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Short communication

# Localization of orexin B and orexin-2 receptor in the rat epididymis

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ABSTRACT

The peptides orexin A (OXA) and orexin B (OXB) derived from the proteolytic cleavage of a common precursor molecule, prepro-orexin, were originally described in the rat hypothalamus. Successively, they have been found in many other brain regions as well as in peripheral organs of mammals and other less evolved animals. The widespread localization of orexins accounts for the multiple activities that they exert in the body, including the regulation of energy homeostasis, feeding, metabolism, sleep and arousal, stress, addiction, and cardiovascular and endocrine functions. Both OXA and OXB peptides bind to two G-coupled receptors, orexin-1 (OX1R) and orexin-2 (OX2R) receptor, though with different binding affinity. Altered expression/activity of orexins and their receptors has been associated with a large number of human diseases. Though at present evidence highlighted a role for orexins and cognate receptors in mammalian reproduction, their central and/or local effects on gonadal functions remain poorly known. Here, we investigated the localization of OXB and OX2R in the rat epididymis. Immunohistochemical staining of sections from caput, corpus and cauda segments of the organ showed intense signals for both OXB and OX2R in the principal cells of the lining epithelium, while no staining was detected in the other cell types. Negative results were obtained from immunohistochemical analysis of hypothalamic and testicular tissues from OX2R knock-out mice ($OX2R^{-/-}$) and OX1R/OX2R double knock-out ($OX1R^{-/-}$; $OX2R^{-/-}$) mice, thus demonstrating the specificity of the rabbit polyclonal anti-OX2R antibody used in our study. On contrary, the same antibody clearly showed the presence of OX2R in sections from hypothalamus and testis of normal mice and rats which are well known to express the receptor. Thus, our results provide the first definite evidence for the immunohistochemical localization of OXB and OX2R in the principal cells of rat epididymis.

## 1. Introduction

Orexin A (OXA) and orexin B (OXB) are two peptides initially discovered in the mammalian hypothalamus (Sakurai et al., 1998; de Lecea et al., 1998) and subsequently found in other regions of the brain and peripheral organs. Both peptides originate from the proteolytic cleavage of a common precursor molecule, prepro-orexin, and bind to two G-coupled receptors, namely orexin-1 (OX1R) and orexin-2 (OX2R) receptor. While OX1R is highly selective for OXA, OX2R shows similar binding affinity for both orexins (Sakurai et al., 1998). The orexins and their receptors will be refereed hereunder as the orexinergic complex.

A huge amount of evidence demonstrated the involvement of orexinergic complex in the regulation of a variety of physiological processes including energy homeostasis, feeding, sleep and arousal, neuroendocrine and autonomic responses, addiction, cognition, mood, and many other (Silvani and Dampney, 2013; Chieffi et al., 2017; Kukkonen, 2013; Ma et al., 2018; Mieda and Sakurai, 2009; Tafuri et al., 2017; Zhou and Leri, 2016). Furthermore, the orexinergic complex plays important roles in the regulation of the mammalian nervous and digestive system, cardiovascular apparatus, pancreas, adrenal glands, and reproductive tract (Celik et al., 2015; Heinonen et al., 2008; Korczynski et al., 2006; Kukkonen, 2017; Rani et al., 2017; Voisin et al., 2003). Disturbances in the release of the two peptides and/or altered expression levels of their receptors contribute to the onset of many human diseases including insomnia, narcolepsy with cataplexy, drug addiction, major mood disorders, obesity, cardiovascular and neuroendrocrine disorders, cancers, and other (Rainero et al., 2011; Alexandre et al., 2014; Burfeind et al., 2016; Ferini-Strambi, 2014; James et al., 2017; Nixon et al., 2015; Song et al., 2015; Szczepanska-Sadowska et al., 2010). Currently, orexin receptors are active targets in

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a number of therapeutic areas (Andrews et al., 2016; Boss and Roch, 2017; Tanaka et al., 2016; Xu et al., 2013).

Although in recent years increasing attention has been drawn to address the role of the orexinergic complex in the mammalian reproductive axis (Celik et al., 2015; Nurmio et al., 2010; Silveyra et al., 2010), the central and/or local effects of orexins on gonadal functions remain to be assessed. Expression of OXA and OX2R in the placenta of cat and dog (Dall'Aglio et al., 2012, 2014), OXA and OXB in the porcine uterus (Nitkiewicz et al., 2012), OX1R and OX2R in the rat and porcine ovaries (Nitkiewicz et al., 2010; Silveyra et al., 2007), and OXA and OX1R in the vestibular glands of the cattle genital tract (Pavone et al., 2009) has been demonstrated. These findings, coupled with functional studies, indicated that orexins and their receptors affect female reproductive functions through the modulation of ovarian steroidogenesis.

As far as it concerns the presence of the orexinergic complex in the male, Karteris et al. (2004) detected OX1R and OX2R in human testis, epididymis, penis, and seminal vesicle. In particular, receptor expression was found in Leydig cells, myoid cells of seminiferous tubules, and Sertoli cells. Orexin receptors have been shown to be expressed in the testes of rat (Jöhren et al., 2001), chicken (Ohkubo et al., 2003), sheep (Zhang et al., 2005) and boar (Russo et al., 2014). The expression of OXA and OX1R in the mouse testis at different stages of postnatal development has been reported (Joshi and Singh, 2016, 2017). Recently, we demonstrated OXA and OX1R immunohistochemical localization in multiple organs of the mammalian male reproductive apparatus, often combined with the expression of mRNAs coding for pre-proorexin and OX1R, and the corresponding proteins. In particular, we provided evidence for the presence of OXA and OX1R in the urethroprostatic complex of the cattle (Russo et al., 2008), epididymis (Liguori et al., 2014; Tafuri et al., 2009) and testis of rat (Assisi et al., 2012; Tafuri et al., 2010) and alpaca (Liguori et al., 2012), and in human normal, hyperplastic and neoplastic prostates (Alexandre et al., 2014; Valiante et al., 2013, 2015). Functional studies suggest a potential involvement of OXA and OX1R in the control of the Leydig cell steroidogenesis as well as in the development of the seminiferous epithelium (Assisi et al., 2012; Barreiro et al., 2005; Tafuri et al., 2010).

By contrast, the expression and role of OXB and OX2R in the male gonads have been poorly investigated. We recently demonstrated the expression of OXB and OX2R in pachytene and secondary spermatocytes and in spermatids through all stages of the seminiferous epithelium cycle of the rat and alpaca testis (Liguori et al., 2017a,b). OXB, in contrast to OXA, was not found to promote steroidogenesis. In order to get more insights into the localization of OXB and OX2R in the reproductive apparatus, in this study we explored the presence of OXB and OX2R in the rat epididymis by immunohistochemistry. To ensure specificity of the anti-OX2R antibody used, we tested the antibody on hypothalamic and testicular tissues from OX2R knock-out ($OX2R^{-/-}$) mice and OX1R/OX2R double knock-out ($OX1R^{-/-}/OX2R^{-/-}$) mice.

## 2. Materials and methods

### 2.1. Antibodies and reagents

Mouse anti-OXB (MAB734) monoclonal antibodies were purchased from R&D System (Abingdon, UK) and their synthetic peptides from Tocris Bioscience (Bristol, UK); rabbit polyclonal anti-OX2R antibody (ab3094), and its blocking peptide (AG794) from Millipore (Bellerica, MA, USA); biotinylated goat anti-mouse (BA-9200) and goat anti-rabbit (BA-1000) secondary antibodies, and peroxidase conjugated avidin–biotin complex (PK-6105) from Vector Laboratories (Burlingame, CA, USA).

### 2.2. Animals

Eight healthy adult Wistar male rats (Charles River, Calco, LC, Italy) were bred in the vivarium of the Department of Veterinary Medicine and Animal Productions of the University of Naples Federico II. The animals were kept with free access to food and tap water, under constant conditions of light and temperature (22 °C). The experimental procedures were approved by the Ethical Committee for Animal Experimentation of our Universities, and were conducted in accordance with the EU Directive 2010/63/EU for animal experiments. The anesthetized animals were sacrificed and epididymes were collected and divided in three segments (caput, corpus and cauda); each segment was transversely cut in small pieces. Specimens were fixed in Bouin's fluid for 24–48 h, and then processed for immunohistochemistry as described below. Anesthetized $OX2R^{-/-}$ and $OX1R^{-/-}/OX2R^{-/-}$ mice were transcardiacally perfused with 4% paraformaldehyde, and their hypothalami and testes were fixed in the same fluid for 24 h after collection. Such materials were cryoprotected in 40% sucrose and successively embedded in OCT to obtain 10 µm cryocut sections.

### 2.3. Immunohistochemistry

The fixed samples from caput, corpus and cauda segments of epididymis were dehydrated in ascending alcohols, embedded in Paraplast, and microtomically cut in 6 µm thick sections. The avidin-biotin immunohistochemical procedure was performed as previously described (De Luca et al., 2014; Pelagalli et al., 2016). Briefly, sections were covered with normal goat serum, and then incubated with the primary antibodies 1:200 dilution, for overnight at 6 °C. Reactions were detected with a goat anti-mouse or goat anti-rabbit secondary antibody. Successively, sections were incubated with peroxidase conjugated avidin-biotin complex (ABC) for 30 min and 3-3′ diaminobenzidine (DAB) was used as final staining. Some sections were counterstained with hematoxylin in order to better localize the immunoreactive structures and the epididymal cytotypes. The preparations were observed by a Nikon Eclipse E 600 light microscope, and photographed by a Coolpix 8400 digital camera.

Sections of hypothalamus and testis from $OX2R^{-/-}$ and $OX1R^{-/-}/OX2R^{-/-}$ mice (Irukayama-Tomobe et al., 2017) in the C57BL6/J background were used as negative controls for immunohistochemistry with anti-OX2R antibody. We used paraplast sections from wild-type mouse hypothalamus and rat testis, both tissues well known to express OX2R (Irukayama-Tomobe et al., 2017; Liguori et al., 2017a), as positive controls.

## 3. Results and discussion

Multiple studies demonstrated that orexins may affect mammalian reproductive functions either centrally or locally. However, the specific tissue localization and the exact mechanism of action of orexins in the epididymal/gonadal complex has not been fully explored. In order to extend our current knowledge on this issue, here we investigated the localization of OXB and OX2R in the rat epididymis by immunohistochemistry. The analysis of the tissue samples showed the presence of OXB immunoreactivity (IR) in the epithelium of all segments (caput, corpus, and cauda) of the epididymis (Fig. 1). The caput of epididymis is characterized by a proximal and a distal portion. In the proximal caput, single or scattered epithelial principal cells were intensely stained (Fig. 1a). The positive material filled the entire profile of the cells from the basal to the apical portion. OXB-IR was also found in the distal caput (Fig. 1b), corpus (Fig. 1c) and cauda (Fig. 1d) of the epididymis. In these segments, the positive material appeared as condensed granules, localized in the supra-nuclear portion of the principal cell cytoplasm. The amount of staining in these segments was much higher than that observed in the proximal caput, and sometimes positive cells were seen to encircle almost entirely the transverse profile of the epididymal tubule, rarely intermingled by negative elements. Throughout the organ the distribution of the positive material was zonal. Negative controls were performed substituting the anti-OXB

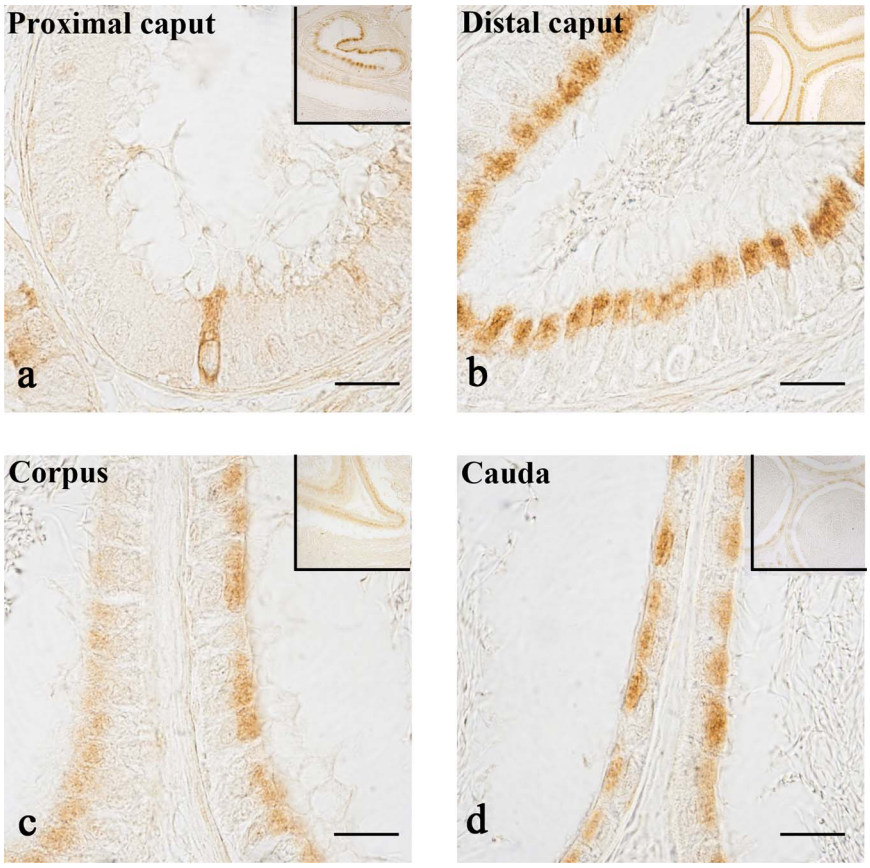


**Fig. 1.** OXB-IR in the rat epididymis. a: OXB-IR was found in principal cells in few tubules (insert) of the proximal caput. The immunoreactive material filled the entire profile of the cell cytoplasm. b–d (inserts): in the distal caput (b, insert), corpus (c, insert) and cauda (d, insert) of the epididymis rows of positive cells were seen to encircle almost completely the transverse profile of the organ. Avidin-biotin immunohistochemical method. Bars: 20 μm.

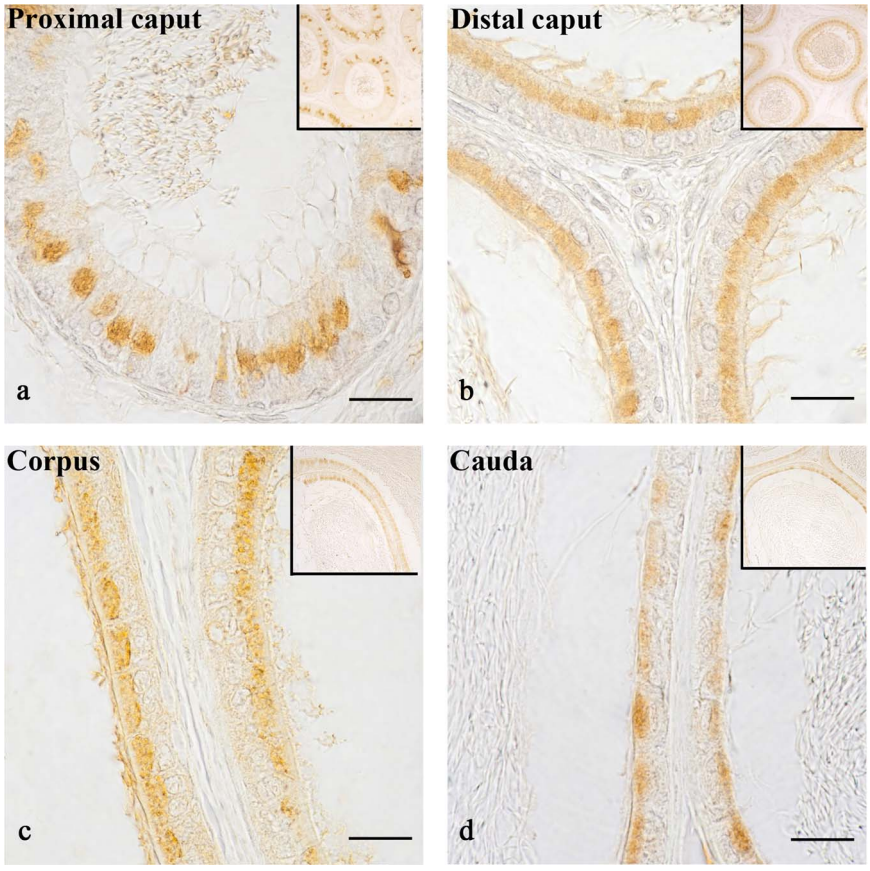


**Fig. 2.** OX2R-IR in the rat epididymis. a: in the proximal caput, the immunoreactive material was granular in shape and contained in the luminal and infranuclear portions of the principal cells (insert). b–d: also in the distal caput (b, insert), corpus (c, insert) and cauda (d, insert) of the epididymis the immunoreactive material was found in the principal cytotype alone. In these cells such material showed always a supranuclear localization. Only few negative cells are intermingled among the positive ones. Avidin-biotin immunohistochemical method. Bars: 20 μm.

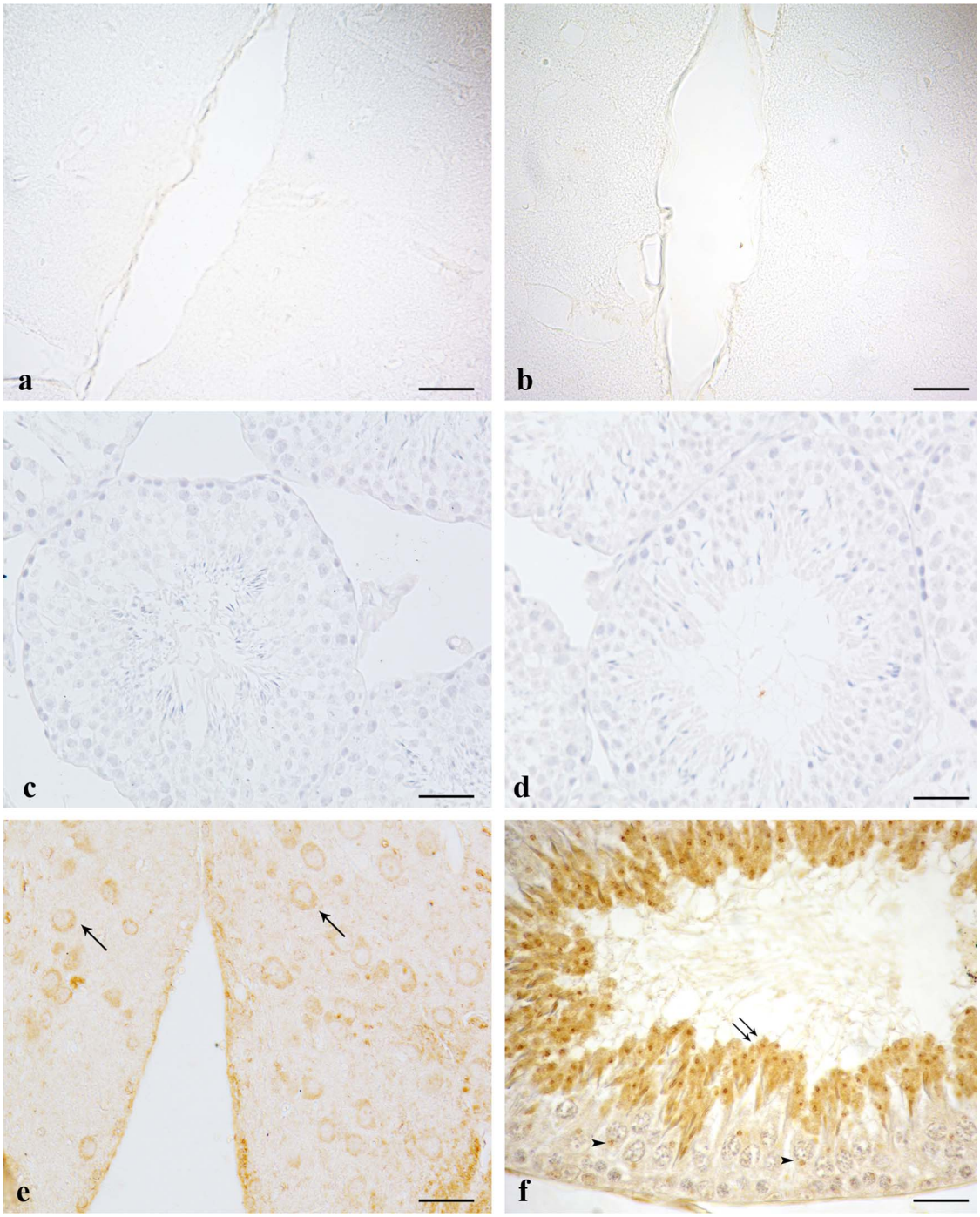


**Fig. 3.** Testing of OX2R antibody specificity in positive and negative controls. a–d: negative controls from the hypothalamus (a,b) and testis (c.d) of $OX2R^{-/-}$ and $OX1R^{-/-}/OX2R^{-/-}$ mice. e,f: positive controls from wild-type mouse hypothalamus (e) and rat testis (f). Positive neurons (arrows) and, immunoreactive pachytene spermatocytes (arrowheads) and elongated spermatids (double arrow), are clearly visible in the respective tissues. Avidin-biotin immunohistochemical method. Bars: 20 µm.

antibody with phosphate buffered saline or with the same antiserum pre-absorbed with an excess (100 µg/ml) of the synthetic peptide. Both controls gave negative results (data not shown). In order to test whether the anti-OXB antibody cross reacted with OXA, we pre-absorbed the anti-OXB antibody with the heterologous peptide (OXA), before staining. The results showed no effect on the observed staining (data not shown). In previous studies in the rat and alpaca testes (Liguori et al., 2017a,b), we stained alternately 3 µm consecutive sections of the gonad with the anti-OXA and anti-OXB antibodies here used, and we found that OXA-IR never co-existed with OXB-IR. These results account for a lacking of cross-reactivity of the anti-OXB antibody here used with the improper antigen OXA.

The profile of OX2R-IR was similar to that described for OXB in the proximal (Fig. 2a) and distal caput (Fig. 2b), corpus (Fig. 2c) and cauda (Fig. 2d) of the epididymis. In the proximal caput, the immuno-reactive material was widely distributed in the cytoplasm of the principal cells (Fig. 2a), and the positive cells appeared more intensely stained than those that contained OXB. The positive material was clustered in the supra-nuclear portion of the cytoplasm in the immunoreactive cells observed along the remaining segments of the epididymis (Fig. 2b–d). Negative controls were performed substituting the anti-OX2R antibody with phosphate buffered saline or with the same antiserum pre-

absorbed with an excess (100 μg/ml) of its blocking peptide. Both controls gave negative results (data not shown). However, due to the debate on the poor selectivity of commercial antibodies against OX2R or even the ability to detect their targets (Chen et al., 2013), we also tested the commercially available rabbit polyclonal anti-OX2R antibody, used in our immunohistochemical analysis, on hypothalamic and testicular tissues from $OX2R^{-/-}$ and $OX1R^{-/-}/OX2R^{-/-}$ mice. Tissues from hypothalamus of wild-type mouse and rat testis were used as positive controls. No staining was observed in the tissues from both types of knockout mice (Fig. 3a–d), while immune-reactive material was detected in mouse hypothalamus and rat testis (Fig. 3e,f). In particular, OX2R-IR was found in neurons of the wild type mouse hypothalamus (Fig. 3e) and in the cytoplasm of pachytene spermatocytes and elongated (mature) spermatids of the rat testis (Fig. 3f). The results obtained demonstrated the specificity of the anti-OX2R antibody used in this work.

Our study highlighted the localization of both OXB and OX2R in the principal cells of the epididymis epithelium, while no staining was observed in the other cell types such as basal, apical and clear cells. Our previous study demonstrated that also OXA and OX1R are localized in the principal cells of rat (Tafuri et al., 2009) and South American camelid alpaca (Liguori et al., 2014) epididymis. The simultaneous presence of both OXA and OXB peptides in the epididymis well correlates with the expression of prepro-orexin mRNA transcripts and the precursor protein molecule previously detected in this tissue (Tafuri et al., 2009; Liguori et al., 2014). Indeed, OXA and OXB may be locally synthesized in the epididymis by the proteolytic cleavage of prepro-orexin.

The supranuclear localization of OXA and OXB in the principal cells suggests that they may act as paracrine signaling molecules which can be rapidly spread to targets even far away. However, the precise molecular mechanism of action of the orexinergic complex in the epididymis functions remains to be addressed. At present, it is worth to note that orexins share neuro-endocrine origin, modality of action, cytoplasmic localization in epididymal principal cells, and binding to G type receptors similar to the peptides secretin (Chow et al., 2004) and pituitary adenilate cyclase-activating peptide (PACAP) (Leung et al., 1998; Zhou et al., 1997) which play major roles in controlling electrolyte transport in the epididymal lining epithelium, thus regulating hydration of the luminal fluid. However, while PACAP acting on the apical side of the epididymis epithelium stimulates chloride but not bicarbonate secretion, secretin acting on both apical and basolateral sides promotes chloride and bicarbonate secretion (Chow et al., 2004). Furthermore, the stimulation by PACAP but not secretin requires local prostaglandin synthesis. Interestingly, both secretin and PACAP exhibit a regional difference in their expression along the epididymal duct, thus suggesting that they may act in a paracrine or autocrine fashion in the regulation of epididymal fluid secretion (Leung et al., 1998)., As a consequence of the above mentioned similarities between orexins and the two peptides secretin and PACAP, at the moment we can only speculate that OXA and OXB might be involved in the absorptive and secretory activity of the epididymis, thus regulating epididymal and sperm functions along the epididymal duct.

## 4. Conclusions

The results of this study provide the first evidence of the immunohistochemical localization of OXB and OX2R receptor in the principal cells of rat epididymis. In the same organ, we had previously demonstrated the expression of prepro-orexin and OX1R mRNA transcripts and the respective proteins as well as OXA immunohistochemical localization. Functional investigations are required to establish the precise roles of the two peptides and their cognate receptors in the physiology of epididymis. A better understanding of whether and how OXB and OXA and their receptors may concur to regulate the environmental conditions that allow the epididymal tubule to fertilize the immature male gamete is a mandatory need to address the involvement of orexinergic complex in the mammalian reproduction.

## Author contributions

AC, AV and NM contributed to the experimental design, the interpretation of results, and critical review of the manuscript draft; CS, GL, ST, and VDP collected the samples, performed the experiments, discussed the results, and contributed to write the manuscript draft; CM and MY provided the specimens from knock out mice, discussed the results, and contributed to review the manuscript draft. All the authors approved the final version of the manuscript.

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