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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 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

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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., 2014Yu 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 mi-

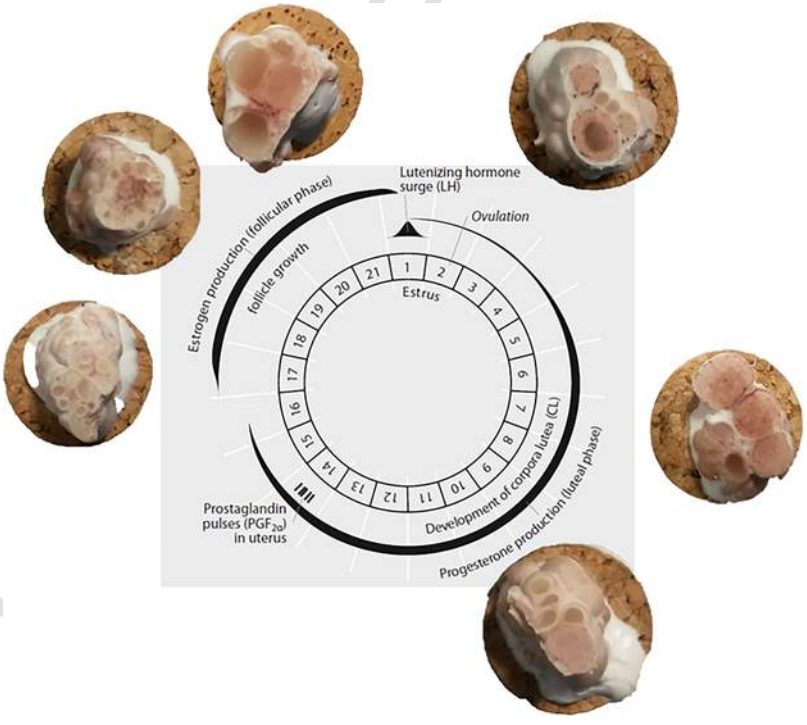

Fig. 1. Exemplary images of swine frozen and cryosectioned ovaries, showing their different morphology during the different stages of the estrous cycle.*Modified from* Kirkwood (1999).

crowave 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 peptide #1 cat. no. OX1R11-P, Alpha Diagnostic International; Orexin A Peptide Synthetic Peptide cat. no. 350307 Abbiotec San Diego CA). The sections were rinsed with PBS for 5 min after each step of the immunolabeling process and finally mounted in buffered glycerol. 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), mounted on a fluorescent microscope (Zeiss Axioskop 2 plus) equipped with epillumination and appropriate filters set for DAPI (excitation wavelength 390–420 nm; emission wavelength 450 nm), to reveal fluorescent nuclear labeling, FITC (excitation wavelength 450–490 nm; emission wavelength 515–565 nm) and Texas Red (excitation wavelength 530–585 nm; emission wavelength 615 nm). Relationships between nuclear (DAPI) distribution and IF staining were examined directly by interchanging filters.

2.2.3. Detection of apoptotic cells

Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) using the detection kit (Invitrogen, ThermoFisher Scientific, Carlsbad USA). Briefly, frozen sections from each ovary were post fixed in 4% paraformaldehyde, rinsed with PBS and permeabilized by incubation with proteinase K. After immersion in the terminal deoxynucleotidyl transferase (TdT) reaction buffer (TdT enzyme, 5-ethynyl-2′-deoxyuridine 5′-triphosphate nucleotides [EdUTP] and biotin-azide), the endogenous peroxidase activity was blocked with 3% hydrogen peroxide prior the incubation with Streptavidin-Peroxidase and finally 3,3′-diaminobezidine tetrahydrochloride (DAB) to obtain the colorimetric reaction. Nuclei were counterstained with Methyl Green 0.5% in 0.1 M sodium acetate buffer (pH = 4.2) For positive controls, paraformaldehyde post fixed and permeabilized slides were preincubated with 1U of DNase I before going forward with the described reaction. For negative controls, slides were incubated with reaction buffer (without TdT enzyme). The slides were scanned as whole-slide (Nanozoomer S60, Hamamatsu, Japan).

3. Results

3.1. Ovaries in follicular phase

3.1.1. Gametogenic follicles

In the follicular cells of primordial, primary, secondary and tertiary gametogenic follicles the fluorescent positive signal due to the immunoreactivity (IR) for OXA and OXR2 appeared diffusely distributed in the cytoplasm. The positive signal for OXR1, instead, principally overlapped the one for nuclear counterstaining (Fig. 2 and Supplementary Fig. S1 in the online version at DOI: 10.1016/j.aanat.2018.04.006). Gametogenic tertiary follicles generally showed the same pattern of IR independently from their size (small, medium and large/preovulatory, see also Ciccimarra et al., 2018). The cells of all these structures appeared TUNEL negative.

In non atretic (gametogenic) and TUNEL negative follicles of large dimensions (diameter >6 mm) (Fig. 3 and Supplementary Fig. S2 in the online version at DOI: 10.1016/j.aanat.2018.04.006), the oocytes showed only weak IF for the antibodies tested. They were surrounded by a layer of granulosa cells (the corona radiata) in which the IF for OXA and OXR2 was stronger and diffused in the cytoplasm, while the one for OXR1 principally overlapped the DAPI staining. The same pattern of IR, albeit slightly weaker, was visible in the cumulus oophorus, and in the granulosa cells forming the follicular wall.

OXR1-IR had the same localization also in the theca interna cells adjacent to those of granulosa, while in some scattered outermost cells and theca externa cells it displayed a cytoplasmic granular appearance and was, therefore, presumably localized at the level of cytoplasmic vesicles. In this case it was colocalized both with OXA-IR

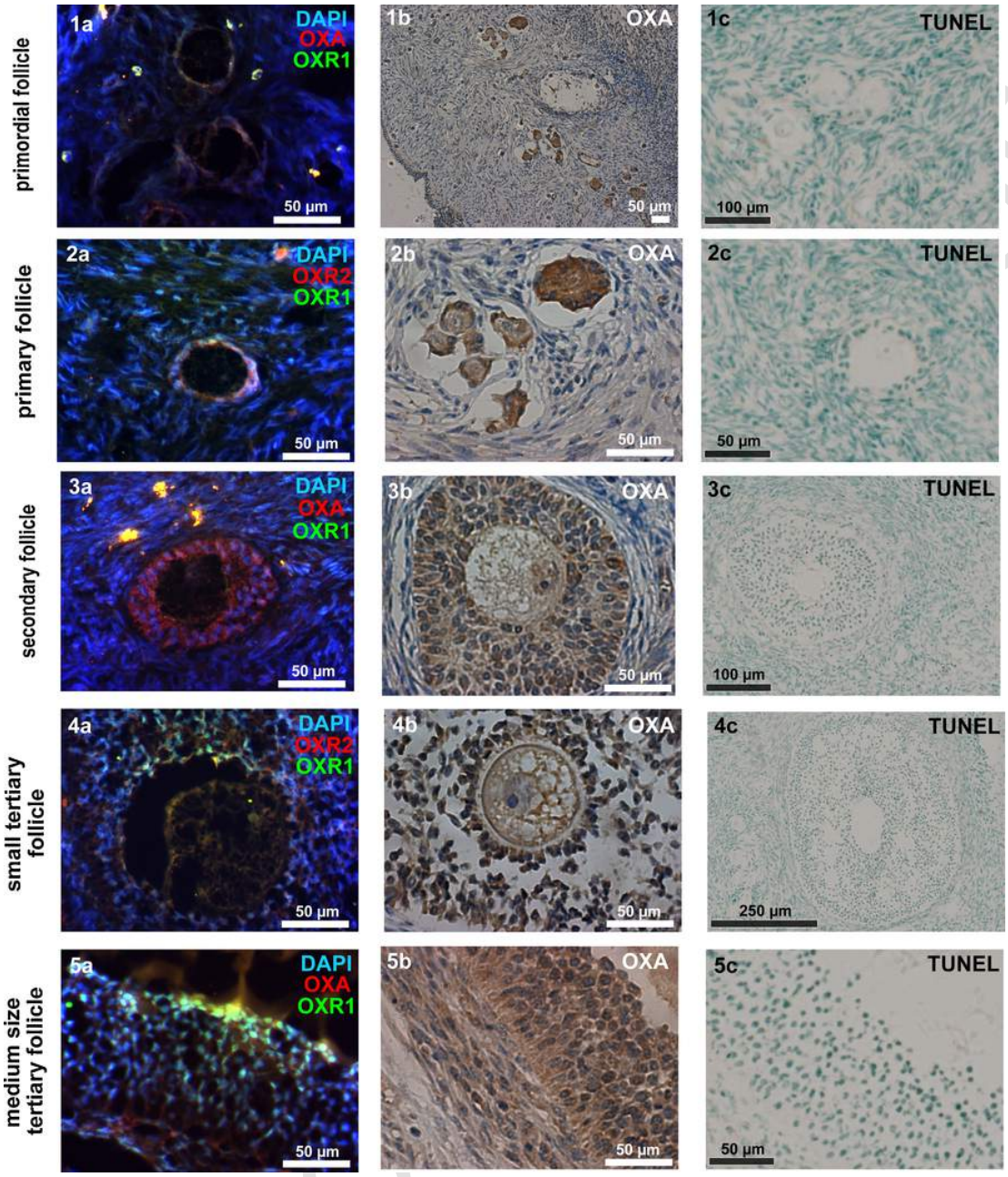

Fig. 2. Localization of OXA and its receptors and TUNEL staining results in different types of porcine ovarian gametogenic follicles during the follicular phase. In the first column images of OXA- or OXR2- (red), OXR1- (green) immunofluorescence and DAPI nuclear counterstaining (blue) are merged. Immunofluorescence images of single and merged channels of the indicated markers of the same specimen are shown in Fig. S1. In the second column the immunoreactivity for OXA has been detected also by HRP/DAB reaction to show better morphological details. The immunofluorescent signals for OXA and OXR2 appear homogenously diffused within the cells, OXR1 immunofluorescence appear instead principally overlapping nuclear counterstaining. After TUNEL staining and nuclear counterstaining with Methyl Green (third column), granulosa cells of all these type of follicles resulted TUNEL negative. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

or OXR2-IR. IR for the antibodies tested was also observed in endothelial cells of vessels surrounding the follicles.

3.1.2. Atretic follicles

In small antral atretic follicles (Fig. 4 and Supplementary Fig. S3 in the online version at DOI: 10.1016/j.aanat.2018.04.006), in which a degenerating oocyte and scattered surrounding granulosa cells were visible, the fluorescent signals due to the IR for OXA and its receptors were, in general, colocalized and homogeneously diffused. Granulosa cells showed also intense colocalized IR of granular appearance in the cytoplasm, while their nuclei showed IR in particular for OXR1. A diffuse IR was visible also in the zona pellucida. Only some scattered granulosa cells in these follicles resulted TUNEL positive.

Granulosa cells of small and medium sized atretic follicles, disaggregating and invading the antrum, resulted instead strongly TUNEL positive. In these cells the IR for OXA had principally granular shape and was localized in the cytoplasm but also, to a lesser extent, at nuclear level. On the contrary, the nuclear IR for OXR1 seems to be more evident even in karyopyknotic cells and in cell debris with con-

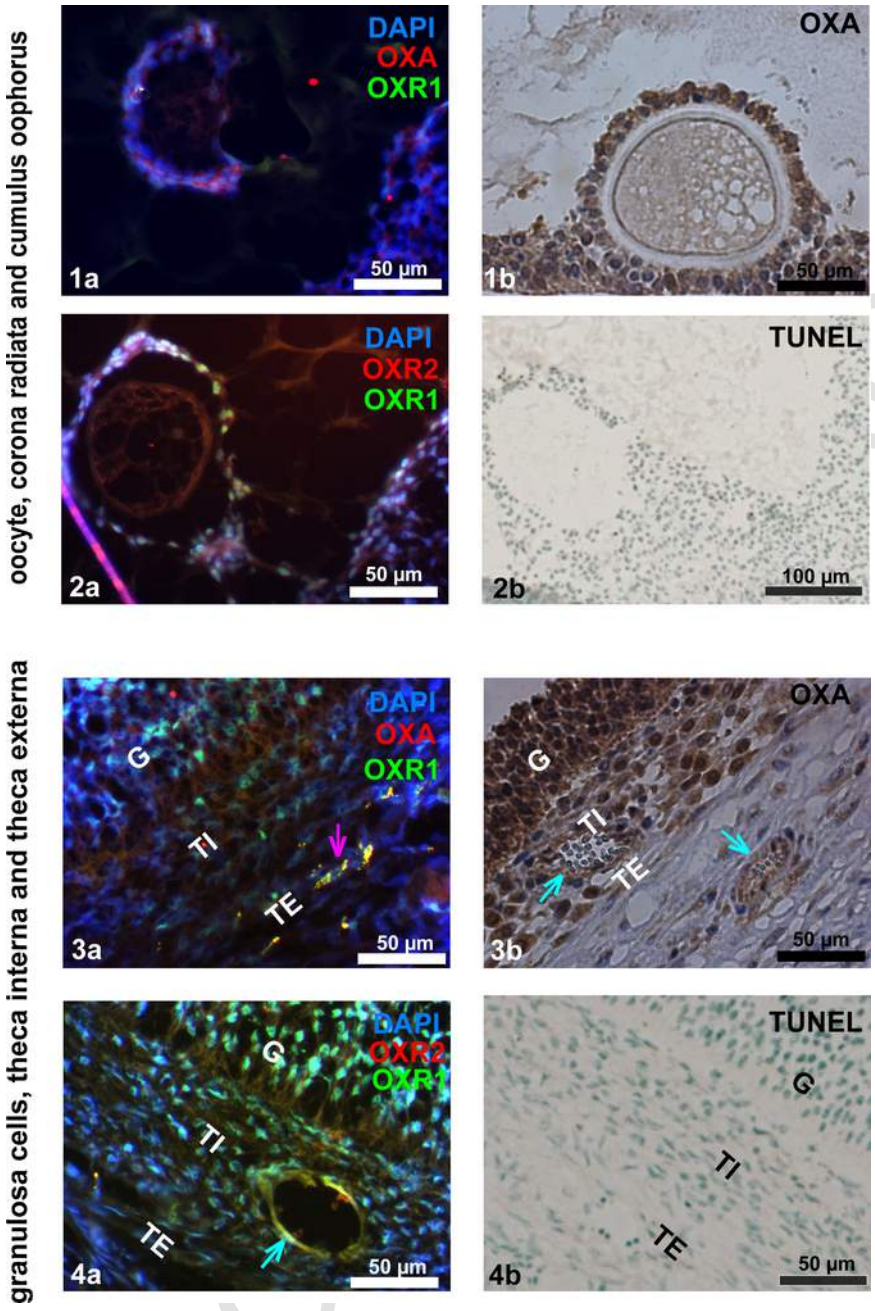

Fig. 3. Localization of OXA and its receptors and TUNEL staining results in porcine large (diameter >6 mm) ovarian follicles. In the first column images of OXA- or OXR2- (red), OXR1- (green) immunofluorescence and DAPI nuclear counterstaining (blue) are merged. Immunofluorescence images of single and merged channels of the indicated markers of the same specimen are shown in Fig. S2. In 1b and 3b the immunoreactivity for OXA has been detected also by HRP/DAB reaction to show better morphological details. In the follicular and theca interna cells, the immunofluorescent signals for OXA and OXR2 appear homogenously diffused within the cells, while OXR1 immunofluorescence principally overlaps nuclear counterstaining. In some scattered theca externa cells OXA- and OXR1-immunoreactivity displayed a granular appearance (pink arrows) and did not overlap DAPI staining. Immunoreactivity for the antibodies tested was also observed in endothelial cells of vessels (3b, 4a; blue arrows). After TUNEL staining and nuclear counterstaining with Methyl Green (2b, 4b), all the cells resulted TUNEL negative. (G) Granulosa cells, (TI) theca interna, (TE) theca externa. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

densed chromatin. The IR for OXR2 was less evident and predominantly colocalized with OXR1-IR.

In atretic follicles showing a collapsing glassy membrane, easily identifiable *theca interna* cells showed the same pattern of IR (both diffused in the cytoplasm and of granular aspect and always colocalized) above described for granulosa cells. Only a small number of these *theca interna* cells resulted TUNEL positive.

In the theca interna of some atretic follicles, roundish cells with diameter ranging between 10–15 µm and bilobated nucleus showed intense fluorescent staining homogeneously diffused in the cytoplasm, when observed with the filters for FITC and Texas Red (Fig. 5 and Supplementary Fig. S4 in the online version at DOI: 10.1016/j.aanat.2018.04.006).

In H&E images the cytoplasm of these cells resulted highly eosinophil. In light microscopy images observed for the detection of

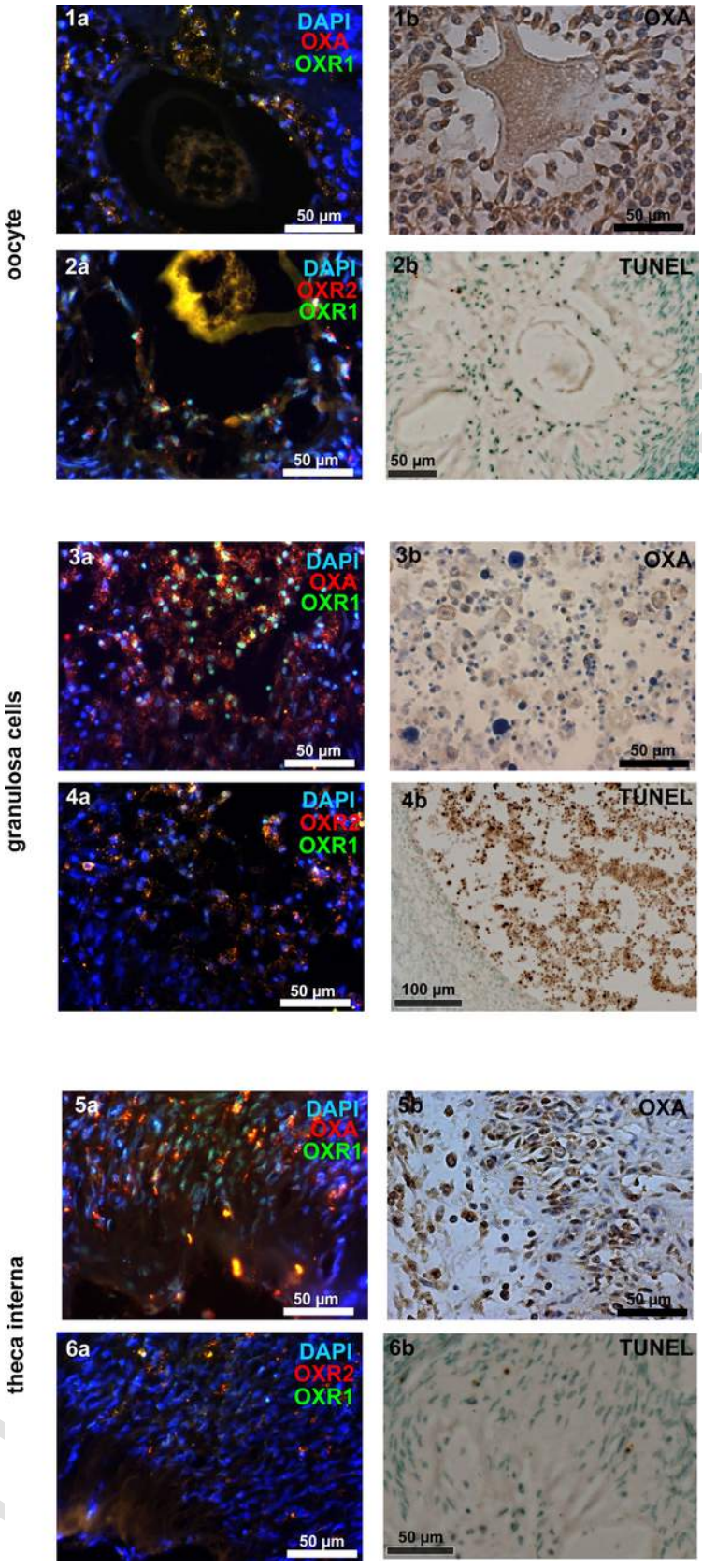
oocyte
granulosa cells
theca interna
1a
DAPI
OXA
OXR1
50 µm
1b
OXA
50 µm
2a
DAPI
OXR2
OXR1
50 µm
2b
TUNEL
50 µm
3a
DAPI
OXA
OXR1
50 µm
3b
OXA
50 µm
4a
DAPI
OXR2
OXR1
50 µm
4b
TUNEL
100 µm
5a
DAPI
OXA
OXR1
50 µm
5b
OXA
50 µm
6a
DAPI
OXR2
OXR1
50 µm
6b
TUNEL
50 µm

Fig. 4. Localization of OXA and its receptors and TUNEL staining results in small and medium sized atretic follicles. In the first column images of OXA- or OXR2- (red), OXR1- (green) immunofluorescence and DAPI nuclear counterstaining (blue) are merged. Immunofluorescence images of single and merged channels of the indicated markers of the same specimen are shown in Fig. S3. The immunoreactivity for OXA has been detected also by HRP/DAB reaction to show better morphological details (1b, 3b, 5b). The degenerating oocyte and the zona pellucida show OXA IR (1b). The surrounding granulosa cells, scattered on the inner surface of the follicular wall, show both intense OXA immunoreactivity (1a, 1b) and cytoplasmic granules contemporaneously immunofluorescent for all the antibodies (1a, 2a) but only occasionally TUNEL positivity (2b). Detached and degenerating granulosa cells show characteristic changes in nuclei (oligonucleosomal fragmentation and karyopycnosis) (3b) and intense TUNEL positivity (4b). They are accompanied by an increase of immunofluorescence of granular appearance with colocalization of OXA and both its receptors (3a, 4a). In follicles in which the basement membrane between the stratum granulosum and the theca interna become very prominent, many theca interna and theca externa cells show the same type of immunoreactivity (5a, 5b, 6a), but only some scattered cells result TUNEL positive (6b). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

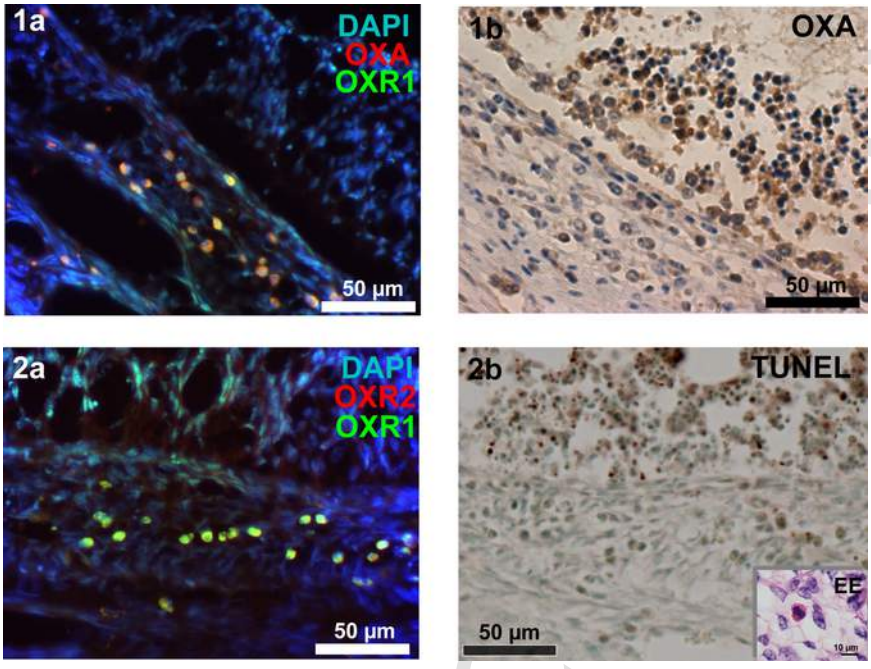

Fig. 5. Presence of leucocytes in the theca interna and externa of atretic follicles. These cells are recognizable as eosinophils in H&E stained sections (lower left corner in Image 2b). In 1a and 2a, images of OXA- or OXR2- (red), OXR1- (green) immunofluorescence and DAPI nuclear counterstaining (blue) are merged. Immunofluorescence images of single and merged channels of the indicated markers of the same specimen are shown in Fig. S4. The immunoreactivity for OXA has been detected also by HRP/DAB reaction to show better morphological details (1b). The immunofluorescence for OXA, OXR2 (2a) and OXR1 (1a, 2a) appears homogeneously distributed and colocalized in their cytoplasm. After TUNEL staining and nuclear counterstaining with Methyl Green these cells show scarce positivity compared to that of granulosa cells (2b). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

OXA IR and TUNEL staining positivity, the cytoplasm of these cells appeared only lightly stained.

3.1.3. Corpora albicantia

In the corpora albicantia, very large numbers of irregular shaped cells exhibited numerous cytoplasmic granulations simultaneously immunoreactive to all antibodies. The granular appearance of the staining proves that it was related to cytoplasmic vesicles. The immunofluorescent cells were scattered throughout the corpus albicans, but in certain areas they also formed small clusters that, at low magnification, appeared as brighter areas. Only a small number of these cells resulted TUNEL positive (Fig. 6 and Supplementary Fig. S5 in the online version at DOI: 10.1016/j.aanat.2018.04.006).

3.1.4. Interstitial cells

Interstitial cells with irregular shape and containing numerous cytoplasmic granulations simultaneously immunoreactive to all the antibodies tested, were abundantly present in the connective tissue both between large pre-ovulatory follicles (Fig. 7 and Supplementary Fig. S6 in the online version at DOI: 10.1016/j.aanat.2018.04.006) (see also Ciccimarra et al., 2018) and, more superficially in the ovary, among developing follicles (see also Fig. 2 and Supplementary Fig. S1 in the online version at DOI: 10.1016/j.aanat.2018.04.006; lines 1–3). The immunofluorescent staining in these cells did not overlap that of DAPI. Only some scattered interstitial cells resulted TUNEL positive.

3.2. Ovaries in luteal phase

3.2.1. Corpora lutea

In the corpora hemorrhagica and corpora lutea (Fig. 8 and Supplementary S7 in the online version at DOI: 10.1016/j.aanat.2018.04.006), the IR for OXA was diffusely present in the cytoplasm and on the nucleus of the luteal cells, while the IR for the receptors, in particular for OXR1, overlapped the DAPI staining, however a certain IR for the two receptors appeared also homogeneously diffused in the cytoplasm. Luteal cells resulted TUNEL negative. In some corpora lutea, in particular in the septa derived by the folding of the theca folliculi, also endothelial cells of blood vessels showed colocalized IR for OXA and its receptors. Moreover, some small scattered irregular shaped cells in the septa derived by the folding of the theca folliculi (presumably small luteal cells), showed colocalized IF for the two receptors. This IF had granular shape and was localized in the cytoplasm. Almost all these cells resulted TUNEL negative.

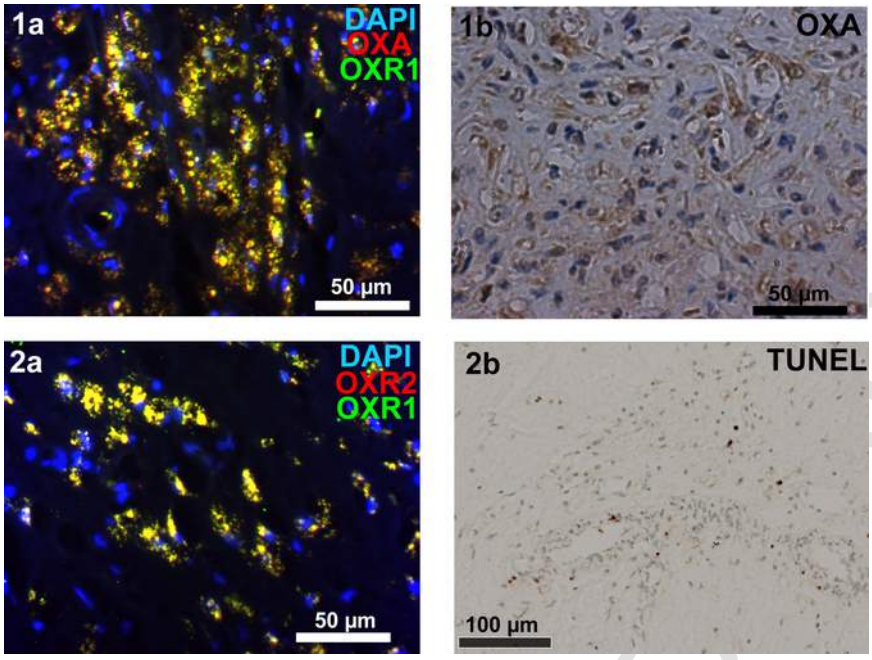

Fig. 6. Localization of OXA and its receptors in some cells of a corpus albicans. In 1a and 2a, images of OXA- or OXR2- (red), OXR1- (green) immunofluorescence and DAPI nuclear counterstaining (blue) are merged. Immunofluorescence images of single and merged channels of the indicated markers of the same specimen are shown in Fig. S5. The immunoreactivity for OXA has been detected also by HRP/DAB reaction to show better morphological details (1b). In the merged images the fluorescent signals for OXA-, OXR1 and OXR2 have granular appearance, are colocalized and do not overlap DAPI counterstaining of the nuclei. After TUNEL staining and nuclear counterstaining with Methyl Green (2b), only some scattered cells resulted TUNEL positive. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

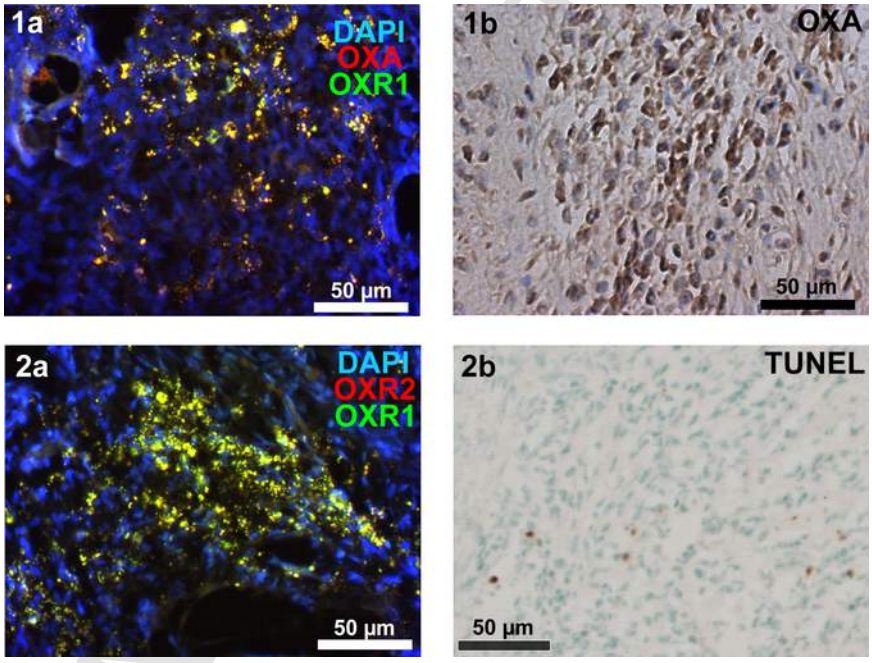

Fig. 7. Localization of OXA and its receptors in interstitial cells of a swine ovary in follicular phase. In 1a and 2a, images of OXA- or OXR2- (red), OXR1- (green) immunofluorescence and DAPI nuclear counterstaining (blue) are merged. Immunofluorescence images of single and merged channels of the indicated markers of the same specimen are shown in Fig. S6. The immunoreactivity for OXA has been detected also by HRP/DAB reaction to show better morphological details (1b). In the merged images, the fluorescent signals for OXA-, OXR1 and OXR2 have granular appearance, are colocalized and do not overlap DAPI counterstaining of the nuclei. After TUNEL staining and nuclear counterstaining with Methyl Green (2b), only some scattered interstitial cells resulted TUNEL positive. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

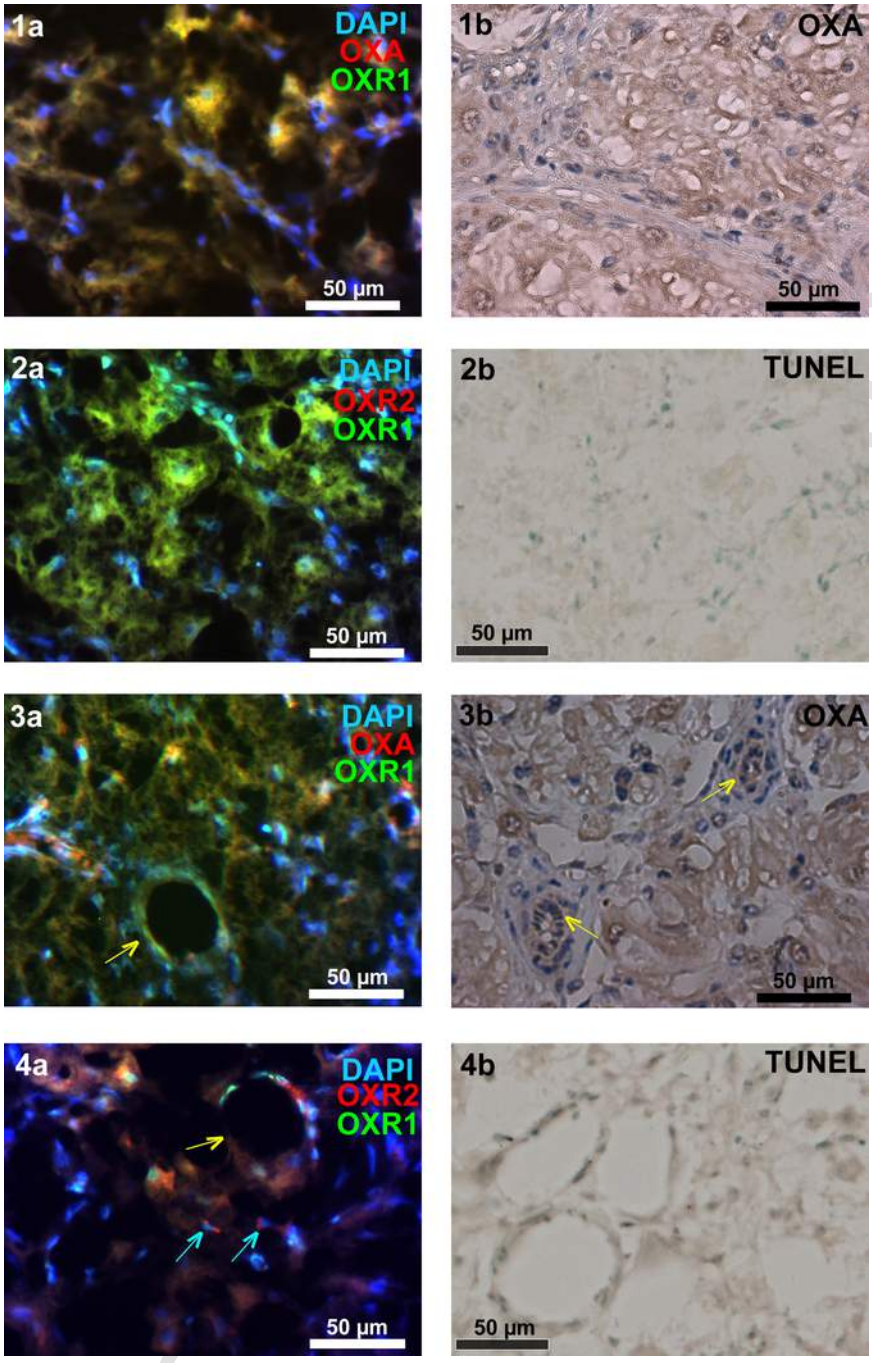

Fig. 8. Localization of OXA and its receptors in the corpus luteum of an ovary in luteal phase. In the first column images of OXA- or OXR2- (red), OXR1- (green) immunofluorescence and DAPI nuclear counterstaining (blue) are merged. Immunofluorescence images of single and merged channels of the indicated markers of the same specimen are shown in Fig. S7. The immunoreactivity for OXA has been detected also by HRP/DAB reaction to show better morphological details (1b, 3b). In luteal cells OXA-, OXR2-immunoreactivity appear both diffusely distributed in the cytoplasm and overlapping nuclear counterstaining. The same distribution is visible for OXR1-immunofluorescence, but in many cells the positive signal on the nucleus is particularly evident (1a, 2a). In the septa derived by the folding of the theca folliculi, also endothelial cells of blood vessels show the same pattern of immunoreactivity (3a, 3b, 4a; yellow arrows). Some small cells of irregular shape, presumably small luteal cells, show colocalized granular cytoplasmic immunofluorescence for the antibodies tested (4a; blue arrows). After TUNEL staining and nuclear counterstaining with Methyl Green (2e, 4e), almost all the cells resulted TUNEL negative. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2.2. Gametogenic and atretic follicles

In ovaries in luteal phase, the cells of small and medium sized tertiary follicles, both gametogenic (healthy) and atretic, showed the same pattern of IR and TUNEL staining that have been described for the follicular phase.

3.2.3. Interstitial cells

Also in ovaries in luteal phase, strong and colocalized IR for both OXA and OXR1 and for OXR1 and OXR2 was detected in interstitial cells (Fig. 9 and Supplementary Fig. S8 in the online version at DOI: 10.1016/j.aanat.2018.04.006). They appeared as irregular shaped

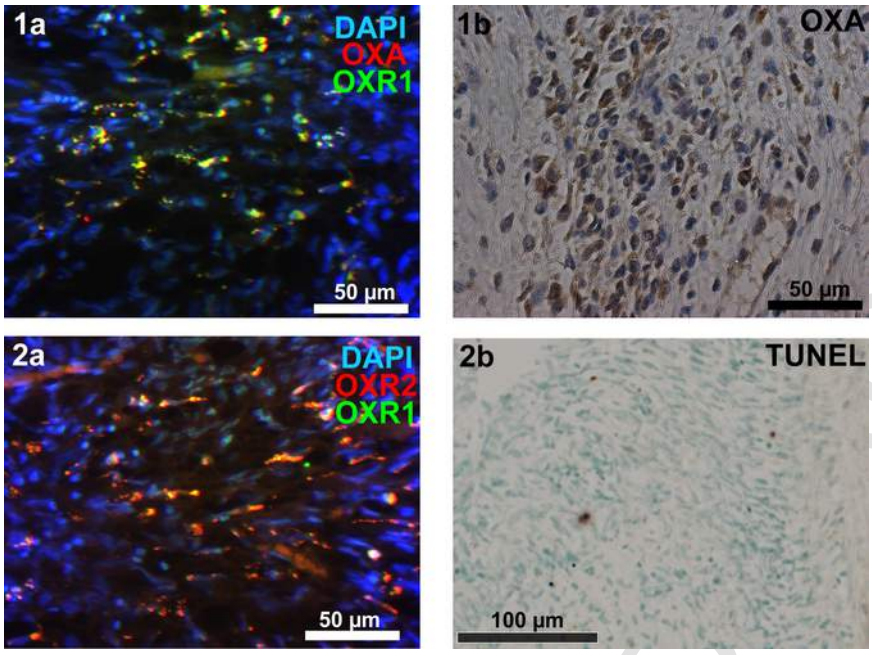

Fig. 9. Localization of OXA and its receptors in the interstitial cells of a swine ovary in luteal phase. In 1a and 2a, images of OXA- or OXR2- (red), OXR1- (green) immunofluorescence and DAPI nuclear counterstaining (blue) are merged. Immunofluorescence images of single and merged channels of the indicated markers of the same specimen are shown in Fig. S8. The immunoreactivity for OXA has been detected also by HRP/DAB reaction to show better morphological details (1b). In the merged images the fluorescent signals for OXA-, OXR1 and OXR2 have granular appearance, are colocalized and do not overlap DAPI counterstaining of the nuclei. After TUNEL staining and nuclear counterstaining with Methyl Green (2b), only some scattered interstitial cells resulted TUNEL positive. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cells and their immunofluorescent staining had granular appearance and did not overlap that of DAPI, being therefore localized at the level of cytoplasmic vesicles. Only a little number of these interstitial cells appeared TUNEL positive.

3.3. Ovarian surface epithelium

The ovarian surface epithelium showed IR for OXA and its receptors, in general colocalized and particularly evident at the nuclear level, however OXA and OXR2 appeared also diffused in the cytoplasm. The surface epithelial cells appeared only occasionally TUNEL positive (Fig. 10 and Supplementary Fig. S9 in the online version at DOI: 10.1016/j.aanat.2018.04.006). The same pattern of IR was observed both in follicular and luteal phase.

As many structures are present in the swine ovary in both follicular and luteal phase and show the same type of localization of OXA and its receptors, we tried to provide a summary of our results independently from the phase of the estrous cycle (Table 1). The subcellular localization of OXA and its receptors seems to vary, depending on the cell physiological state of functional activity or degeneration.

4. Discussion

The results of the present study provide new evidence on the presence and distribution of OXA and its receptors in the swine ovary during both phases of the ovarian cycle. For the first time their immunolocalization and colocalization were compared with the detection of damaged DNA within the cells. The investigation carried out on sections of the whole ovary allowed the study not only of large follicle and corpora lutea isolated from the rest of the organ, but also of follicles at any stage of development, with and without signs of atresia, corpora albicantia, interstitial cells, endothelial cells and ovarian surface epithelium in their original localization.

The variability of the mRNA expression for prepro-orexin, OXR1 and OXR2 and the presence of the related proteins during the phases of the oestrus cycle, had already been proved in the whole ovary (Ning et al., 2008) but without making any distinction between luteal and follicular cells. In more recent years, Nitkiewicz et al. (2010, 2014) designed a better in vivo study, also proving that in vitro administration of orexins influenced the secretion of steroid hormones by ovarian luteal, granulosa and theca interna cells. Polish authors used 7–8 months of age (i.e. mature gilts) experimental animals, whose P4 plasma levels and behavior in the presence of an intact boar were daily monitored. This allowed to confirm the phase of the estrous cycle determined on the basis of ovarian morphology. In these works, however, only corpora lutea or granulosa and theca interna cells of large follicles isolated from the rest of the ovary were analyzed.

We chose to perform this study observing sections of whole organs collected in a local slaughterhouse. This did not allow us to know the exact day of the animal's estrous cycle, but we planned that for our analysis, it was sufficient to have an estimate of the functional phase of the estrous cycle performed on the basis of cyclic changes in the anatomy of the ovary, as widely described in literature (Akins and Morrissette, 1968; McDonald, 1975; Maxson et al., 1985; Babalola and Shapiro, 1988; Oberlender et al., 2014).

Observing panoramic views of whole organs, we realized that large follicles and corpora lutea represented only a small part of the structures that actually expressed OXA and its receptors and that degenerating or regressing cells in the ovary showed different pattern of immunoreactivity compared to cells in a normal state of metabolic activity. As it is known that apoptosis occurs during follicular atresia (Derecka et al., 1995; Sugimoto et al., 1998; Guthrie et al., 1995; Yu et al., 2014; Yu et al., 2004) and luteal regression (Hoyer, 1998; Aboelenain et al., 2015), we decided to compare the expression of OXA and its receptors with the detection of damaged DNA within

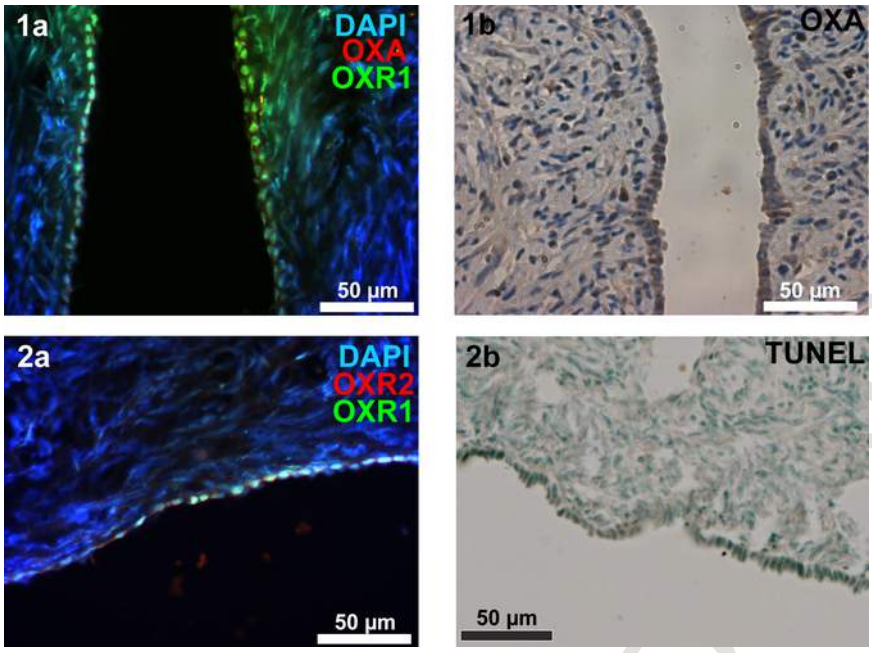

Fig. 10. Localization of OXA and its receptors in the ovarian surface epithelium cells. In 1a and 2a, images of OXA- or OXR2- (red), OXR1- (green) immunofluorescence and DAPI nuclear counterstaining (blue) are merged. Immunofluorescence images of single and merged channels of the indicated markers of the same specimen are shown in Fig. S9. The immunoreactivity for OXA has been detected also by HRP/DAB reaction to show better morphological details (1b). OXA- OXR1- and OXR2-immunoreactivity appear colocalized, and particularly strong in correspondence of the nucleus. After TUNEL staining and nuclear counterstaining with Methyl Green, the vast majority of the cells resulted TUNEL negative (2b). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the cells. Also this part of the study introduces some novelty, because orexins have already been proved to be active on apoptosis and cell growth inhibition (Voisin et al., 2006; Laburthe et al., 2010), but only in cancer cell lines.

The positive signal for OXA and OXR2 IR appeared diffusely distributed in the cytoplasm of the cells of gametogenic (health) follicles and corpora lutea, while the one for OXR1 predominantly overlapped the nuclear counterstaining. The presumable association of this receptor with the membranes of the nuclear envelope and the functional significance of these localizations within gametogenic follicles has already been discussed in our previous work (Ciccimarra et al., 2018) and in another manuscript dealing with the localization within corpora lutea cells, that has been recently accepted for publication (Basini et al., 2018). In general, our results have strengthened the ones of in vitro tests performed by Nitkiewicz et al. (2010, 2014) suggesting that OXA not only stimulates follicular and luteinic cells, but is also produced by them. It would act through a both autocrine and paracrine mechanism of action and would stimulate follicular cells of growing follicles to proliferate and produce estradiol. Instead, in luteal cells, the orexinergic system would be involved in the regression processes, as it has no effect on cellular growth, inhibits progesterone production and reduces the angiogenesis within corpora lutea.

On the other hand, in atretic follicles, either with few or many TUNEL positive cells, corpora albicantia and interstitial cells, OXA and both its receptors appear colocalized and associated with cytoplasmic vesicles, even though OXR1 IR often remains also associated with the nuclear envelope. We would like to stress that, according to Barone (2003), ovarian interstitial cells derive from epithelioid cells of the theca interna of follicles, in particular of the atretic ones. The expression of OXA and its receptors by all these types of cells had never been considered before, but the fluorescent signal, due to the colocalized IR observed, in all these degenerating cells appeared very strong and, interestingly, localized in different cellular compartments, compared to (health) non degenerating cells. It has already been proved that individual GPCRs can be differently localized, not only between the plasma membrane and the various intracellular compartments, but also between different plasma membrane domains, as they move along dynamic trafficking pathways, subjected to physiological regulation (Hislop and von Zastrow, 2011). Orexin receptors signaling cascades are, in fact, multifaceted, highly tunable (Kukkonen and Leonard 2014) and differently distributed (Assisi et al., 2012), depending on the environment in which they are operating. They are able to activate classical plasticity-regulating cascades (Kukkonen and Leonard, 2014), among which the ones contributing to differentiation towards steroid producing cells (Ramanjaneya et al., 2008; Ramanjaneya et al., 2009; Wenzel et al., 2009; Leonard and Kukkonen, 2014).

A continuous stimulation of both orexin receptors subtypes, although with not extremely high orexin levels, may also induce programmed cell death (Laburthe et al., 2010). This has been demonstrated in both recombinant Chinese hamster ovary (CHO) cells and primary cancer cells (Ammoun et al., 2003, 2006; Rouet-Benzineb et al., 2004; Voisin et al., 2006; Voisin et al., 2011; Leonard and Kukkonen, 2014). However, our data demonstrate that such persistent exposure of orexin receptors to orexins take physiologically place in normal ovarian follicle cells turning into luteal cells and, subsequently, into cells of the corpus albicans. This might be one of the mechanisms underlying the cyclical transformations that occur in the ovary.

Colocalized IR for OXA and OXR1 was also observed in the ovarian surface epithelium and in endothelial cells of vessels surrounding follicles (see also Ciccimarra et al., 2018) and corpora lutea. The functional significance of this localizations remains to be explained. However, also these two epithelia undergo cyclic modifications to be regulated. Ovarian follicles growth and ovulation depend on the development of an adequate blood vessels network capable of

Table 1
Immunolocalization of OXA, OXR1 and OXR2 in the different structures of the porcine ovary and TUNEL positivity.

Structure	Type of cells	Immunoreactivity localization		TUNEL staining
		Diffused[a]	Granular[b]	
Gametogenic follicle				
	Oocyte (nucleus not observed)	+/−		
	Granulosa cells	+		
	Theca interna cells	+		
	Theca externa cells	+		
Atretic follicles				
	Degenerating oocyte (nucleus not observed)	+		+
	Granulosa cells	+	++	+++
	Theca interna cells	+	++	++
	Theca externa cells	+	++	++
	Leucocytes	+ (less intensity on the nucleus)		+
Corpus luteum				
	Theca lutein cells	+	+ (only in some cells)	
	Granulosa lutein cells	+		
Corpus albicans			++ (groups of clustered cells)	++
Interstitial cells			++	++
Endothelium		+		
Ovarian surface epithelium		+		+

Intensity of the immunoreactivity indicated by + (least) to +++ (highest)

[a] OXA- OXR1- and OXR2-IR homogeneously diffused in the cytoplasm, OXR1-IR principally evident on the nucleus.

[b] OXA- OXR1- and OXR2-IR colocalized on cytoplasmic vesicles.

supporting the proliferative and endocrine function of the follicular cells (Martelli et al., 2009). Moreover, a conspicuous neovascularization and vascular remodeling, characterized by the appearance of blood vessels with evident smooth musculature, are involved in corpus luteum formation after ovulation (Martelli et al., 2006), while vasculature regression is associated with luteolysis. A link between orexins and the control of circulation has already been suggested by Silveyra et al. (2007) and also our study group recently demonstrated the inhibitory effect of OXA on vessel growth, highlighting its role in corpus luteum regression (Basini et al., 2018).

The orexinergic system could also play a role in the modulation of the cyclic plasticity of the surface ovarian epithelium. During the ovulatory process, the DNA integrity of the surface cells circumjacent to the ovarian rupture site is compromised (Murdoch and McDonnel, 2002), while, after ovulation, the surface cells contribute to postovulatory repair of the ovarian cortex proliferating, migrating over the site of follicular rupture and deposing a basement membrane. A clonal expansion of epithelial cells with damaged (unrepaired) DNA may lead to carcinogenesis (Murdoch and McDonnel, 2002) that, in this case, could be related with the expression of the orexinergic system.

Strong colocalized IR for OXA and its receptors was observed also in eosinophils invading the theca interna extravascular compartment of atretic follicles. The accumulation of eosinophils in this area had already been described, but only in the sheep (Cavender and Murdoch, 1988) and buffalo (Ramadan et al., 2001) ovary during the ovulatory period. The presence of leukocytes is to be expected in the wall of degenerating follicles, as the ovulatory process has been compared with inflammation because several classical inflammatory mediators appear to participate in this process. Moreover, the cells capable of phagocytosis of apoptotic bodies and cell debris include not only neighboring granulosa cells with normal ultrastructure (Tajima et al., 2002), but also cells of mesenchymal origin (Sugimoto et al., 1998). We cannot exclude that the fluorescence observed could be due to eosinophil granules autofluorescence. However, the absorbance and emission spectra of eosinophil granule extract have excitation peaks at 360 nm and 450 nm and a single emission peak at 520 nm (Weil and Chused, 1981). It is also possible that the antibodies used in the present investigation have cross-reacted with some epitope mimicking hypocretin which could be present within these cells. If not, the functional significance of this finding remain to be further investigated. It is conceivable that it might be related with the presence of other mediators often in functional alliance with orexin, like tumor necrosis factor, interleukin-1 and insulin that are ubiquitous, pleiotropic and phylogenetically ancient. They are involved in innate immunity, but have also physiological and disease roles that, at least in literature, overshadow their immune functions. They are often termed proinflammatory cytokines and, depending on their concentration, they modulate normal physiology (including physiological sleep, the innate arm of the immune system) and inflammatory disease processes and progression (Clark and Vissel, 2014).

Finally, the use of the TUNEL staining on sections of the whole ovary has also allowed us to confirm the results of previous studies on the occurrence of apoptosis during follicular atresia in the follicular walls of the porcine ovary (Derecka et al., 1995). These studies have also been performed on granulosa and theca interna layers isolated from follicles and indicated that intranucleosomal DNA fragmentation takes place in granulosa, but not (or on a small scale) in theca interna cells of atretic follicles. So the death of the cells of the different layers of porcine ovarian follicle seems to be induced by different processes. The presence of OXA colocalized with its two receptors seems to be a common factor that is both related to the stimulation of apoptosis in granulosa cells, and of theca internal cells death occurring during follicular atresia.

5. Conclusions

The present work demonstrates that OXA and its two receptors subtypes are present in all the different structures composing the swine ovary, albeit in different ways, in both phases of the ovarian cycle, confirming the pleiotropic characteristics of this neuropeptide. In general, OXA and OXR2 seem to be diffusely distributed within “health”, proliferating and steroid producing cells, while associated to cytoplasmic vesicles in degenerating cells, independently if apoptotic or not. OXR1 IR, instead, is often associated to 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, OXR1 expression is present also in the cytoplasm, in particular on vesicles, and appears 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. They could help to explain the various biological actions of OXA.

The results of the present study may help studies of follicle development in pig as part of in vitro oocyte maturation and fertilization programs in livestock.

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