3, 10 to 12, 14 to 16 and 17 to 19 of the estrous cycle and (c) between anterior and posterior pituitary glands on days 2 to 3, 10 to 12, 14 to 16 and 17 to 19 of the cycle. Results are means ± s.e.m. (\( n = 5 \)). Bars with different superscripts are significantly different. Uppercase letters indicate \( P < 0.05 \); **\( P < 0.01 \), ***\( P < 0.001 \).
Figure 2 A comparison of prepro-orexin (PPO) protein concentration determined by Western blotting analysis in porcine anterior (a) and posterior (b) pituitary glands between days 2 to 3, 10 to 12, 14 to 16 and 17 to 19 of the estrous cycle and (c) between anterior and posterior pituitary glands on days 2 to 3, 10 to 12, 14 to 16 and 17 to 19 of the cycle. Upper panels: representative immunoblots (molecular marker (MM)); lower panels: densitometric analysis of PPO protein relative to actin protein. Values are expressed as means ± s.e.m. in arbitrary optical density units (n = 5). Bars with different superscripts are significantly different. Uppercase letters indicate P < 0.05; ***P < 0.001.

Figure 3 A comparison of orexin A (OXA) protein immunoreactivity in porcine anterior (a) and posterior (b) pituitary glands between days 2 to 3, 10 to 12, 14 to 16 and 17 to 19 of the estrous cycle and (c) between anterior and posterior pituitary glands on days 2 to 3, 10 to 12, 14 to 16 and 17 to 19 of the cycle. Values are expressed as means ± s.e.m. in arbitrary optical density units (n = 5). Bars with different superscripts are significantly different. Uppercase letters indicate P < 0.05; ***P < 0.001.
and lowest ($P < 0.05$) on days 10 to 12 and 17 to 19 of the cycle (Figure 4a). Similarly to the OXA protein, the highest ($P < 0.05$; from about 1.2 to 1.3 times higher) content of OXB protein was noted in NP on days 10 to 12 (Figure 4b). OXB protein concentrations were higher ($P < 0.05$) in AP than in NP on days 2 to 3 and 14 to 16 of the cycle, whereas OXB protein expression levels were higher in NP than in AP on days 10 to 12 (Figure 4c).

**Discussion**

Our study was the first experiment to demonstrate the expression of PPO mRNA and PPO, OXA and OXB proteins in both AP and NP of cyclic gilts. To our knowledge, this is the first study that quantifies PPO gene expression and the concentrations of orexin peptides in the porcine pituitary during the estrous cycle. The expression of PPO mRNA was higher during the follicular phase in both AP and NP. The highest PPO protein content was noted on days 2 to 3 of the estrous cycle in AP and on days 10 to 12 and 17 to 19 in NP. In AP, OXA immunoreactivity was highest on days 10 to 12 and 14 to 16 of the cycle, and the lowest immunoreactivity levels were reported during the follicular phase of the cycle. In NP, OXA protein content was highest on days 10 to 12 and lowest on days 14 to 16 and 17 to 19. OXB immunoreactivity was highest on days 2 to 3 in AP and on days 10 to 12 in NP. The lowest content of OXB protein in AP was observed during mid-luteal and the follicular phases of the cycle. The above results indicate that gene and protein expression levels were determined by the stage of the estrous cycle.

The differences between the expression patterns of PPO and OXA or OXB proteins may be due to the fact that the PPO is a common precursor of both orexins with different half-lives. It is worth noting that the expression of the PPO gene was lower in AP than in NP, whereas the highest PPO protein concentrations were noted in AP. The cause of the above variations remains unknown.

To date, OXA and OXB have been identified in the pituitaries of rats (Date *et al.*, 2000) and humans (Blanco *et al.*, 2003). The concentrations of both peptides, measured by reverse-phase HPLC, were 13-fold higher in NP than in AP of rats. OXB protein concentrations were higher than OXA levels in AP and NP. The results of immunohistochemical analysis revealed the presence of OXA and OXB fibers in NP, but orexin fibers were not determined in AP or the intermediate pituitary (Date *et al.*, 2000). Immunoreactivity to OXA was detected in a high number of human lactotrophs, and it was determined in a smaller percentage of thyrotrophs, somatotrophs and gonadotrophs. Corticotrophs were negative for OXA. By contrast, OXB immune-positive cells were present in all corticotrophs, whereas the remaining cell types were negative. Immunostaining for both peptides was weak in human NP (Blanco *et al.*, 2003). The above results indicate that NP is the main source of orexins in rats, whereas human lactotrophs are the main source of OXA and corticotrophs are the main source of OXB. The presence of OX1R and OX2R was demonstrated in rat (Date *et al.*, 2000; Jöhren *et al.*, 2001, 2003), human (Blanco *et al.*, 2001), sheep (Zhang *et al.*, 2005) and porcine pituitaries (Kaminski *et al.*, 2010a). In rats, OX1R and OX2R genes were found in AP and NP. Intense hybridization signals were detected in the pituitary.
GnRH neurons express OX1R. In pigs, around 30% of GnRH pituitary cells (Martynska et al., 2011). OXA dose dependent (Barb and Matteri, 2005). In immature female rats, and GnRH-induced LH from AP cells in a dose-dependent manner (Kaminski et al., 2010b). The presence of orexins in AP and NP provides evidence that the pituitary gland is capable of synthesizing the discussed hormones locally. The local production of orexins and the presence of specific receptors suggest that OXA and OXB exert a direct effect on the pituitary with possible autocrine and/or paracrine action of neuropeptides in the gland.

Some lines of evidence suggest that orexins regulate pituitary hormone secretion and control the pituitary branch of the hypothalamic–pituitary–gonadal (HPG) axis. In a study of prepubertal gilts, OXB stimulated the secretion of basal and GnRH-induced LH from AP cells in a dose-dependent manner (Barb and Matteri, 2005). In immature female rats, OXA stimulated the release of LH from primary culture of pituitary cells (Martynska et al., 2011). OXA dose dependent inhibited the secretion of GnRH-induced LH from pituitary cells in proestrus females rats (Russell et al., 2001). Orexins control the LH secretion by not acting directly on pituitary cells, but also by influencing GnRH neurons at the hypothalamic level. Campbell et al. (2003) reported that 75% to 85% of hypothalamic GnRH neurons in rats have direct synaptic contacts with orexin fibers and ~85% of GnRH neurons express OX1R. In pigs, around 30% of GnRH neurons were in close contact with OXB immunoreactive fibers (Su et al., 2008). An in vivo experiment demonstrated that OXA has a dual effect on LH release. The stimulatory effect was observed after injection into the rostral preoptic area, and the inhibitory effect was noted when OXA was injected into the medial preoptic area or the median eminence of the hypothalamus (Small et al., 2003). In vitro studies have shown that OXA can enhance GnRH release from hypothalamic explants harvested from intact male rats and proestrus female rats (Russell et al., 2001). A stimulatory effect of OXA on GnRH transcription and release from immortalized GT1-7 hypothalamic neurons was also observed by Sasson et al. (2006). In addition, i.c.v. injected orexins modulated LH secretion in an ovarian-steroid-dependent manner (Pu et al., 1998; Small et al., 2003). Orexins enhanced LH release in estradiol- and progesterone-pretreated ovx rats, but they suppressed LH secretion in unprimed rats (Pu et al., 1998). Both the stimulatory and inhibitory effects of OXA were antagonized by SB334867A, a selective OX1R antagonist (Small et al., 2003).

In addition to exerting an influence on the HPG axis, the orexin system is characterized by a gender-specific pattern of expression. Significantly higher levels of hypothalamic PPO mRNA (Jöhren et al., 2002) and OXA protein (Taheri et al., 1999) were reported in female than in male rats. OX1R mRNA expression in the pituitary was much higher in male than in female rats. The expression of OX1R mRNA was enhanced in the hypothalamus of female rats (Jöhren et al., 2001). The expression of PPO and orexin receptors genes as well as OXA concentrations in the hypothalamus were regulated by the estrous cycle, pregnancy, parturition and lactation (Russell et al., 2001; Wang et al., 2003). Thus, it seems that gonadal steroid hormones may regulate the expression of orexin system. In ovx rats, the expression of the pituitary OX1R gene was suppressed after estradiol treatment (Jöhren et al., 2003). On the other hand, in a study by Silveyra et al. (2007b) the expression of PPO, OX1R and OX2R genes in rat hypothalami and pituitaries was enhanced during proestrus. In the case of both receptors, mRNA concentrations increased in rat ovaries during proestrus, and the above coincided with the hormone peaks (Silveyra et al., 2007a). These data are in line with our present and recent findings that indicate that the expression of orexins, orexin receptors genes and proteins fluctuate throughout the estrous cycle. In our previous study, higher expression levels of OX1R and OX2R genes and proteins in porcine hypothalamic structures during the follicular phase could suggest that estrogens have a stimulatory effect on both receptors (Kaminski et al., 2010b). In the porcine pituitary, enhanced levels of OX1R mRNA during the luteal phase and higher concentrations of PPO and OX2R mRNAs during the follicular phase of the cycle imply that OX1R gene expression is stimulated by progesterone, whereas the expression of PPO and OX2R genes is influenced by estradiol (Kaminski et al., 2010a; present study). Similarly, variation in the expression of OX1R and OX2R genes in the porcine ovary during luteal and follicular phases of the cycle may imply that progesterone and estradiol affect orexin receptor expression (Nikitiewicz et al., 2010). Summarizing, the above studies clearly suggest the impact of the physiological hormonal milieu on the orexergic system.

Conclusions
In this study, the expression of the PPO gene and the presence of PPO, OXA and OXB proteins were determined in the porcine pituitary. mRNA and protein concentrations are influenced by the phase of the estrous cycle. Our present and previous findings expand our knowledge of the role played by orexin system in the control of the pituitary gland.

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Supplementary material
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Orexin gene and proteins in the porcine pituitary


References


