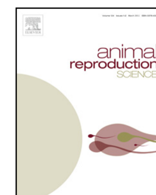
Animal Reproduction Science 148 (2014) 53–62

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci



Expression of orexins and their precursor in the porcine ovary and the influence of orexins on ovarian steroidogenesis in pigs

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ARTICLE INFO

Article history:
Received 14 October 2013
Received in revised form 16 March 2014
Accepted 23 March 2014
Available online 9 May 2014

Keywords:
Orexin
Corpus luteum
Granulosa cells
Theca interna cells
Steroid hormones
Pig

ABSTRACT

Orexins A and B are hypothalamic neuropeptides associated with homeostasis and the reproductive system. The aim of the study was to compare the expression of the prepro-orexin gene and the intensity of orexins immunoreactivity in the porcine ovary (corpora lutea, granulosa and theca interna cells) during four different stages of the oestrous cycle (days: 2–3, 10–12, 14–16 and 17–19) and to examine the *in vitro* effect of orexins on the secretion of steroid hormones by porcine luteal, granulosa and theca interna cells. The highest expression of prepro-orexin mRNA was observed in theca interna cells on days 17–19 of the oestrous cycle. The highest content of immunoreactive orexin A was noted in corpora lutea on days 10–12 and the highest level of immunoreactive orexin B on days 14–16 of the cycle. Immunoreactive orexin A concentrations were higher in theca interna cells than in granulosa cells, whereas similar levels of immunoreactive orexin B were observed in both cell types. Under *in vitro* conditions, at the concentration of 10 nM, orexins A and B inhibited FSH-induced oestradiol secretion by granulosa cells. The obtained results suggest that the pattern of orexin peptide expression in the porcine ovary is related to the animals' hormonal status. Our findings imply that orexins can affect porcine reproductive functions through modulation of ovarian steroidogenesis.

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1. Introduction

Orexins A and B, also known as hypocretin 1 and 2, are hypothalamic neuropeptides which were discovered simultaneously in 1998 by two independent laboratories (Sakurai et al., 1998; de Lecea et al., 1998). Both orexins are derived by proteolytic cleavage from a common 130-amino acid (aa) precursor molecule, prepro-orexin (PPO). Orexin A (OXA) is a 33-aa peptide, while orexin B (OXB) consists of 28 amino acids. The amino-acid sequence of orexin A is highly conserved among mammalian species. The amino acid sequence of OXA is identical in pigs, humans, rats and cows, and the sequence of OXB in pigs and humans differs by only one amino acid residue (Sakurai et al., 1998; Dyer et al., 1999). The biological action of hormones is mediated by binding two distinct, but structurally similar G protein-coupled receptors: orexin receptor type 1 (OX1R) and orexin receptor type 2 (OX2R). Orexin A is a more selective ligand for OX1R, whereas OX2R binds both orexins with similar affinity (Sakurai et al., 1998; de Lecea et al., 1998).

Orexins were originally discovered in the lateral hypothalamus, a structure involved in the control of food intake and energy balance (Sakurai et al., 1998; de Lecea

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http://dx.doi.org/10.1016/j.anireprosci.2014.03.015
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et al., 1998; Fabris et al., 2004). Orexin-producing neurons distribute their projections widely to other structures of the hypothalamus and the central nervous system to influence multiple physiological functions, such as control of arousal and sleep and wakefulness, and neuroendocrine homeostasis. Neuroendocrine homeostasis is achieved through regulation of the central branches of the hypothalamic–pituitary–adrenal axis (Spinazzi et al., 2006) and the hypothalamic–pituitary–gonadal axis (Porka-Heiskanen et al., 2004; Barreiro et al., 2005; Silveyra et al., 2007; Kaminski et al., 2010a,b; Nitkiewicz et al., 2010). The effects of orexins on the gonadal axis seem to be mediated through their action on GnRH neurons (Russell et al., 2001). GnRH neurons were found to express OX1R, and approximately 85% or 35% neurons had synaptic connections with orexinergic neurons in rats (Campbell et al., 2003) and pigs (Su et al., 2008), respectively. By modifying GnRH secretion, orexins indirectly affect pituitary and gonadal hormone production. It has been also shown that central administration of orexin A stimulates LH secretion (Kok et al., 2004).

The role of orexins in controlling the female reproductive system remains largely unexplored. A limited number of articles describing the direct influence of orexins on the reproductive system focused on the expression of orexin receptors in the ovaries (Silveyra et al., 2007; Nitkiewicz et al., 2010). Based on stimulatory effect of orexins on adrenal (Nanmoku et al., 2002; Ziolkowska et al., 2005) and testicular (Barreiro et al., 2004) steroidogenesis, there are suggestions that they may act in a similar manner in the ovary. The above was demonstrated by Cataldi et al. (2012) who described the influence of orexins on progesterone secretion by ovarian cells in rats. However, there is an absence of published data on the presence of orexin system components in the ovaries of animals other than rats, the influence of the animals' hormonal status on orexin expression in the gonads and the possible impact of orexins on ovarian (luteal and follicular) steroidogenesis.

The aim of this study was to investigate the expression of the prepro-orexin gene by real-time PCR, to determine the presence of immunoreactive orexins A and B in the porcine ovary (corpora lutea by immunohistochemistry, granulosa and theca interna cells by immunocytochemistry) and to compare gene and protein expression levels during different stages of the oestrous cycle in pigs. The *in vitro* effect of orexins on the secretion of steroid hormones (progesterone, oestradiol, androstenedione, testosterone) by ovarian luteal, granulosa and theca interna cells was also studied in the presence and absence of selective antagonists of orexin receptors and porcine gonadotrophins.

2. Materials and methods

2.1. Experimental animals

All experiments were carried out in observance of the ethical standards of the Animal Ethics Committee at the University of Warmia and Mazury in Olsztyn. The experimental material comprised mature gilts (Large White & Polish Landrace) from a private breeding farm, aged 7–8 months, weighing 130–140 kg. The gilts were divided into four experimental groups as follows: days 2–3, 10–12, 14–16, and 17–19 of the oestrous cycle. Diets were balanced (crude protein, metabolizable energy, exogenous amino acids and minerals) in accordance with the nutrient requirements of domestic pigs. Individuals were given free access to water. Females were monitored daily for oestrous behavior in the presence of an intact boar. The day of the second oestrus was designated as day 0 of the oestrous cycle. The phase of the oestrous cycle was also determined based on ovarian morphology (Akins and Morrissette, 1968). The ovaries were removed and placed on ice within minutes after slaughter. Dissected corpora lutea (CLs) from different stages of the oestrous cycle (days 2–3 – corpora haemorrhagica, 10–12 – mature CLs and 14–16 – regressing CLs) were either immediately frozen in liquid nitrogen and stored at −80 °C until RNA and protein analysis or, similarly to the ovaries from days 17 to 19 of the cycle, placed in cold PBS buffer and transported to the laboratory where luteal, follicular granulosa and theca interna cells were isolated.

2.2. Measurement of plasma progesterone level

To confirm correctness of the evaluation of the oestrous cycle phase, level of progesterone (P_4) was determined according to the RIA method described by Ciereszko et al. (1998). Cross-reactivities of the antiserum against P_4 have been published previously (1982). 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 P_4 were 2 pg per tube. The plasma level of P_4 on days 2–3, 10–12, 14–16, and 17–19 was as follows: 4 ± 2 ng/ml, 19 ± 3.4 ng/ml, 8 ± 2.2 ng/ml, 0.2 ± 0.03 ng/ml, respectively, and correspond with earlier published data pertaining to the steroid concentration in pig plasma during the oestrous cycle (Henricks et al., 1972). Intra-assay coefficients of variations of the P_4 were 1.46% (inter-assay coefficients of variations were not calculated because the assays were done in one assay).

2.3. Isolation of luteal, granulosa and theca interna cells

Luteal cells were isolated by the method described by Kaminski et al. (1999). Dissected corpora lutea from ovaries on days 2–3, 10–12 and 14–16 were minced into small fragments and dispersed in F-12 medium containing bovine serum albumin fraction V (BSA; 1%) and antibiotics. Corpora lutea were enzymatically dissociated in 0.125% trypsin solution (4–6 times) in 37 °C, centrifuged (300 × *g*, 10 min, 4 °C) and washed three times. Isolated luteal cells were filtered through nylon mesh (40 μm in diameter) and resuspended in fresh F-12 medium. The cells were counted using a hemocytometer, and their viability (∼90%) was determined by 0.4% trypan blue dye exclusion.

Granulosa and theca interna cells – precursors for large and small luteal cells, respectively – were isolated from large follicles (diameter > 6 mm) without signs of atresia. Granulosa cells were aspirated with a syringe and additionally washed out with a strong stream of media directed to the internal wall of the follicle (Kaminski et al., 2004).

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