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Immunolocalization of Orexin A and its receptors in the different structures of the porcine ovary☆

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ABSTRACT

Orexins are neuropeptides with pleiotropic functions, involved in the coordination of multiple versatile physiological processes, in particular related to food intake and several aspects of the reproductive process. Their actions are carried out through the bond with the related Orexin 1 (OXR1) and Orexin 2 (OXR2) G-protein-coupled receptors. Studies on the expression of the orexinergic system in the female genital organs are scarce and limited to preovulatory gametogenic follicles and corpora lutea isolated from the rest of the ovary. As the description of only these structures is insufficient to provide a complete picture of the organ, the present study is aimed to give a panoramic view of all the ovarian structures and cells expressing Orexin A (OXA) and its receptors in their original localization. Double labeling immunofluorescent methods, applied on frozen sections of the whole organ in both follicular and luteal phase, were used to highlight the particular distribution and colocalization of the proteins. For a better recognition of cellular morphology and a better distinction between gametogenic (healthy) and atretic follicles, also a single labeling immunolocalization of OXA on formalin fixed paraffin embedded tissues and a TUNEL staining were performed. The results indicate that OXA and its two receptors subtypes are expressed in all the different structures composing the swine ovary, albeit in different ways, in both phases of the ovarian cycle. In general, OXA and OXR2 appear diffusely distributed within "health", proliferating and steroid producing cells, while has granular appearance, being presumably associated to cytoplasmic vesicles, in degenerating cells, independently if apoptotic or not. The immunoreactivity for OXR1, instead, is often associated with the nuclear envelope but it is also detectable, to a lesser extent, diffusely distributed in the cytoplasm of growing or steroid producing cells. When cells undertake the path leading to degeneration, also OXR1 immunoreactivity assumes a granular appearance in the cytoplasm and is colocalized with OXA and OXR2. Different roles for the two receptors in the same cell and a different regulation of their expression remain to be investigated. Their comprehension could help studies of follicle development in pig, as part of in vitro oocyte maturation and fertilization programs in livestock.

1. Introduction

Orexin (or hypocretin) A (OXA) and B (OXB) are two neuropeptides, initially identified in the hypothalamus, derived by proteolytic cleavage from a single 130 amino acid precursor called prepro-orexin (PPO) (Sakurai et al., 1998; De Lecea et al., 1998). They have pleiotropic functions, being involved in the coordination of multiple versatile physiological processes such as food intake, energy balance, reproductive activity, sleep and wakefulness. Their action is mediated by two receptors coupled to the G(q) proteins (GPCRs), called receptor 1 (OXR1), highly specific for Orexin A, and receptor 2 (OXR2) having the same affinity for OXA and OXB. Initially, orexins were considered to be produced and localized only in the central nervous system, but in recent years their presence has been found both in the nerve component and in non-neuronal cells of many peripheral organs (Sakurai et al., 1998; De Lecea et al., 1998; Fabris et al., 2004; Spinazzi et al., 2006; Barreiro et al., 2005; Silveyra et al., 2007; Kaminski et al., 2010).

In the genital organs in particular, the presence of orexins has been studied especially in the male, while, as far as we know, there is a small amount of published data on the presence of components

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of the orexinergic system and their possible impact on ovarian steroidogenesis in non-rodent animals (Cataldi et al., 2012). We chose to analyze their presence in the swine ovary, because it is particularly suitable for reproduction studies, having multiple ovulations, several follicles which develop and mature together and large follicles and corpora lutea reaching such dimensions that they can be easily isolated without the help of a microscope.

Ning et al. (2008) and Nitkiewicz et al. (2010, 2014) have already performed some studies on the expression of PPO mRNA and of orexins and their receptors in the porcine ovary. Their levels resulted closely associated with the animal's hormonal status during the estrous cycle. However, Ning et al. (2008) demonstrated it throughout the whole ovary, without making any distinction between the various structures that compose it. Instead Nitkiewicz et al. (2010, 2014), used experimental animals, whose age and phase of the estrous cycle were known, but analyzed only corpora lutea or granulosa and theca interna cells of large follicles isolated from the rest of the ovary. We therefore thought another study to be useful which would give a better morphological description of the cells expressing OXA and its receptors in their original localization, highlighting the particular distribution and eventual colocalization of the proteins. We have already published data on large follicles (Ciccimarra et al., 2018) and another study on corpora lutea has been recently accepted for publication (Basini et al., 2018). However, the description of only these structures is still insufficient to provide a complete picture of the ovary. In fact, during the development of follicles, only a limited number of them are selected for ovulation, while most of the structures observed at any stage of the ovarian cycle are represented by atretic follicles and bodies that started the degeneration process at any stage of development. Primary aim of the present study was therefore to give a panoramic view of the presence, distribution and colocalization of OXA and its two receptors in all the structures composing the ovary. For this, immunohistochemical (IHC) and double labeling immunofluorescence (IF) methods were used on sections of the whole organ. Moreover, as orexins have been proved to be active on apoptosis and cell growth inhibition in cancer cell lines (Voisin et al., 2006; Laburthe et al., 2010), and it is known that apoptosis occurs also during follicular atresia (Derecka et al., 1995; Guthrie et al., 1995; Sugimoto et al., 1998; Yu et al., 2004) and luteal regression (Hoyer, 1998; Aboelenain et al., 2015), the secondary aim of the study was to compare the expression of OXA and its receptors with the detection of damaged DNA within the cells through a TUNEL assay.

2. Materials and methods

2.1. Collection of ovaries

Swine ovaries were collected at a local slaughterhouse from 20 Large White cross-bred gilts, (parity = 0, 8–9 months aged, weighing about 180 kg), within a few minutes after death. Immediately after collection, ovaries were placed into cold PBS (4 °C) supplemented with penicillin (500 IU/ml), streptomycin (500 μg/ml) and amphotericin B (3.75 μg/ml), maintained in a freezer bag and transported to the laboratory within 1 h.

The stages of the estrous cycle were unknown, so we performed evaluations on the phase of the estrous cycle based on the ovarian morphology (Fig. 1) as described in literature (Akins and Morrissette, 1968; McDonald, 1975; Maxson et al., 1985; Babalola and Shapiro, 1988; Guthrie et al., 1995; Oberlender et al., 2014).

2.2. Immunohistochemistry

2.2.1. Single labeling immunohistochemistry

The ovaries of ten pigs (five ovaries were selected in follicular phase and five in luteal phase, so that all the ovarian structures described in literature could be present) were treated with routine preparation of immunohistochemistry. Briefly, tissues were fixed in 10% buffered formalin and embedded in paraffin. Sections of 5 μm thick were utilized for routine staining (haematoxylin and eosin, (H&E)) and IHC studies using mouse monoclonal antibody anti-OXA (MAB763 R&D Systems, Minneapolis, MN, USA).

Antigen retrieval was carried out by dipping the sections in 0.01 M sodium citrate buffer, pH 6.0, and heating them in a microwave oven (15 min at 750 W). The sections were consecutively incubated in: (1) 3% hydrogen peroxide for 15 min to block endogenous peroxidase; (2) primary antibody anti-OXA, dil. 5 μg/ml, overnight at 4 °C; (3) LSAB2 System-HRP rabbit/mouse (Dako, Santa Clara, CA, USA) for 20 min; (4) 3,3-diamino-benzidine (Dako) for 5 min. Between each step the sections were washed with PBS. The sections were counterstained with Mayer's haematoxyline solution. The samples were then photographed using a Zeiss Axiocam MRc5 digital camera and by means of the digital image processing software AxioVision release 4.5 (Carl Zeiss, MicroImaging GmbH, Germany).

2.2.2. Double labeling immunofluorescence

Ovaries collected from the other ten animals (five ovaries were selected in follicular phase and five in luteal phase), were sectioned in two or three approximately equal parts, to allow a better fixation. They were then fixed for 6–8 h in 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.2) at 4 °C, rinsed overnight in phosphate-buffered saline (PBS: 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2) and stored at 4 °C in PBS containing 30% sucrose and sodium azide (0.1%). The following day, the tissues were transferred to a mixture of PBS-30% sucrose-azide and Killik cryostat embedding medium (Bio-Optica, Milan, Italy) at a ratio of 1:1 for an additional 24 h before being embedded in 100% embedding medium. Each specimen was then frozen in isopentane, cooled in liquid nitrogen and stored at −80 °C until further processing. Subsequently the samples were serially sectioned on a Microm HM 505 E Cryostat into 16 μm thick sections that were thawmounted onto poly-L-lysine-coated glass microscope slides (Menzel-Glaser, Braunschweig, Germany). Serial cryostat sections from each ovary were stained by a double labeling IF method. The first section was used to assess the presence and the distribution of cells expressing OXA and OXR1. In the following section the expression of both receptors OXR1 and OXR2 was evaluated. After air-drying at room temperature (RT) for 30 min to decrease a nonspecific binding, the sections were incubated with a solution containing 0.25% Triton X-100 (Sigma–Aldrich, MO, USA), 1% bovine serum albumin (BSA, Sigma–Aldrich, MO, USA) and 10% normal goat serum (Sigma–Aldrich, MO, USA) in phosphate buffered saline (PBS: 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2) for 1 h (RT), to reduce non-specific background staining. They were then incubated (overnight, 4 °C) with a combination of the primary antisera (OXR1 = Rabbit anti-Rat Orexin-1 receptor, dil. 10 μg/ml, cat. no. OX1R11-A, Alpha Diagnostic International; OXA = Mouse anti Human/Mouse/Rat Orexin A, dil. 5 μg/ml, cat. no. MAB763, R&D Systems; OXR2 = Mouse anti Human Orexin-2 receptor, dil. 24 μg/ml, cat. no. MAB52461, R&D Systems); further incubated with a mixture of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (cat. no. F0382, Sigma, dil. 1:40) and biotinylated-goat anti mouse IgG (cat. no. RPN1001, Amersham Pharmacia Biotech, dil. 1:100) (1 h; RT) and then incubated with Texas Red-conjugated streptavidin (cat. no. RPN1233, Amersham Pharmacia Biotech, dil. 1:100) (1 h; RT). Finally the sections were covered with 300 nM DAPI (4′,6-diamidino-2-phenylindole) stain solution for 5 min for nuclear counterstaining.

For negative control, the primary antibody was omitted, and tissues were incubated in 0.01 M PBS, or alternatively, by pre-absorbing the primary antiserum with an excess (100 μg/ml) of the related antigen (Rat Orexin-1 Receptor Control/blocking pep-