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Rapid communication

Expression of orexin A and its receptor 1 in the rat epididymis

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ABSTRACT

The hypothalamic peptide orexin A (oxA) derives from the proteolytic cleavage of the precursor molecule prepro-orexin. It binds with the high affinity G-protein-coupled orexin receptor 1 (OX1R). Here, we report the detection of oxA and OX1R in the principal cells of the rat caudal epididymis by immunohistochemistry. Both oxA and OX1R immunolabelling showed cytoplasmic supranuclear localization, filling the apical portion of the cells. The expression of prepro-orexin and OX1R mRNA transcripts in the rat epididymis was assessed by reverse-transcriptase polymerase chain reaction, while the presence of both these proteins in the tissue was confirmed by Western blotting analysis. Our findings provide the evidence for the presence of oxA and OX1R in the rat epididymis, and demonstrate that both proteins are locally synthesised, thus suggesting a role for oxA in governing the fertilizing capability of the immature male gamete.

1. Introduction

Orexin A (oxA) and orexin B are two peptides expressed in the lateral hypothalamus and adjacent regions [1,2]. They are produced from a common precursor molecule, prepro-orexin, by usual proteolytic processing, and bind two G-protein-coupled receptors, termed orexin receptor 1 (OX1R) and orexin receptor 2 (OX2R). While OX2R shows similar affinity for both neuropeptides, OX1R is highly selective for oxA [1].

The originally detected localization of orexins in the lateral hypothalamic area, a region with a key role in the control of food intake and energy balance, suggested their regulatory function in feeding behaviours [1] and energy homeostasis [3–6]. Subsequently, the observation that orexinergic fibers also project into locus coeruleus and the raphe nucleus [2], two centres known to regulate arousal, suggested the involvement of orexins in the regulation of sleep–wake cycle [7]. Orexin knockout mice exhibit a phenotype similar to human narcolepsy [8]. Furthermore, orexins stimulate the cardiovascular system via activation of the central sympathetic outflow [9].

In addition to these central functions, multiple evidence demonstrate that orexins may also function outside the central nervous system [3,10]. The neuropeptides and their receptors are expressed in a wide range of peripheral tissues, including the intestine, pancreas, adrenals, kidney, adipose tissue and reproductive tract [11–16]. Thus, endocrine, paracrine and neurocrine roles for orexins in the periphery have been established, including the regulation of insulin release and intestinal motility, the control of pancreatic hormone secretion and glucose metabolism, the activation of both central and peripheral branches of the hypothalamus–pituitary–adrenal axis [10,15–18]. Orexins have also been implicated in sexual behaviours [19].

In the context of the orexin action on reproductive functions, it is noteworthy the finding that testis, after the brain, represents the tissue holding the highest orexin expression levels reported so far [12,20]. Barreiro et al. [18] provided evidence for the regulated expression of oxA in the rat testis and its potential involvement in the control of steroidogenesis and seminiferous tubule functions. We recently demonstrated the expression of oxA and its receptor OX1R in the urethro-prostatic complex and vestibular glands of cattle [21,22]. Furthermore, the expression of orexin receptor mRNA transcripts has been assessed in the testis of rats [12], sheep [23] and chicken [24] and in the testis, seminal vesicles, penis and epididymis of humans [25]. However, the precise mechanism of action of the oxA and its cognate receptors in the peripheral organs is far to be fully elucidated.

In order to further highlight the sites of action of orexins in the control of male reproductive axis, in this study we investigated the localization of oxA and its receptor OX1R in the rat epididymis by immunohistochemistry. The expression of prepro-orexin and OX1R mRNAs and of these proteins in the tissue was analyzed by reverse-transcriptase polymerase chain reaction (RT–PCR) and Western blotting analyses, respectively.

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2. Material and methods

2.1. Antibodies and chemicals

Horseradish peroxidase conjugated anti-rabbit or anti-goat IgG were purchased from Sigma Chemical Co. (St. Louis, MO, USA); goat polyclonal anti-oxA (sc-8070) and anti-OX1R (sc-8073) antibodies, and their respective blocking peptides (sc-8070 P and sc-8073 P) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); rabbit polyclonal anti-prepro-orexin antibody (AB3096), its blocking peptide (AG774), and monoclonal anti-tubulin antibody (MAB1637) from Chemicon International Inc. (Temecula, CA, USA); biotinylated secondary antibodies and avidin–biotin complex (PK-6105) from Vector Laboratories (Burlingame, CA, USA); Triazol from Invitrogen (Carlsbad, CA, USA); the enhanced chemiluminescence (ECL) kit, the GFX PCR DNA and Gel Purification Kit (code 27-9602-01) from Amersham (Little Chalfont, UK); the DC protein assay kit from Bio-Rad Laboratories (Hercules, CA, USA). The primers for rat prepro-orexin, OX1R, and β-actin were provided by Primm (Milan, Italy), and the kit for PCR and RT–PCR by Promega (Madison, WI, USA).

2.2. Animals

Ten healthy adult Wistar male rats bred in the vivarium of the Department of Biological Structures, Functions and Technologies (University of Naples Federico II) were used. The animals were kept in groups of five rats per cage with free access to food and tap water, under constant conditions of light and temperature (22 °C). Experimental procedures were approved by the University Ethical Committee for animal experimentation and were conducted in accordance with the European Union normative for care and use of experimental animals.

The animals were killed by decapitation, and the epididymes were collected. The organs were divided in a cranial portion, an intermediate- and a caudal-one. The samples were fixed in Bouin's fluid for 18–24 h, and successively processed for immunohistochemistry. For the experiments involving protein and mRNA analyses, epididymes were immediately removed after rat decapitation, frozen in liquid nitrogen, and stored at −80 °C until processed.

2.3. Immunohistochemistry

The fixed samples were dehydrated in ascending alcohols, embedded in Paraplast, and microtomically cut in 5 μm thick sections. The sections were stained by the immunohistochemical avidin–biotin method. The primary antibodies used in the specific steps were polyclonal, raised in goat and directed against oxA and OX1R. Both antibodies were diluted 1:200 and applied on sections overnight at 6 °C. Sometimes, before staining, the sections were immersed in a citrate buffer (pH 6.0) and heated in a microwave oven for 10′ at 750 W, in order to reveal antigens masked by the fixation and/or other steps of the technical procedure. The dye 3-3′ diaminobenzidine (DAB) was used as final staining. The preparations were observed by a Nikon Eclipse E 600 light microscope, and photographed by a Colpix 8400 digital camera.

Negative controls were obtained substituting in the specific step the primary antisera with phosphate buffered saline (PBS) or with the same antisera pre-absorbed with an excess (100 μg/ml) of their relative antigen. They resulted always negative.

2.4. RNA extraction and RT–PCR analysis

Total RNA was extracted from epididymis samples by using Triazol solution. The RNA was re-suspended in 50 μl diethyl pyrocarbonate treated water, and stored at −80 °C until used. Synthesis of cDNAs for the detection of prepro-orexin and ox1R mRNAs was performed by using a reverse transcription (RT) system. The following specific

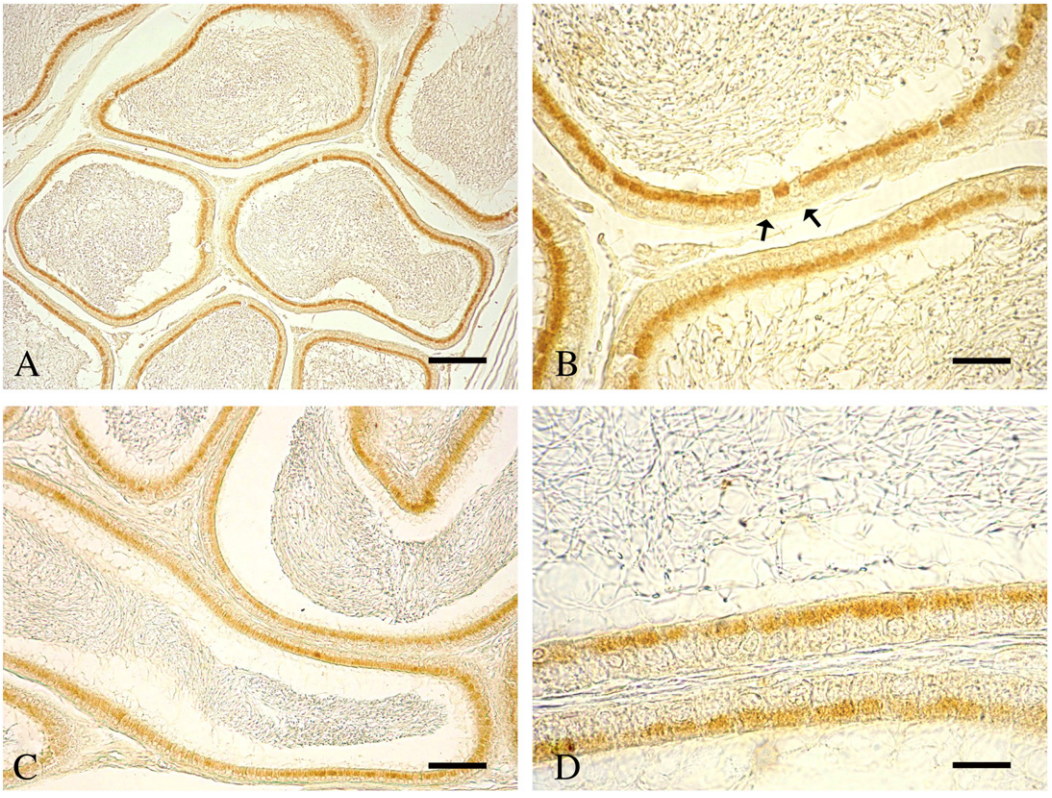

Fig. 1. OxA- and OX1R-immunoreactivity in the rat epididymis. A–D: OxA (A, B) and OX1R (C, D) are localized in the principal cells of some adjacent epididymal tubules, and clearly show a cytoplasmic apical localization. In (B) the positive epithelial border is interrupted by the presence of two negative cells (arrows) corresponding to the clear cytotype. Avidin–biotin immunohistochemical technique, DAB staining. Scale bars. (A): 30 μm; (B, C): 20 μm; (D): 100 μm.

primers were used: forward 5′-ATCCTTCCTCTACAAAGGTCTCC-3′ and reverse 5′-CTTGCCCAGCGTGAGGAT-3′ for prepro-orexin; forward 5′-AGGCTGCGGTCATGGAAT-3′ and reverse 5′-TTCCTGACCAGGGCTGAC-3′ for OX1R. These primers were designed in such a way that the forward and the reverse primers span different exons, so that the amplification product obtained from the cDNA would be of different length from that obtained from any contaminant genomic DNA comprising intronic sequences. Furthermore, to definitely rule out the possibility of amplifying genomic DNA, one PCR was carried out prior RT of the RNA. As internal control for RT and reaction efficiency, amplification of β-actin mRNA was carried out in parallel in each sample, using the primer pair: forward 5′-GGCACCCAGCACAATGAA-GAT-3′ and reverse 5′-CCTTCACCGTTCCAGTTTTTA-3′. The PCR products were separated on a 2% agarose gel, and visualized by ethidium bromide using a 1 kb DNA ladder to estimate the band sizes. As a negative control for all reactions, distilled water was used in place of cDNA. The bands were cut off from the gel, purified and sequenced by Primm [GenBank accession no.: AB084625 for prepro-orexin, and AB092488 for OX1R].

2.5. Western blotting analysis

The tissue samples were homogenized by an Ultraturrax L-407 at 4 °C with 5 ml/1.5 g tissue of buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulphate (SDS), 1% Nonidet P-40, 1 mM phenylmethylsulphonyl fluoride, 0.1 U/ml aprotinin, 10 μg/ml leupeptin, and 1 mM Na_3VO_4. Homogenates were centrifuged at 15,000 *g* for 10 min at 4 °C. Supernatants were divided into small aliquots, and stored at −80 °C until used. The amount of total proteins in each sample was determined by the Bio-Rad DC protein assay.

Homogenated samples containing equal amount of proteins (100 μg) were boiled for 5 min in SDS buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue, and 5% β-mercaptoethanol), and run on a 12.5% SDS/polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose using a Mini trans-blot apparatus (Bio-Rad Laboratories) according to the manufacturer's instructions. Membranes were blocked for 1 h at room temperature with TBS-T buffer (150 mM NaCl, 20 mM Tris HCl, pH 7.4, 0.1% Tween 20) containing 5% milk. The blots were incubated overnight with polyclonal antibodies directed against prepro-orexin or OX1R, both diluted 1:1000 in TBS-T containing 2.5% milk. After the incubation, the membranes were washed three times with TBS-T and incubated for 1 h with horseradish peroxidase conjugated anti-rabbit or anti-goat (IgG) Ig diluted 1:3000 in TBS-T containing 2.5% milk. The proteins were visualized by ECL. To ensure specificity, pre-absorption of prepro-orexin or OX1R antibodies with their relative control peptides (AG774 and sc-8073 P, respectively) was performed before Western blotting. To monitor loading of gel lanes, the blots were stripped by incubation for 30 min at 70 °C with a solution containing 2% SDS, 100 mM β-mercaptoethanol in 62.5 mM Tris–HCl, pH 6.8, and re-probed using anti-tubulin monoclonal antibody.

3. Results and discussion

The localization of oxA and OX1R in the rat epididymis was assessed by immunohistochemistry. Immunoreactivity for oxA and OX1R was found in the cells forming the epithelium lining the tubules in the caudal portion of the rat epididymis (Fig. 1A–D). Such cells, belonging to the principal cytotype [26], appeared to be very numerous in some zones of the cauda. They regularly lined the circular profile of the tubules and were intermingled by few isolated negative cells. The positive material showed the aspect of fine granules grouped in clusters and exhibited cytoplasmic sopranuclear localization, thus almost entirely filling the apical portion of the cells. The histological pictures obtained by using the anti-oxA (Fig. 1A, B) and anti-OX1R (Fig. 1C, D) antibodies were roughly similar as far as the abundance of positive cells, the localization of the positive material, and the intensity of staining. No immunoreactivity for oxA and OX1R was observed in the cells corresponding to cytotypes different from the principal, such as basal, apical and clear cells.

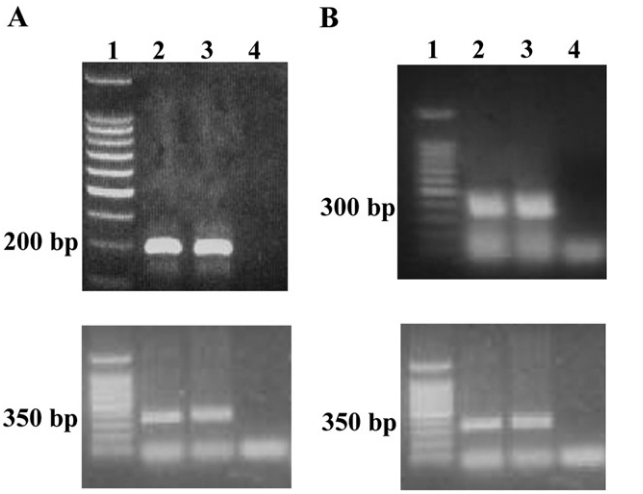

Fig. 2. RT–PCR analysis for the expression of prepro-orexin and OX1R mRNAs in the rat epididymis. A. Prepro-orexin mRNA transcripts from a rat brain tissue used as positive control (lane 2), and from epididymis (lane 3). Lane 1 corresponds to the DNA ladder. Negative control is shown in lane 4. B. OX1R1 mRNA transcripts from a rat brain tissue used as positive control (lane 2), and from epididymis (lane 3). Lane 1 corresponds to the DNA ladder. Negative control is shown in lane 4. A and B bottom, expression of β-actin mRNA transcripts (internal control).

These results provide the first evidence for oxA and OX1R immunoreactivity in the principal cells of the caudal portion of the rat epididymis. Previous studies demonstrated oxA immunoreactivity in rat testis, with an evident signal in Leydig cells and spermatocytes [18], while immunoreactive staining for OX1R was reported in Leydig cells, myoid cells of the seminiferous tubules, and Sertoli cells of human testis [25].

Although the possibility that systemic oxA (i.e. from the blood) arrives to the epididymis cannot be ruled out, the presence of oxA and OX1R in the principal cells of the epididymis cauda suggests that these peptides may be also locally synthesized. Thus, the expression of prepro-orexin and OX1R mRNAs in the rat epididymis was analyzed by RT–PCR. This analysis resulted in the amplification of specific DNA fragments of 200-bp for prepro-orexin (Fig. 2A, top, lane 3) and of 300-bp for OX1R (Fig. 2B, top, lane 3) in the rat epididymis samples as well as in a whole rat brain tissue used as a positive control (Fig. 2, Panels A and B, top, lanes 2). A 350-bp transcript was obtained from the amplification of β-actin cDNA in all tested samples (Fig. 2, Panels A and B, bottom). This result definitely demonstrates that epididymis is able to synthesize prepro-orexin and OX1R. Thus, the proteolytic processing of prepro-orexin originates the mature oxA detected in the tissue by immunohistochemistry. Our finding is consistent with the previously reported presence of prepro-orexin and OX1R mRNAs in human epididymis [25].

The presence of both proteins, prepro-orexin and OX1R, in the rat epididymis was confirmed by Western blotting, using, respectively, a rabbit polyclonal antibody raised against a 17 amino acid peptide mapping near the C-terminus of mouse prepro-orexin, and a goat polyclonal antibody raised against a peptide mapping near the C-terminus of OX1R of rat origin. The detected prepro-orexin showed a molecular mass of 16 kDa (Fig. 3A, upper blot, lane 2), while OX1R a molecular mass of 50 kDa (Fig. 3B, upper blot, lane 2), thus demonstrating that prepro-orexin and OX1R detected in the rat epididymis are structurally similar to the commonly known proteins detected in the brain or other tissues of mammals [1,2,12,13,21,22]. The specificity of the response was confirmed by pre-incubation of the prepro-orexin and OX1R antibodies with their respective blocking

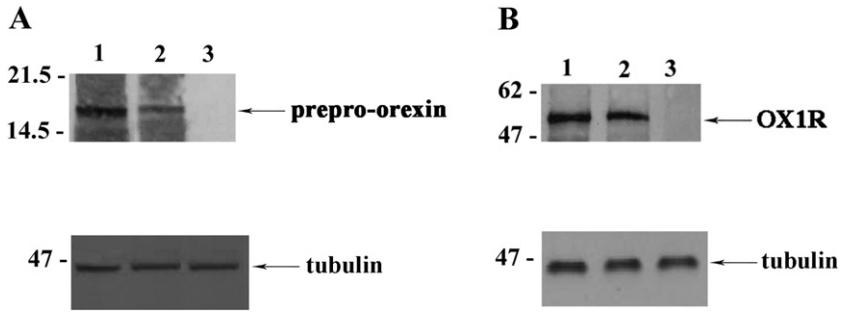

Fig. 3. Expression of the proteins prepro-orexin and OX1R in the rat epididymis. A. Western blotting analysis by using antiserum directed against prepro-orexin. B. Western blotting analysis by using antiserum directed against OX1R. Lanes 1, homogenate from a whole rat brain (positive control); lanes 2, homogenate from rat epididymis; lanes 3, negative control (epididymis homogenates treated with the antiserum directed against prepro-orexin or OXR1R pre-absorbed with their respective control peptide). The upper blots were stripped and re-probed with an anti-tubulin monoclonal antibody to ensure equal loading of proteins in all lanes (A and B, lower blots). Molecular mass markers are indicated on the left. Similar results were obtained from four separate experiments of identical design.

peptide. There was no expression of prepro-orexin and OX1R in these preparations (Fig. 3, Panels A and B, upper blots, lanes 3), whereas the presence of both proteins was detected in the whole rat brain homogenate which was used as positive control (Fig. 3, Panels A and B, upper blots, lanes 1). The stripping of the upper blots and their re-probing with a mouse monoclonal anti-tubulin antibody demonstrated equal loading of proteins in all lanes (Fig. 3, Panels A and B, lower blots).

The expression of oxA and OX1R in the rat epididymis strongly supports the biological relevance for these peptides in the function of mammalian genital tract. The oxA acting on this target is not only of systemic origin and spread by the blood circulation, but it is also locally originated. The sources for the peptide in the male genital tract include the neuroendocrine cells of the urethral epithelium [21] and the Sertoli cells of the testis [18]. Thus, the principal cytotype of caudal epididymis represents an additional genital source of oxA, since prepro-orexin mRNA transcripts were detected in this tissue. In addition to producing oxA, either Sertoli cells of the testis or the principal cytotype of caudal epididymis also express the receptor OX1R.

The principal cells lining the epididymal tubules are actively engaged in the transport of electrolytes and water. These processes lead to the formation of a specialized fluid milieu in which spermatozoa acquire their fertilizing capacity and motility [27]. The bioactive substances secretin [28] and pituitary adenilate cyclase-activating peptide (PACAP) [29] play major roles in controlling electrolyte transport in the epididymis. Similarly to secretin and PACAP, the peptide oxA is expressed in the principal cytotypes of the epididymis, shows apical localization in the cytoplasm of these cells, and binds G-coupled receptors. These findings suggest a concerted action of oxA with the activity of the other two neurotransmitters/modulators secretin and PACAP in the control of transepithelial electrolyte and fluid transport within the reproductive tissue.

In conclusion, our results provide the first immunohistochemical localization of oxA and its receptor OX1R in the principal cells of the caudal epididymis of mammals, and demonstrate that the peptide and OX1R are locally synthesized. Although further studies are needed to establish the precise molecular mechanism of action of oxA in the regulation of reproductive functions, our findings suggest that oxA may concur to regulate the environmental conditions of epididymal tubules which determine the fertilizing capability of the immature male gamete.

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