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Rapid communication

Novel localization of orexin A in the tubular cytotypes of the rat testis

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ABSTRACT

The hypothalamic peptides orexin A (OXA) and orexin B (OXB), deriving from the proteolytic cleavage of the precursor molecule prepro-orexin, have also been localized in multiple cerebral areas and peripheral organs. They regulate food intake, arterial blood pressure, heart rate, sleep/wake cycle, sexual behavior, arousal, and the hypothalamic/hypophyseal axes. Prepro-orexin mRNA expression and OXA-immunoreactivity were previously detected in the rat testis at different ages of postnatal development, with strong peptide signal in Leydig cells and spermatocytes. In this study, OXA-immunoreactivity was found in Sertoli cells and spermatids of rat testis. Hematoxylin-counterstained sections revealed OXA positive spermatids in the stages of the germinal epithelium cycle ranging from the VIIth to the XIVth. The expression of prepro-orexin mRNA and of the protein in the testis tissue was ascertained by reverse-transcription polymerase chain reaction and Western blotting analysis, respectively. Although the functional role of OXA in the male genital tract still remains to be elucidated, our findings provide the first evidence that Sertoli cells, belonging to the tubular compartment of testis, represent an important source of OXA, thus suggesting the potential involvement of the peptide in the control of seminiferous epithelium development.

1. Introduction

The peptides orexin A (OXA) and orexin B (OXB), firstly discovered in the rat hypothalamus, derive from the proteolytic cleavage of the precursor molecule prepro-orexin [1,2]. OXA is a 33 amino acid peptide (3562 Da) with N-terminal pyroglutamyl residue and two intrachain disulphide bonds, whereas OXB is a 28 amino acid linear peptide (2937 Da). They bind two G-coupled receptors, receptor 1 (OX1R) and receptor 2 (OX2R), showing different binding affinity: OX1R is highly selective for OXA, while OX2R is a non selective receptor which binds both OXA and OXB.

Although orexins are primarily expressed in the lateral hypothalamus, the brain area controlling food intake, orexinergic fibers project toward multiple cerebral regions [3,4]. Thus, in addition to a key role in food intake [5], orexins regulate arterial blood pressure and heart rate [6], sleep/wake cycle [7], sexual behavior and arousal [8], and plasma corticosterone levels [9]. They regulate the hypothalamic/hypophyseal axes involved in the regulation of adrenal and gonadal functions [10,11]. Moreover, a role for orexins in the pathogenesis of narcolepsy in humans and mice has been established [3,12].

Recent studies demonstrated that both orexins and their receptors are expressed in peripheral organs belonging to the gastrointestinal [13,14] and genital [15–18] tracts of mammals. In particular, OXA and OX1R have been localized in the rat testis [16,19,20], in endocrine and exocrine cells of the cattle urethro-prostatic complex [21], and in the principal cells of the rat epididymis [17]. The expression of mRNAs encoding for prepro-orexin and both orexin receptors has been reported in the testis, penis, epididymis and seminal vesicles of several mammals [15,16,22] and of the fowl [23]. However, the functional role of orexins and their cognate receptors in the male genital tract remains to be elucitaded.

With regard to the potential function of OXA in mammalian testis, the elegant work by Barreiro et al. [16] demonstrated prepro-orexin mRNA expression and OXA-immunoreactivity in the rat at different ages of postnatal development, with strong peptide signal in Leydig cells and spermatocytes. They provided evidence for a potential involvement of OXA in the control of steroidogenesis and germ cell development.

In this study, the presence of OXA in the Sertoli cells of rat testis and in spermatids at different developmental stages of the germinal epithelium cycle was investigated by immunohistochemical staining. The expression of prepro-orexin mRNA and of the protein in the testis tissue was also evaluated by reverse-transcription polymerase chain reaction (RT-PCR) and Western blotting analysis, respectively.

2. Materials and methods

2.1. Antibodies and chemicals

Horseradish peroxidase conjugated anti-goat IgG was purchased from Sigma Chemical Co. (St. Louis, MO, USA); goat polyclonal anti-

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OXA (sc-8070) antibody, and its blocking peptide (sc-8070 P) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); rabbit polyclonal anti-prepro-orexin antibody (AB3096), its blocking peptide (AG774), and mouse 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 (Milan, Italy); the 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 and β-actin were purchased by Primm (Milan, Italy), and the kit for PCR and reverse-transcription (RT)-PCR by Promega (Madison, WI, USA).

2.2. Animals and tissue sample collecting

Eight adult male rats bred in the vivarium of the Department of Biological Structures, Functions and Technologies at the University of Naples Federico II were used. The animals were kept in number of two per cage with free access to pellet food and fresh tap water under constant conditions of temperature (22 °C) and light. The experimental procedure was approved by the University Committee for animal experimentation and followed the rules of the European Union normative for care and use of experimental animals. The anesthetized rats were killed by decapitation, and the testes collected soon after the death. Samples were fixed in Bouin's fluid for 48 h, dehydrated in ascending alcohols, and embedded in Paraplast for immunohistochemical processing. Sections (6 μm thick) were cut by a microtome, collected on slides and stained. For biochemical analyses, samples from the testis tissues were frozen in liquid nitrogen, and stored at −80 °C until processed.

2.3. Immunohistochemistry

Dewaxed and re-hydrated tissue sections were stained by the avidin–biotin immunohistochemical procedure. The primary goat polyclonal antibody directed against OXA, diluted 1:200, was applied on sections overnight at 5–6 °C. Sometimes, before the immunohistochemical reaction, an antigen unmasking procedure was carried out by dipping the sections in 0.01 M sodium citrate buffer, pH 6.0, and heating them in a microwave oven for 10 min at 750 W. The dye 3,3-diamminobenzidine was utilized for the final staining. Many sections were counterstained with hematoxylin in order to identify the evolution stage of the germinative epithelium, according to the procedure described by Clermont and Perey [24]. The preparations were observed by a Nikon Eclipse E600 light microscope, and photographs were taken by a Colpix 8400 digital camera.

2.4. RNA extraction and RT-PCR analysis

Total RNA was extracted from testis 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 was performed by using the Promega RT system. The following specific primers were used: forward 5′-ATCCTTCCTCTACAAAGGTCTCC-3′ and reverse 5′-CTTGCCCAGCGTGAGGAT-3′ for prepro-orexin. 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′-GAGGCTCAGAGCAAGAGAGG-3′ and reverse 5′-TGACATCTCGCACAATCTCC-3′. As a negative control for all reactions, distilled water was used in place of cDNA. 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. The bands were cut off from the gel, purified using Amersham GFX PCR DNA and gel purification kits, and sequenced by Primm [GenBank accession no.: NM_013179 for rat prepro-orexin, and NM_031144 for rat β-actin].

2.5. Western blotting

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 a Bio-Rad DC protein assay. Samples containing equal amount of proteins 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 Bio-Rad mini trans-blot apparatus according to the manufacturer's instructions. Membrane was 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 blot was incubated overnight with the rabbit polyclonal antibody directed against prepro-orexin, diluted 1:1000 in TBS-T containing 2.5% milk. Then, the membrane was washed three times with TBS-T and incubated for 1 h with horseradish peroxidase conjugated anti-rabbit (IgG) Ig diluted 1:3000 in TBS-T containing 2.5% milk. The proteins were visualized by ECL. To ensure specificity, pre-absorption of the anti-prepro-orexin antibody with its relative control peptide (AG774) was performed before Western blotting. To monitor loading of gel lanes, the blot was stripped by incubation for 30 min at 70 °C with a solution containing 2% SDS, 100 μM β-mercaptoethanol in 62.5 mM Tris–HCl, pH 6.8, and re-probed using anti-tubulin monoclonal antibody.

3. Results

3.1. Localization of OXA in Sertoli cells and spermatids of rat testis: immunohystochemical analysis.

In the rat testis samples, OXA-immunoreactivity was selectively detected in Sertoli cells (Fig. 1 A–C), and in both round and oblong-shaped spermatids (Fig. 1 D–E). Positive Sertoli cells were well evident in transverse sections of seminiferous tubules, although sometimes only a portion of the cell was visible. The Sertoli cells showed fine granular reactivity generally localized at the base of the cell, but sometimes rising up to the whole cytoplasm, so making visible the entire profile of cellular irregular shape.

Positive round and elongated spermatids were much more numerous than the OXA-containing Sertoli cells, and their positivity was always localized in the acrosomial complex. An accurate examination of hematoxylin-counterstained sections revealed that positive spermatids were present in the stages of the germinal epithelium cycle ranging from the VIIth to the XIVth (Fig. 2). No OXA immunostaining was ever seen in the first portion of the cycle, i.e. from the first to the sixth stage (data not shown).

No differences were detected between normal and unmasked preparations with respect to the staining intensity of the positive structures. Negative controls were obtained by substituting, in the specific step, the primary antiserum with phosphate buffered saline or with the same antiserum pre-adsorbed with an excess (100 μg/ml) of the relative antigen. They resulted always negative (data not shown).

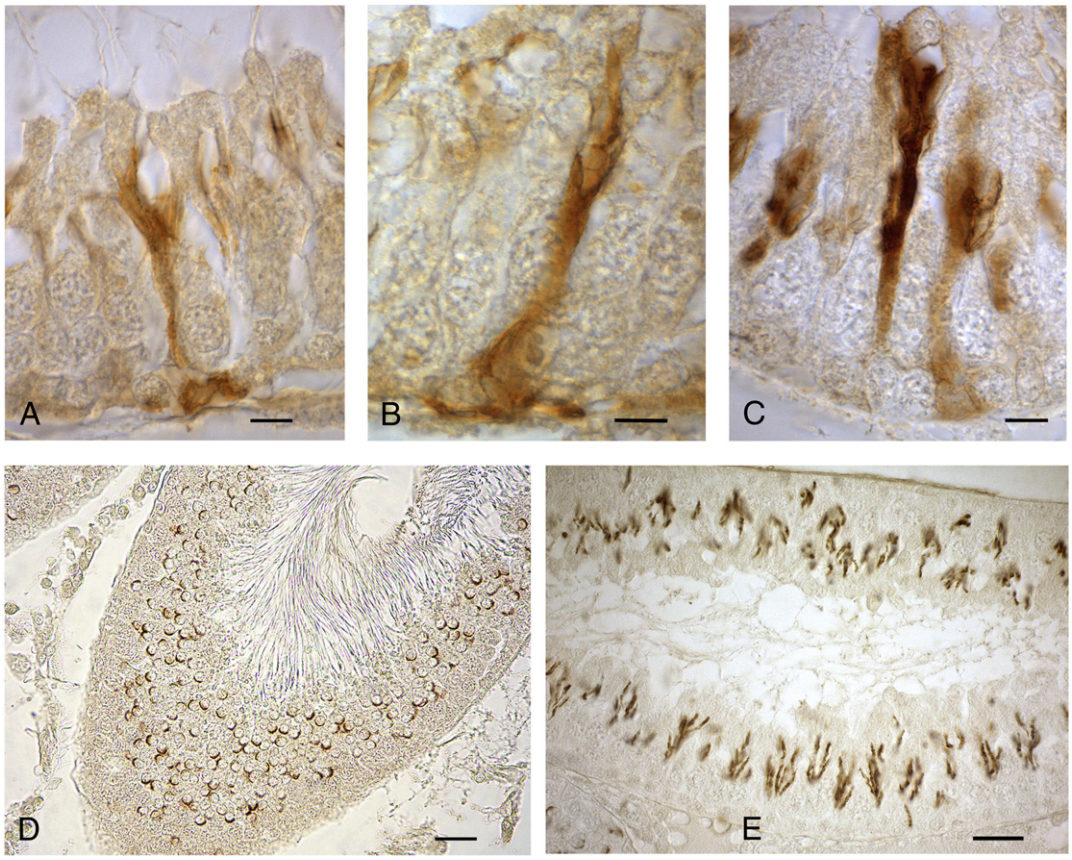

Fig. 1. Sections of seminiferous tubules of the rat testis showing OXA immune-reactive cytotypes. A, B, and C: OXA-containing Sertoli cells arising from the tubular membrane towards the lumen. The entire cellular profile is visible for some of them. D, E: small, round (D) and elongated (E) spermatids intensely stained by the anti-OXA antibody. Note that the whole population of such cytotypes in the two sections appears to respond to the immune reaction. Avidin–biotin immunostaining. Bars A, B, and C: 20 μm. D, E: 10 μm.

3.2. Prepro-orexin mRNA and protein expression in the rat testis: biochemical analyses

The expression of prepro-orexin mRNA was analyzed by RT-PCR. This analysis resulted in the amplification of specific DNA fragments of 180-bp for prepro-orexin (Fig. 3, Panel A, top, lane 3) in the rat testis samples as well as in a whole rat brain tissue used as a positive control (Fig. 3, Panel A, top, lane 2). A 469-bp transcript was obtained from the amplification of β-actin cDNA in all tested samples (Fig. 3, Panel A, bottom). No amplification products were obtained when distilled water was used in place of cDNA (negative control) (Fig. 3, Panel A, lane 4, top and bottom).

The presence of prepro-orexin in the rat testis was confirmed by Western blotting, using a rabbit polyclonal antibody raised against a 17 amino acid peptide mapping near the C-terminus of mouse prepro-orexin. The detected prepro-orexin showed a molecular mass of 16 kDa (Fig. 3, Panel B, upper blot, lane 2). The specificity of the response was confirmed by pre-incubation of the prepro-orexin antibody with its respective blocking peptide. There was no expression of prepro-orexin in these preparations (Fig. 3, Panel B, upper blot, lane 3), whereas the presence of the protein was detected in the whole rat brain homogenate which was used as positive control (Fig. 3, Panel B, upper blot, lane 1). The stripping of the upper blot and its reprobing with a mouse monoclonal anti-tubulin antibody demonstrated equal loading of proteins in all lanes (Fig. 3, Panel B, lower blot).

4. Discussion

Multiple studies suggest that orexins might play an important role in regulating the functions of mammalian male genital tract. The neuroendocrine cells of the urethro-prostatic complex, the principal cells of the epididymis, and the testis have been shown to provide a source of OXA in this tract [16,17,20]. However, the testis is likely the tissue that, after the brain, holds the highest orexin expression levels reported so far [21,25]. A strong OXA-immunoreactivity was detected in Leydig cells and spermatocytes of rat testis throughout postnatal development [16]. This study provides the first evidence that Sertoli cells may represent an important source of OXA in the mammalian testis, thus confirming previous hypothesis that OXA-producing cells belong to the tubular compartment rather than to the interstitial-one [16]. In our investigation, the expression of the OXA precursor protein, prepro-orexin, and of prepro-orexin mRNA in the rat testis was also established, although the specific cytotypes expressing the protein were not isolated.

Interestingly, while Barreiro et al. [16] found OXA-immunoreactivity in only two cytotypes of rat testis, namely the Leydig cells and spermatocytes, in our investigation OXA positivity was found in two different cytotypes such as Sertoli cells and spermatids. Moreover, while in the first report positive spermatocytes were present along the whole evolutive cycle of the germinative epithelium, with the exception of the V and VI stages, we observed the presence of positive spermatids only in the second half of the cycle, precisely from the VIIth to the XIVth stage. Although this discrepancy could be due to the different procedures and antibodies used in the two investigations, however, the rapid turnover of OXA could be also a probable explanation. The production of endocrine-like substances by the testis might occur rarely, although in large amount. Their synthesis could be induced by a molecule expressed by the same cytotype(s) in some areas of the organ. This is consistent with the results obtained in our previous study on the presence of OXA and its cognate receptor 1 in the rat epididymis showing that the two peptides are expressed in almost all principal cells of restricted areas of the organ [17].

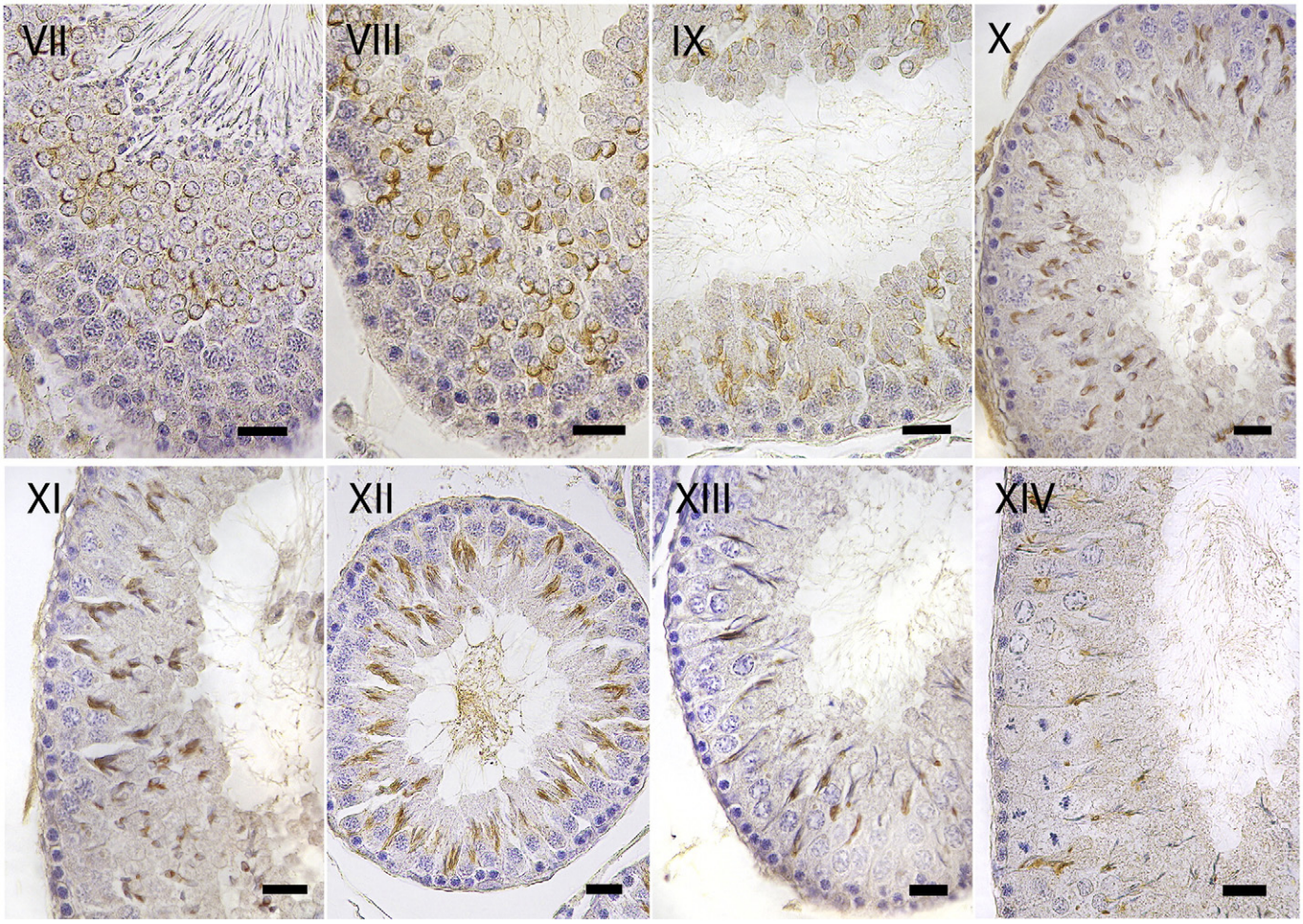

Fig. 2. Sections of seminiferous tubules of the rat testis showing progressive stages of the evolutive cycle of the germinal epithelium. Different shapes of strongly positive spermatids, from the perfectly round to the extremely elongated, characterize the transition from the VIIth to the XIVth stage of the cycle. Avidin–biotin immunoreaction counterstained by hematoxylin. Bars: 20 μm.

Despite the definite sources of OXA in the rat testis, numerous peptide targets in the male reproductive system has been described [15,19,22]. However, the possibility that at least a part of the OXA normally circulating in the blood of men [21] and rats [11] is of genital origin cannot be ruled out. The data reported so far lead to hypothesize that OXA exerts multiple functions in the mammalian male genital tract. In the tubular compartment of the rat testis, OXA produced by the Sertoli cells may act in an autocrine way on the same cell, thus inhibiting the production of the Mullerian-inhibiting substance, which has a steroidolitic action on the Leydig cells, and of stem cell factor, which stimulates the proliferation of the germinative epithelium [16]. Thereby, OXA has a steroidogenic activity, although with moderate intensity, and anti-proliferative action with respect to the tubular germ cells.

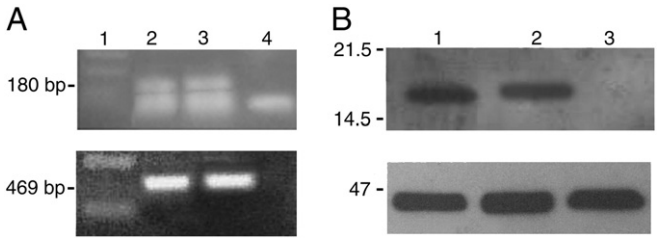

Fig. 3. Expression of prepro-orexin in the rat testis. Panel A. RT-PCR analysis for the expression of prepro-orexin mRNA in the rat testis. Prepro-orexin mRNA transcripts from a rat brain tissue used as positive control (lane 2), and from testis (lane 3). Lane 1 corresponds to the DNA ladder. Negative control (no cDNA input) is shown in lane 4. The a specific bands at the basis of lanes 2 and 3 correspond to the primer dimers and are probably due to the low temperature used in the PCR run. The bottom of Panel A reports the β-actin mRNA transcripts (internal control). Panel B. Western blotting analysis for the expression of prepro-orexin in the rat testis. Lane 1, homogenate from a whole rat brain (positive control); lane 2, homogenate from rat testis; lane 3, negative control (testis homogenates treated with the antiserum directed against prepro-orexin pre-absorbed with its control peptide). The upper blot was stripped and re-probed with an anti-tubulin monoclonal antibody to ensure equal loading of proteins in all lanes (lower blot). Molecular mass markers are indicated on the left. Similar results were obtained from four separate experiments of identical design.

In the tubular compartment of testis which contains the highest levels of prepro-orexin and OX1R mRNAs [16], in addition to its autocrine activity, OXA could also act by a paracrine fashion because some germinal cytotypes have been found to contain immunoreactivity for OX1R both in men [15] and rats [19]. In particular, the presence of OX1R mRNA was established in all the stages of the germinal epithelial cycle in the rat, thus suggesting that OXA produced by Sertoli cells could be spread towards the germinal cells. The action of OXA on these cells remains unknown. Studies are in progress in our laboratory to identify the rat germinal cytotype(s) containing OX1R immunoreactivity.

On the other hand, the action of OXA in the interstitial compartment of testis is unclear although Leydig cells might produce OXA. This cytotype has been found to contain OXA-immunoreactivity in the rat [16] and OX1R mRNA in the man [15]. Of course, an endocrine way of OXA diffusion in the testis cannot be ruled out, especially in the interstitial compartment which is rich in blood vessels.

As a final remark, it is useful underline that all testicular functions are regulated by three kinds of biologically active substances: i) the hypophyseal gonadotropins regulating the main functions of the male gonads such as steroidogenesis and sperm cell development; ii) a pool of substances including OXA which are produced by the testis and regulate testicular functions acting independently on many targets. These substances show different chemical structure, sources and targets and their modalities of action are autocrine and/or paracrine rather than endocrine. Some of them exert a fine tuning of the gonadotropin functions, and appear replaceable to each other when necessary [26–28], however, their role is still largely unknown; iii) few substances belonging to the second group that could be clustered in a small category being produced by both the hypothalamic/hypophyseal system and testis. These latter substances regulate the production of gonadotropins, both indirectly and directly influencing testicular functions. They include OXA [29], nitric oxide [30], aspartic acid [31], grelin [16], and leptin [32]. On such basis, it is likely that male gonad activity might depend not only by the gonadotropin secretion but also by the auto-regulation capability of the same testis.

Although further studies are required to establish the molecular mechanism of action of OXA in the mammalian male genital tract, our findings, providing evidence that Sertoli cells represent an important source of OXA in the rat testis, strongly suggest the involvement of the peptide in the control of seminiferous tubule functions.

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