General and Comparative Endocrinology 242 (2017) 66–73

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Expression of orexin B and its receptor 2 in rat testis

Giovanna Liguori [a], Luigi Michele Pavone [b], Loredana Assisi [c], Emilia Langella [d], Simona Tafuri [a], Nicola Mirabella [a], Anna Costagliola [a], Alfredo Vittoria [a,*]

[a] Department of Veterinary Medicine and Animal Productions, University of Naples Federico II, Via F. Delpino 1, 80137 Naples, Italy
[b] Department of Molecular Medicine and Medical Biotechnology, University of Naples Federico II, Via Pansini 5, 80131 Naples, Italy
[c] Department of Biology, University of Naples Federico II, Via Mezzocannone 6, 80134 Naples, Italy
[d] School of Agricultural, Forestry, Food and Environmental Sciences, University of Basilicata, Viale dell'Ateneo Lucano 10, 85100 Potenza, Italy

ARTICLE INFO

Article history:
Received 22 June 2015
Revised 23 November 2015
Accepted 25 November 2015
Available online 26 November 2015

Keywords:
Orexin B
Orexin 2 receptor
Testis
Rat
Steroidogenesis

ABSTRACT

The peptides orexin A (OxA) and orexin B (OxB) deriving from a common precursor molecule, prepro-orexin, by proteolytic cleavage, bind the two G-coupled OX_1 and OX_2 receptors. While OX_1 selectively binds OxA, OX_2 shows similar affinity for both orexins. Firstly discovered in the hypothalamus, orexins and their receptors have been found in other brain regions as well as in peripheral tissues of mammals, thus resulting involved in the regulation of a broad variety of physiological functions. While the functional localization of OxA and OX_1 in the mammalian genital tract has been already described, the expression of OxB and OX_2 and their potential role in the reproductive functions remain to be explored. Here, we investigated the presence of OxB and OX_2 in the rat testis by immunohistochemical and biochemical analyses. The results definitely demonstrated the localization of OxB and OX_2 in pachytene and second spermatocytes as well as in spermatids at all stages of the cycle of the seminiferous epithelium. The expression of both OX_2 mRNA and protein in the rat testis was also established by RT-PCR and Western blotting, respectively. The analysis of the molecular mechanism of action of OxB in the rat testis showed that OxB, in contrast with OxA, is unable to promote steroidogenesis. These results translate into the regulation of diverse biological actions by OxA and OxB in the male gonad.

1. Introduction

At the end of the last century, two independent research groups discovered in the rat hypothalamus two novel peptides called hypocretins/orexins, namely orexin A (OxA) and orexin B (OxB) (de Lecea et al., 1998; Sakurai et al., 1998). Both neuropeptides derive from a common 130 amino acid precursor molecule, prepro-orexin, by proteolytic cleavage. Orexins bind two G-coupled membrane receptors: OX_1 which selectively binds OxA, and OX_2 which shows similar affinity for both orexins. In general, OX_1 is thought to couple to G_q, and OX_2 can signal through G_q or G_i/G_o, but coupling mechanisms seem to differ by cell type (Randeva et al., 2001; Karteris and Randeva, 2003). Both receptors are strongly conserved across mammals, with rat OX_1 and OX_2 displaying 94% and 95% sequence homology, respectively, with human receptors (Sakurai et al., 1998).

In the central nervous system, the cell bodies producing orexins are localized in a narrow zone within the lateral hypothalamus and project mainly to brain regions involved in the regulation of feeding, sleep, neuroendocrine homeostasis, and autonomic functions (Kukkonen et al., 2002; Ohno and Sakurai, 2008; Nixon et al., 2015). Orexins and their receptors are also found outside the central nervous system, e.g., in the gastrointestinal tract, pancreas, cardiovascular system, adrenal glands, and reproductive apparatus, where they orchestrate multifaceted physiological functions (Shahid et al., 2012; Kukkonen, 2013; Li et al., 2014). The ablation of orexin receptors or an impairment of their signaling leads to narcolepsy phenotypes in animal models (Chemelli et al., 1999; Lin et al., 1999; Thannickal et al., 2000). However, many other human pathological conditions have been correlated to the orexin system dysfunctions, including drug addiction, alcohol-seeking behaviour, anxiety/panic states, and cardiovascular, metabolic and inflammatory diseases (Matsuki and Sakurai, 2008; Szczepanska-Sadowska et al., 2010; Xu et al., 2013).

In recent years, much research efforts have been focused to investigate the expression and the physiological role of orexins in the mammalian genital tract. We previously demonstrated the expression of OxA, prepro-orexin and OX_1 in the principal cells of the epididymis and testis of rat and alpaca (Tafuri et al., 2009,

* Corresponding author.
E-mail address: avittori@unina.it (A. Vittoria).

http://dx.doi.org/10.1016/j.ygcen.2015.11.015
0016-6480/

2010; Assisi et al., 2012; Liguori et al., 2012, 2014), in the urethroprostatic complex and the vestibular glands of cattle (Russo et al., 2008; Pavone et al., 2009), and in the human normal and hyperplastic prostate (Valiante et al., 2013). Expression of OX_1 mRNA has also been found in the sheep testis (Zhang et al., 2005) and in the human seminal vesicles, penis and epididymis (Karteris et al., 2004). By contrast, only few reports describe the presence of OxB and OX_2 in the male reproductive system of mammals. The peptide OxB was detected in the rat testis by a radioimmuno assay (Mitsuma et al., 2000), and the presence of prepro-orexin and OX_2 mRNAs was demonstrated in the human testis, epididymis, penis, seminal vesicles (Karteris et al., 2004) and prostate (Malendowicz et al., 2011). However, other investigations failed to detect OX_2 mRNA expression in sheep (Zhang et al., 2005) and rat testis (Jöhren et al., 2001; Barreiro et al., 2004; Zheng et al., 2014).

Overall, these findings raise the possibility of additional peripheral actions of orexins and their receptors in the control of reproductive function, in addition to their role as central neuroendocrine modulators of the reproductive axis (Nurmio et al., 2010). Indeed, an action of OxA in the control of testicular steroidogenesis in rats was demonstrated (Barreiro et al., 2004, 2005; Assisi et al., 2012; Zheng et al., 2014). However, the expression of OxB and OX_2 in the male gonads and the elucidation of their potential role in the reproductive functions is still an open issue. In this study, the localization of OxB and OX_2 in the rat testis was investigated by immunohistochemistry. Biochemical analyses such as RT-PCR and Western blotting were carried out to establish the presence of OX_2 at molecular level in the tissue. Furthermore, in order to get insights into the potential role of OxB in the gonad, its ability to stimulate steroidogenesis was also evaluated.

2. Material and methods

2.1. Antibodies and chemicals

Mouse anti-OxA (MAB763) and anti-OxB (MAB734) monoclonal antibodies were obtained from R&D Systems (Abingdon, UK) and their synthetic peptides from Tocris Bioscience (Bristol, UK); rabbit polyclonal anti-OX_2 antibody (ab3094), its blocking peptide (AG794) from Millipore (Billerica, Massachusetts, USA); horseradish peroxidase conjugated anti-rabbit IgG (A-0545) from Sigma Chemical Co. (St. Louis, MO, USA); biotinylated goat anti-mouse (BA-9200) and goat anti-rabbit (BA-1000) secondary antibodies and avidin–biotin complex (PK-6105) from Vector Laboratories (Burlingame, CA, USA). The peptide OxB (003-32) was purchased from Phoenix Pharmaceuticals Inc. (Karlsruhe, Germany), the luteinizing hormone (LH) from sheep pituitary (L5269), and bovine serum albumin (BSA) from Sigma Chemical Co.; the enhanced chemiluminescence (ECL) kit, the GFX PCR DNA and gel purification kit from Amersham (Little Chalfont, UK); the DC protein assay kit from Bio-Rad Laboratories (Hercules, CA, USA). The primers were provided by Primm (Milan, Italy), and the kit for PCR and RT-PCR by Promega (Madison, WI, USA); EIA kit for testosterone determination from Adaltis (Bologna, Italy).

2.2. Animals

Twelve healthy adult Wistar male rats obtained from Charles River (Calco, Lecco, Italy) were bred in the vivarium of the Department of Veterinary Medicine and Animal Productions at the University of Naples Federico II (Naples, Italy). The animals were kept in groups of two rats for cage 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 University, and were conducted in accordance with the European Union normative for care and use of experimental animals. The anesthetized animals were killed by decapitation, and their testes collected soon after death. Samples from twelve testes of different animals were fixed in Bouin fluid for 24–48 h, and processed for immunohistochemistry. Fragments from other testes were frozen in liquid nitrogen, and stored at −80 °C until used for biochemical analyses.

2.3. Immunohistochemistry

The Bouin-fixed samples were dehydrated in ascending alcohols, embedded in Paraplast, and cut in 6 μm thick sections by microtome. In order to obtain consecutive stained microscopic fields, series of 3 μm thick sections were carefully cut and mounted on numbered slides. Mouse monoclonal anti-OxA and anti-OxB and rabbit polyclonal anti-OX_2 primary antibodies diluted 1:200 were applied on sections, and incubated overnight at 6 °C. Before staining, some sections were dipped in a citrate buffer, pH 6.0, and heated in a microwave oven for 10 min at 750 W to reveal masked antigens. Then, sections were incubated with biotinylated goat anti-mouse and goat anti-rabbit secondary antibodies for 30 min. The 3-3′ diaminobenzidine (DAB) was used as final staining. Some sections were counterstained with hematoxylin in order to better localize the immunoreactive structures and identify the stage of germ epithelium developing cycle according to the procedure described by Clermont and Perey (1957). The preparations were visualized by a Nikon Eclipse E 600 light microscope, and photographed by a Nikon Colpix 8400 digital camera.

In order to verify the manufacturer statement that the two antibodies directed against OxA and OxB do not cross react with the heterologous antigen, each antibody was pre-absorbed with the heterologous peptide before staining. In both cases, the positivity resulted to be unaffected (data not shown). Moreover, the anti-OxA and anti-OxB antibody were alternately applied on 3 μm thick consecutive sections, and couples of adjacent sections were carefully observed to study the tissue distribution of the two immunostainings. Indeed, their potential coexistence would indicate an improper antibody/antigen reaction.

2.4. RNA extraction and RT-PCR analysis

Total RNA was extracted from rat testis samples by using Trizol solution, re-suspended in 50 μl diethyl pyrocarbonate treated water, and stored at −80 °C until used. Synthesis of cDNAs for OX_2 detection was performed by using the Promega reverse-transcription (RT) system. The following specific primers were used: 5′-TGTCCAGCACCAAATTGGAG-3′ (forward) and 5′-ACCATC GGTCCAAGGCAATG-3′ (reverse). These primers were designed in such a way that forward and reverse ones span different exons, so that the amplification product obtained from the cDNA would be of different length to that obtained from any contaminant genomic DNA comprising intronic sequences. To definitely rule out the possibility of amplifying genomic DNA, one PCR was carried out prior to RT of the RNA. As internal control for RT and reaction efficiency, amplification of rat β-actin mRNA was carried out in each sample using the primer: 5′-GAGGCTCAGAGCAAGAGAGG-3′ (forward) and 5′-TGACATCTCGCACAATCTCC-3′ (reverse). As a negative control distilled water was used. All PCRs were carried out using *Taq* DNA polymerase (Life Technologies, Inc., Monza, Italy) with 200 ng cDNA for each amplification. Each of 30 cycles of amplification consisted of a denaturing phase of 1 min at 94 °C, annealing phase of 30 s at 65 °C, and an extension of 1 min at 72 °C. Amplified 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 numbers: NM_013074 for rat OX_2; NM_031144 for rat β-actin).

2.5. Western blotting

Testis 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 g/100 ml deoxycholic acid, 0.1 g/100 ml sodium dodecyl sulfate (SDS), 1% (v/v) Nonidet P-40, 1 mM phenylmethyl-sulfonyl fluoride, 10 μg/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. The total amount of proteins in the obtained supernatant was determined by the Bio-Rad DC protein assay. 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), run on a 12.5% SDS/polyacrylamide gel, and transferred to nitrocellulose using a Mini trans-blot apparatus (Bio-Rad Laboratories). 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 the anti-OX_2 antibody 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 IgG diluted 1:3000 in TBS-T containing 2.5% milk. The proteins were visualized by ECL. 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 reprobed using anti-γ-tubulin monoclonal antibody.

2.6. Evaluation of testicular production of testosterone

The removed testes were decapsulated and cut into pieces of approximately equal size (mean weight/piece: 250 ± 7 mg). Testicular slices (2 slices/well) were kept in 2 ml of Krebs–Ringer bicarbonate buffer (KRB), pH 7.4, containing 10 mM glucose, 100 μM bacitracin, 0.1% ascorbic acid, and 0.1% BSA, for 60 min at 37 °C in an atmosphere of 95% O_2 and 5% CO_2, with constant shaking at 60 cycles/min. Then, the medium was substituted with 2 ml of fresh KRB buffer containing increasing concentrations (10^{-9}–10^{-6} M) of OxB, and the samples were incubated for further 12 h. Negative controls were treated with medium alone, while positive controls were obtained by incubating testis slices for 12 h with LH (500 ng/g tissue) or OxA (1 nM) (Barreiro et al., 2005; Liguori et al., 2012). At the end of the incubation, the medium was aspirated, and divided in aliquots which were processed to assess testosterone levels. To this purpose, the medium was vigorously mixed with ethyl ether (1:10, v/v) and the ether phase was withdrawn after centrifugation at 3000×g for 10 min. Three extractions were performed. Pooled ether extracts were dried by evaporation, and the residue was dissolved in a 0.5 ml sodium phosphate buffer 0.05 M (pH 7.5) containing BSA at a concentration of 10 mg/ml. Such solutions were utilized for testosterone immunoassay, as previously described (Liguori et al., 2012). The following limits of testosterone detection were obtained: sensitivity 6 pg, intra-assay variability 5.3%, inter-assay variability 7.5%. The rate of testosterone recovery from testis samples was about 80%. The results were expressed as normalized values /g incubated tissue.

2.7. Statistical analysis

Data obtained from *in vitro* assays were compared by analysis of variance followed by Duncan's test for multi-group comparison and Student's *t*-test for between-group comparison. The experiments were performed in triplicate and data were expressed as means ± S.D. (standard deviation). The level of significance was taken at $p < 0.01$ and $p < 0.05$.

3. Results and discussion

3.1. Immunohistochemical detection of OxB and OX_2 in rat testis

The localization of OxB and OX_2 receptor in the rat testis was firstly investigated by immunohistochemistry. The analysis of the tissue samples showed the presence of OxB-immunoreactivity (IR) in pachytene spermatocytes (Fig. 1a) and in spermatids under progressive maturation, i.e., in young (round) (Fig. 1b), intermediate (oval) (Fig. 1c), and mature (elongated) (Fig. 1d) cells. In the spermatocytes, OxB-IR had always the shape of a unique, round and compact cluster of granules localized in the inner portion of the cytoplasm close to the nuclear membrane. In the round spermatids, OxB-IR was found in the perinuclear area appearing as a weakly stained punctiform granule. During the cell development, such granule enlarged, became more deeply stained and, loosing contact with the nuclear membrane, moved towards the caudal portion of the cell which progressively shaped oval and, finally, elongated. In mature spermatids, OxB-IR sometimes can acquire also the aspect of a diffused halo contained within the thin tail of the cell (Fig. 2c). Cells showing OxB-IR were found in the second portion of the cycle of the seminiferous epithelium from the VIIth up to the XIVth stage (Fig. 1e).

The use of series of 3 μm thick sections alternately stained by anti-OxB and anti-OxA antibodies demonstrated that OxB-containing cells never contained OxA in our preparations (Fig. 2a and b). On the other hand, control experiments carried out pre-absorbing anti-OxB or anti-OX_2 antibody with an excess (100 μg/ml) of their corresponding antigens, always resulted to be negative (Fig. 2c and d). Leydig cells clearly showing OxA labeled material (Fig. 2e) were chosen as positive control of the relative antibody (Barreiro et al., 2005; Liguori et al., 2012).

OX_2-IR was found in pachytene (Fig. 3a) and second spermatocytes (Fig. 3b), and round (Fig. 3c), oval (Fig. 3d) and elongated (Fig. 3e) spermatids. The positive structures contained in the first two cell types closely resembled those described above in pachytene spermatocytes. OX_2-IR appeared as a small granule closely adherent to the nuclear membrane in young spermatids belonging to the IIth–Vth stage interval of the cycle of the seminiferous epithelium, and progressively became a semilunar, elongated structure encircling portion of the nucleus in round/oval spermatids belonging to the VIth–IXth stage interval. From the Xth stage onwards, the positive material had a granular shape, lost contacts with the nucleus and was contained in the middle or terminal portion of the progressively elongating cell. OX_2-IR-containing cell types were present along the whole cycle of the seminiferous epithelium (Fig. 3f).

These results provide the first immunohistochemical localization of OxB in the male genital tract of mammals. They also demonstrate the presence of OX_2 in rat testis in line with a previous study showing the localization of OX_2 in human testis (Karteris et al., 2004).

3.2. Expression of OX_2 mRNA and the protein in rat testis

The presence of OxB in the rat testis is supported by previous findings showing the expression of the OxB precursor molecule prepro-orexin in this tissue (Jöhren et al., 2001; Tafuri et al., 2010). By contrast, OX_2 mRNA and the protein expression was never demonstrated before. Thus, in order to confirm the immunohistochemical localization of OX_2 observed in rat testis, we

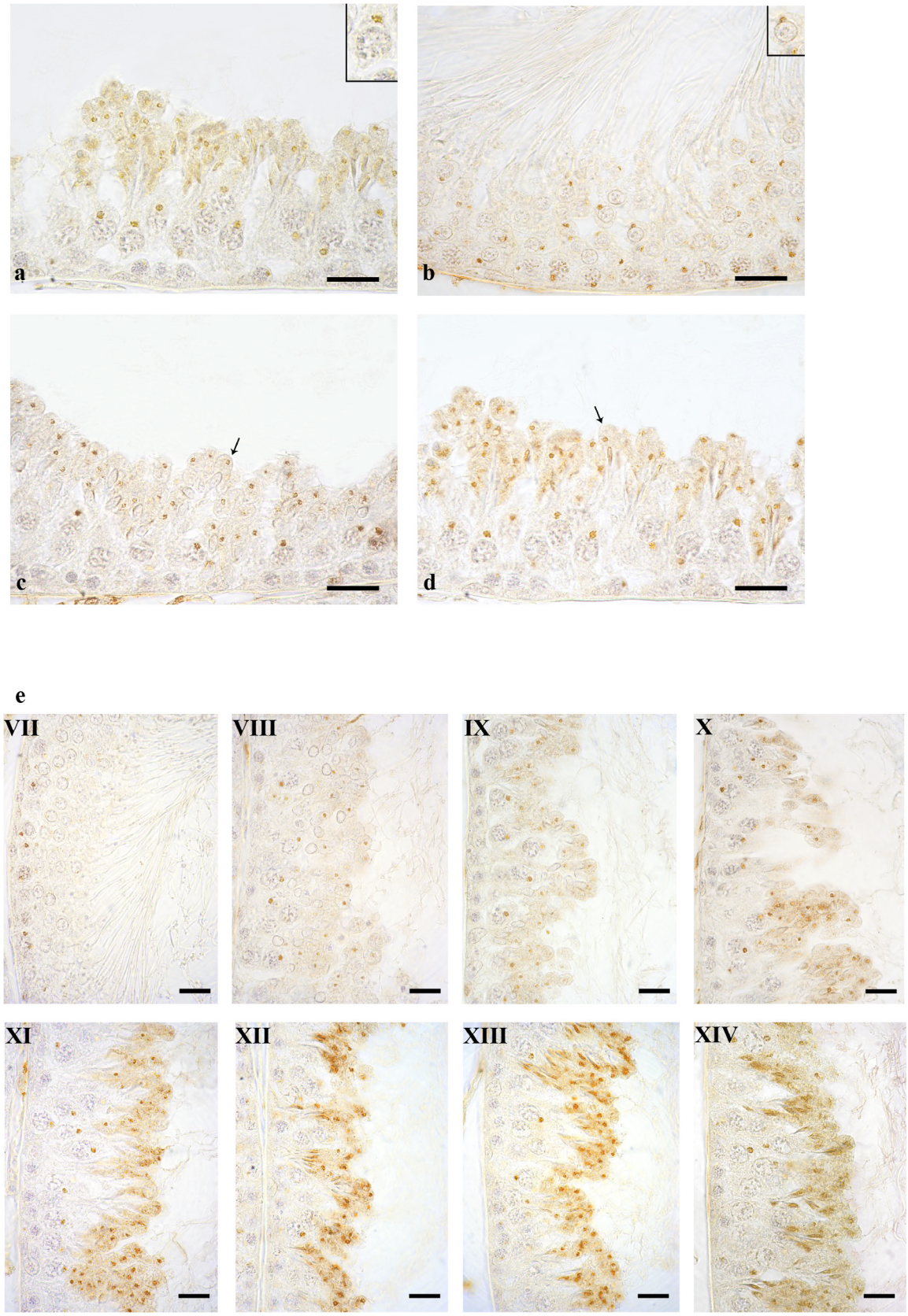

Fig. 1. OxB-immunoreactivity in cytotypes of the rat testis. (a) A round cluster of reactive material is contained in the perinuclear cytoplasm of some pachytene spermatocytes. (b)–(d) Differently sized and round shaped positive material is present in the cytoplasm of round (b), oval (c) and elongated (d) spermatids whose stage of development is clearly shown by the progressive elongation of their nuclei. In (c) and (d) positive granules are visible in the intermediate/terminal portion of the cytoplasm of transversely cut cells. Arrows point two longitudinally cut cells showing both nucleus and positivity in the same plane. The inserts in (a) and (b) show higher magnifications of a pachytene spermatocyte and a round spermatid contained in the respective microphotographs. A round shaped labeled structure is present in the adluminal portion of the cytoplasm of both cells. (e) OxB-immunoreactive cytotypes are present in the second half (from the VIIth to XIVth stages) of the cycle of the seminiferous epithelium. Avidin–biotin immunohistochemical staining. Bars: 20 μm.

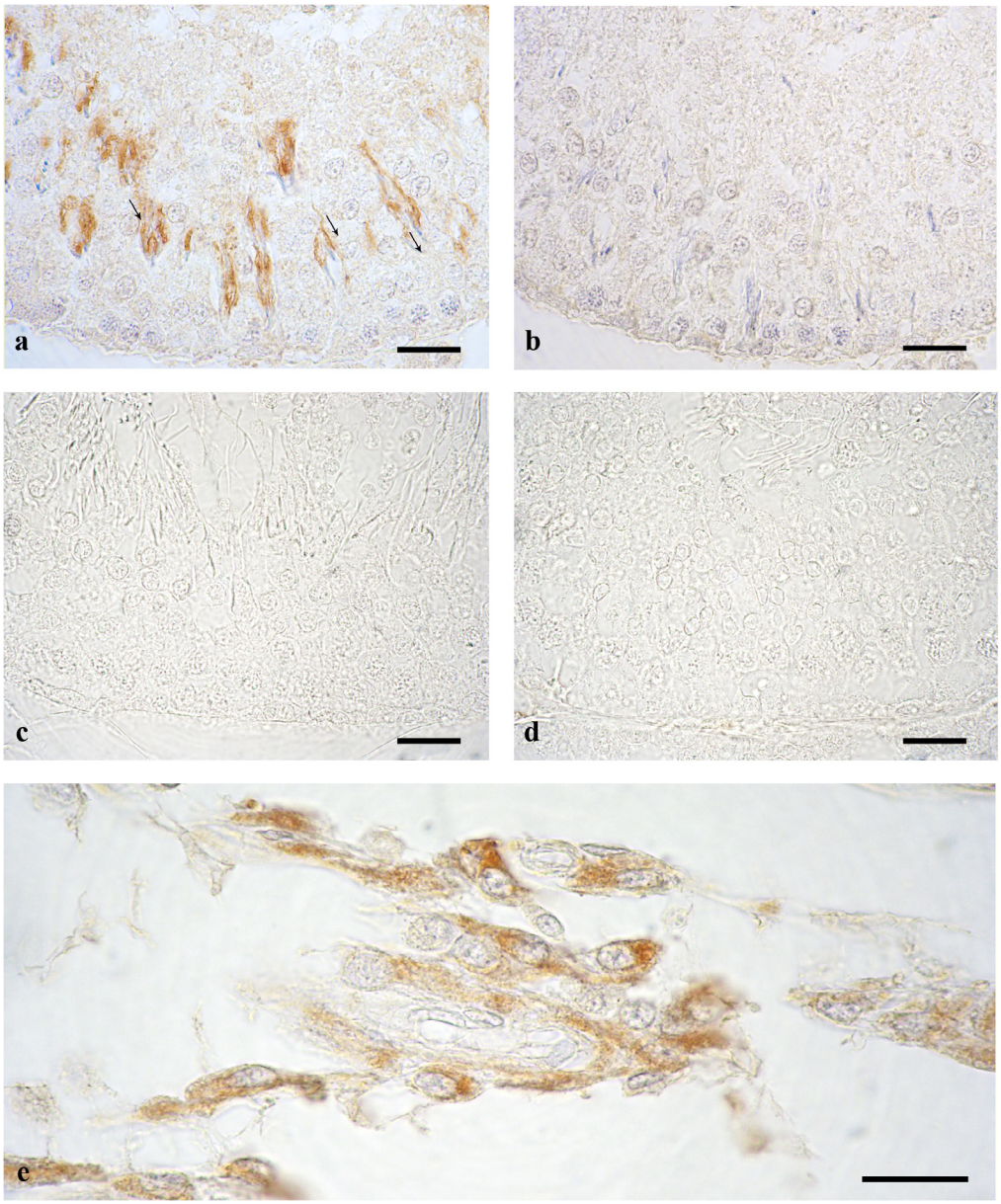

Fig. 2. Anti-OxB and anti-OxA antibodies specificity and controls. (a) and (b) A couple of 3 μm thick consecutive sections has been stained by anti-OxB (a) and anti-OxA (b) antibodies. OxB-immunoreactive material is present in elongated spermatids (a), whereas no trace of staining is visible in the consecutive section (b). (c) and (d) Negative controls of the specificity of the antibodies directed against OxB and OX_2 were obtained absorbing the antibodies with an excess of the relative antigens. The two immunological complexes were used to stain the sections reported in (c) and in (d), respectively. No staining is visible in these sections. (e) Leydig cells showing OxA-containing fine granular material have been chosen as a positive control of the relative antibody functionality. Avidin–biotin immunohistochemical method. Bars: 20 μm.

investigated the expression of OX_2 in the tissue at both mRNA and protein levels. The expression of mRNA coding for OX_2 was analyzed by RT-PCR and such analysis resulted in the amplification of specific DNA fragments of 459 bp (Fig. 4A, lane 2). A 469 bp transcript was obtained from the amplification of β-actin cDNA in tested samples (Fig. 4A bottom, lane 2). No amplification products were obtained when distilled water was used in place of cDNA (negative control) (Fig. 4A, lane 3).

The presence of OX_2 protein in the rat testis was confirmed by Western blotting, using a rabbit polyclonal antibody raised against a 19 aminoacid sequence mapping near the C-terminus of OX_2 of rat origin. The detected OX_2 protein showed the expected molecular mass of approximately 40 kDa (Fig. 4B, lane 1) (Randeva et al., 2001; Karteris et al., 2004; Dehan et al., 2013). The specificity of the response was confirmed by the pre-incubation of the OX_2 antibody with its respective blocking peptide. There was no expression of OX_2 in these preparations (Fig. 4B, lane 2). The stripping of the upper blot and its re-probing with a monoclonal anti-γ-tubulin antibody demonstrated equal loading of proteins in all lanes (Fig. 4B, bottom).

These results together with the immunohistochemical localization definitely demonstrate the expression of OX_2 in the rat testis. The discrepancy between our findings and the results of previous studies (Jöhren et al., 2001; Barreiro et al., 2004; Zheng et al., 2014) can be ascribed to two main factors: (1) the use of different methodological experimental conditions, including tissue sampling, primers and running cycles selected for RT-PCR, primary antibody specificity, etc.; (2) the high turnover of cellular production and/or internalization of neuroendocrine substances such orexins and their receptors. Indeed, opposing results are reported on the localization of orexins and their receptors in other peripheral organs such as the adrenal glands (Karteris et al., 2001;

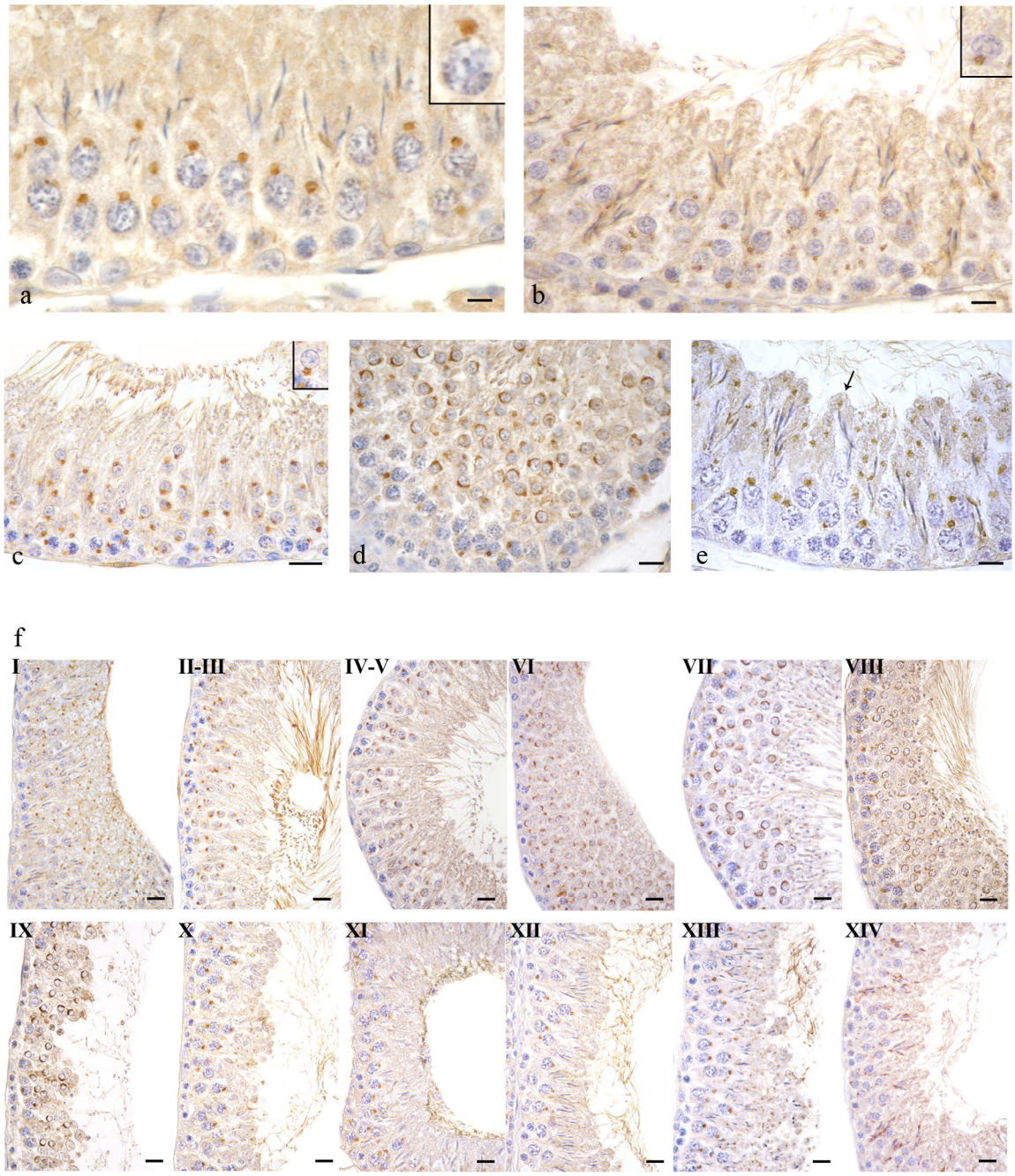

Fig. 3. OX_2-immunoreactivity in cytotypes of the rat testis. (a) and (b) In pachytene (a) and second (b) spermatocytes, the immunoreactive material is shaped as a round granule close in contact with the nuclear membrane and often localized (a) in proximity of the adluminal portion of the cell nuclei. (c)–(e) Along the development of the spermatids the positive material progressively changes in shape and localization. It is punctiform in aspect in young cells (c), encircles the nucleus of older elements as a semilunar structure (d) and becomes a coarse, round granule contained in the adluminal cytoplasm of elongated spermatids (e). Arrow (e) points a longitudinally cut spermatid showing both nucleus and positivity in the same plane. The inserts in (a), (b) and (c) show higher magnifications of a pachytene spermatocyte, a second spermatocyte and a round spermatid contained in the respective microphotographs. The three cells show in perinuclear position a single, round shaped structure resulting from the close packaging of stained microgranules. (f) OX_2-containing cytotypes are clearly seen along the whole cycle of the seminiferous epithelium. Avidin–biotin immunohistochemical method. Bars: 25 μm.

Mazzocchi et al., 2001; Blanco et al., 2002; Spinazzi et al., 2005; Ziolkowska et al., 2005).

3.3. OxB is unable to affect testosterone secretion in rat testis

The expression of functional orexin receptors in the human Leydig cells has suggested a potential role for the orexins in steroidogenesis (Karteris et al., 2004). Indeed, our and other groups demonstrated that OxA binding to OX_1 receptor stimulates testosterone production in the mammalian testis, probably counteracting the anti-steroidogenic effect of Müllerian inhibiting substance (Barreiro et al., 2005; Assisi et al., 2012; Liguori et al., 2012). Furthermore, orexins have been shown to stimulate corticosterone and cortisol production from dispersed rat and human adrenocortical cells through activation of the adenylate cyclase-dependent signaling cascade (Malendowicz et al., 1999). In this study, we tested the ability of OxB to regulate testicular androgenesis *in vitro*. Testis slices were absorbed with increasing concentrations

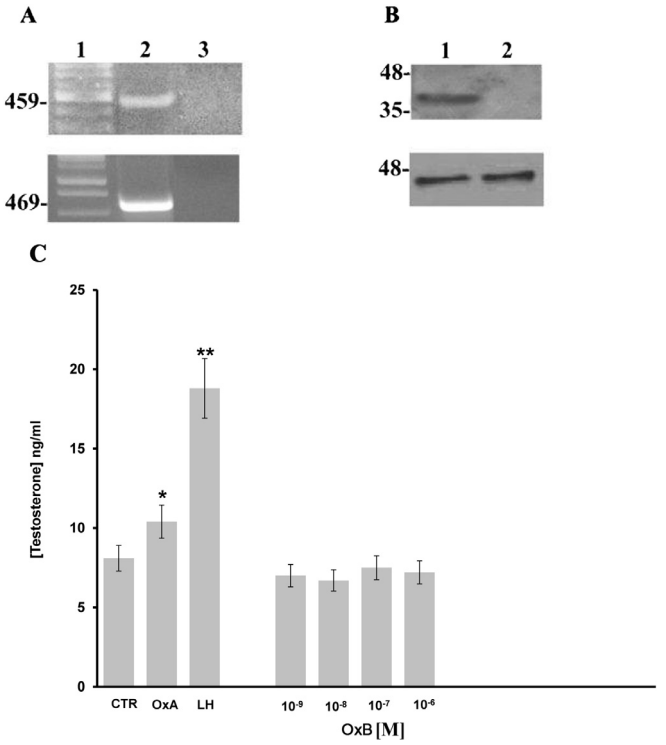

Fig. 4. (A and B) Expression of OX_2 mRNA and the protein in the rat testis homogenates. (A) RT-PCR analysis. Lane 1, DNA ladder; lane 2, OX_2 mRNA transcripts in rat testis sample, lane 3, negative control (no cDNA input). The bottom reports the β-actin mRNA transcripts in the samples (internal control). (B) Western blotting analysis. Lane 1, homogenate from rat testis tissue; lane 2 testis homogenate treated with the antisera directed against OX_2 pre-absorbed with its respective control peptide (negative control). The blots were stripped and re-probed with an anti-γ-tubulin monoclonal antibody to ensure equal loading of proteins in all lanes (lower blot). Molecular mass markers are indicated on the left. Similar results of those reported in (A) and (B) were obtained from four separate experiments of identical design. (C) Effect of OxB on testosterone secretion. Freshly collected rat testis slices were treated with increasing concentrations (from 10^{-9} to 10^{-6} M) of OxB, or 1 nM OxA or LH (500 ng/g tissue), and incubated for 12 h. Control tissue (CTR) was treated with PBS. Testosterone levels are expressed as ng/ml of tissue extract. The data reported represent the mean ± S.D. of three different determinations performed in duplicate. $^{*}p < 0.05$ and $^{**}p < 0.01$, versus control.

of OxB, and testosterone synthesis and release after a 12 h lasting time was measured. Some other tissue slices were incubated with OxA (1 nM) or LH (500 ng/g tissue). While OxA significantly enhanced testosterone production, OxB at all tested concentrations didn't show any effect on testosterone synthesis as compared to control (Fig. 4C). On the other hand, the steroidogenic effect of 12 h long lasting absorption of LH demonstrated that the responsiveness of the tissue used in our *in vitro* settings is warranted. These results are consistent with previous findings showing that OxA is able to enhance glucocorticoid secretion in rat and human adrenal cortices, while OxB was found to be either less potent or ineffective (Kagerer and Jöhren, 2010). Although further studies are needed to fully establish the activity of OxB in the mammalian testis, our results demonstrate that, in contrast with the definite evidence that OxA induces steroidogenesis in the rat testis (Barreiro et al., 2005; Assisi et al., 2012; Zheng et al., 2014), the role of OxB does not imply a direct involvement in the testicular steroidogenesis.

4. Conclusions

This study provides the first evidence for the localization of OxB and OX_2 receptor in the rat pachytene and second spermatocytes and spermatids at all stages of the cycle of the seminiferous epithelium. We also demonstrate the expression of both OX_2 mRNA and the protein in the rat testis, while the presence of OxB in the tissue is supported by the detection of prepro-orexin previously demonstrated by us and other authors (Jöhren et al., 2001; Tafuri et al., 2010). The analysis of the mechanism of action of OxB in the rat testis shows the inability of the peptide in promoting steroidogenesis. These results likely translate into the regulation of diverse biological actions by the two orexins and their receptors in the male gonad.

Acknowledgments

This work was supported by a grant from the University of Naples Federico II – Italy (contract n. U.BI.CO. 11941). We thank Dr. E. Cirillo for administrative help, and Mr. A. Calamo for technical assistance.

References

Assisi, L., Tafuri, S., Liguori, G., Paino, S., Pavone, L.M., Staiano, N., Vittoria, A., 2012. Expression and role of receptor 1 for orexins in seminiferous tubules of rat testis. Cell Tissue Res. 348, 601–607.

Barreiro, M.L., Pineda, R., Navarro, V.M., Lopez, M., Suominen, J.S., Pinilla, L., Señaris, R., Toppari, J., Aguilar, E., Diéguez, C., Tena-Sempere, M., 2004. Orexin 1 receptor messenger ribonucleic acid expression and stimulation of testosterone secretion by orexin-A in rat testis. Endocrinology 145, 2297–2306.

Barreiro, M.L., Pineta, R., Gaytan, F., Archanco, M., Burrell, M.A., Castellano, J.M., Hakovirta, H., Nurmio, M., Pinilla, L., Aguilar, E., Toppari, J., Dieguez, C., Tena-Sempere, M., 2005. Pattern of orexin expression and direct biological actions of orexin-A in rat testis. Endocrinology 146, 5164–5175.

Blanco, M., Garcìa-Caballero, T., Fraga, M., Gallego, R., Cuevas, J., Forteza, J., Beiras, A., Diéguez, C., 2002. Cellular localization of orexin receptors in human adrenal gland, adrenocortical adenomas and pheochromocytomas. Regul. Pept. 104, 161–165.

Chemelli, R.M., Willie, J.T., Sinton, C.M., Elmquist, J.K., Scammell, T., Lee, C., Richardson, J.A., Williams, S.C., Xiong, Y., Kisanuki, Y., Fitch, T.E., Nakazato, M., Hammer, R.E., Saper, C.B., Yanagisawa, M., 1999. Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. Cell 98, 437–451.

Clermont, Y., Perey, B., 1957. The stages of the cycle of the seminiferous epithelium of the rat: practical definitions in PA-Schiff-hematoxylin and hematoxylin-eosin stained sections. Rev. Can. Biol. 16, 451–462.

de Lecea, L., Kilduff, T.S., Peyron, C., Gao, X., Foye, P.E., Danielson, P.E., Fukuhara, C., Battenberg, E.L., Gautvik, V.T., Bartlett 2nd, F.S., Frankel, W.N., van den Pol, A.N., Bloom, F.E., Gautvik, K.M., Sutcliffe, J.G., 1998. The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. Proc. Natl. Acad. Sci. U.S.A. 6, 322–327.

Dehan, P., Canon, C., Trooskens, G., Rehli, M., Munaut, C., Van Criekinge, W., Delvenne, P., 2013. Expression of type 2 orexin receptor in human endometrium and its epigenetic silencing in endometrial cancer. J. Clin. Endocrinol. Metab. 98, 1549–1557.

Jöhren, O., Neidert, S.J., Kummer, M., Dendorfer, A., Dominiak, P., 2001. Prepro-orexin and orexin receptors mRNAs are differentially expressed in peripheral tissues of male and female rats. Endocrinology 142, 3324–3331.

Kagerer, S.M., Jöhren, O., 2010. Interactions of orexins/hypocretins with adrenocortical functions. Acta Physiol. (Oxf.) 198, 361–371.

Karteris, E., Randeva, H.S., 2003. Orexin receptors and G-protein coupling: evidence for another "promiscuous" seven transmembrane domain receptor. J. Pharmacol. Sci. 93, 126–128.

Karteris, E., Randeva, H.S., Grammatopoulus, D.K., Jaffe, R.B., Hillhouse, E.W., 2001. Expression and coupling characteristics of the CRH and orexin type 2 receptors in human fetal adrenals. J. Clin. Endocrinol. Metab. 86, 4512–4519.

Karteris, E., Chen, J., Randeva, H.S., 2004. Expression of human prepro-orexin and signaling characteristics of orexin receptors in the male reproductive system. J. Clin. Endocrinol. Metab. 89, 1957–1962.

Kukkonen, J.P., 2013. Physiology of the orexinergic/hypocretinergic system: a revisit in 2012. Am. J. Physiol. Cell Physiol. 304, C2–C32.

Kukkonen, J.P., Holmqvist, T., Ammoun, S., Akerman, K.E., 2002. Functions of the orexinergic/hypocretinergic systems. Am. J. Physiol. 283, C1567–C1591.

Li, J., Hu, Z., de Lecea, L., 2014. The hypocretins/orexins: integrators of multiple physiological functions. Br. J. Pharmacol. 171, 332–350.

Liguori, G., Assisi, L., Squillacioti, C., Paino, S., Mirabella, N., Vittoria, A., 2012. Presence, distribution and steroidogenic effect of the peptides orexin A and receptor 1 for orexins in the testis of the South America camelid alpaca (*Vicugna pacos*). Gen. Comp. Endocrinol. 179, 137–142.

Liguori, G., Paino, S., Mirabella, N., Squillacioti, C., De Luca, A., Vittoria, A., 2014. Expression of orexin A and its receptor 1 in the epididymis of the South American camelid alpaca (*Vicugna pacos*). Anat. Histol. Embryol. 43, 42–47.

Lin, L., Faraco, J., Li, R., Kadotani, H., Rogers, W., Lin, X., Qiu, X., de Jong, P.J., Nishino, S., Mignot, E., 1999. The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. Cell 98, 365–376.

Malendowicz, L.K., Tortorella, C., Nussdorfer, G.G., 1999. Orexins stimulate corticosterone secretion of rat adrenocortical cells, through the activation of the adenylate cyclase-dependent signaling cascade. J. Steroid Biochem. Mol. Biol. 70, 185–188.
Malendowicz, W., Szyszka, M., Ziolkowska, A., Rucinski, M., Kwias, Z., 2011. Elevated expression of orexin receptor 2 (HCRTR2) in benign prostatic hyperplasia is accompanied by lowered serum orexin A concentrations. Int. J. Mol. Med. 27, 377–383.
Matsuki, T., Sakurai, T., 2008. Orexins and orexin receptors: from molecules to integrative physiology. Results Probl. Cell Differ. 46, 27–55.
Mazzocchi, G., Malendowicz, L.K., Gottardo, L., Aragona, F., Nussdorfer, G.G., 2001. Orexin A stimulates cortisol secretion from human adrenocortical cells through activation of the adenylate cyclase-dependent signaling cascade. J. Clin. Endocrinol. Metab. 86, 778–782.
Mitsuma, T., Hirooka, Y., Kayma, M., Mori, Y., Yokoi, Y., Izumi, M., Rhue, N., Ping, J., Adachi, K., Ikai, R., Kawai, N., Nakayashiki, A., Nogimori, T., 2000. Radioimmunoassay for hypocretin-2. Endocr. Regul. 34, 23–27.
Nixon, J.P., Mavanji, V., Butterick, T.A., Billington, C.J., Kotz, C.M., Teske, J.A., 2015. Sleep disorders, obesity, and aging: the role of orexin. Ageing Res. Rev. 20, 63–73.
Nurmio, M., Tena-Sempere, M., Toppari, J., 2010. Orexins and the regulation of the hypothalamic-pituitary-testicular axis. Acta Physiol. (Oxf.) 198, 349–354.
Ohno, K., Sakurai, T., 2008. Orexin neuronal circuitry: role in the regulation of sleep and wakefulness. Front. Neuroendocrinol. 29, 70–87.
Pavone, L.M., Tafuri, S., Avallone, L., Staiano, N., Vittoria, A., 2009. Expression of orexin A and its receptor 1 in the vestibular glands of the cattle genital tract. Anat. Rec. 292, 202–206.
Randeva, H.S., Karteris, E., Grammatopoulos, D., Hillhouse, E.W., 2001. Expression of orexin-A and functional orexin type 2 receptors in the human adult adrenals: implications for adrenal function and energy homeostasis. J. Clin. Endocrinol. Metab. 86, 4808–4813.
Russo, F., Pavone, L.M., Tafuri, S., Avallone, L., Staiano, N., Vittoria, A., 2008. Expression of orexin A and its receptor 1 in the bovine urethroprostatic complex. Anat. Rec. 291, 169–174.
Sakurai, T., Amemiya, A., Ishii, M., Matsuzaki, I., Chemelli, R.M., Tanaka, H., Williams, S.C., Richardson, J.A., Kozlowski, G.P., Wilson, S., Arch, J.R., Buckingham, R.E., Haynes, A.C., Carr, S.A., Annan, R.S., McNulty, D.E., Liu, W.S., Terrett, J.A., Elshourbagy, N.A., Bergsma, D.J., Yanagisawa, M., 1998. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behaviour. Cell 92, 573–585.
Shahid, I.Z., Rahman, A.A., Pilowsky, P.M., 2012. Orexin and central regulation of cardiorespiratory system. Vitam. Horm. 89, 159–184.
Spinazzi, R., Rucinski, M., Neri, G., Malendowicz, L.K., Nussdorfer, G.G., 2005. Preproorexin and orexin receptors are expressed in cortisol-secreting adrenocortical adenomas, and orexins stimulate in vitro cortisol secretion and growth of tumor cells. J. Clin. Endocrinol. Metab. 90, 3544–3549.
Szczepanska-Sadowska, E., Cudnoch-Jedrzejewska, A., Ufnal, M., Zera, T., 2010. Brain and cardiovascular diseases: common neurogenic background of cardiovascular, metabolic and inflammatory diseases. J. Physiol. Pharmacol. 61, 509–521.
Tafuri, S., Pavone, L.M., Lo Muto, R., Basile, M., Langella, E., Fiorillo, E., Avallone, L., Staiano, N., Vittoria, A., 2009. Expression of orexin A and its receptor 1 in the rat epididymis. Regul. Pept. 5, 1–5.
Tafuri, S., Lo Muto, R., Pavone, L.M., Valiante, S., Costagliola, A., Staiano, N., Vittoria, A., 2010. Novel localization of orexin A in the tubular cytotypes of the rat testis. Regul. Pept. 164, 53–57.
Thannickal, T.C., Moore, R.Y., Nienhuis, R., Ramanathan, L., Gulyani, S., Aldrich, M., Cornford, M., Siegel, J.M., 2000. Reduced number of hypocretin neurons in human narcolepsy. Neuron 27, 469–474.
Valiante, S., Liguori, G., Tafuri, S., Campese, R., Monaco, R., Paino, S., Laforgia, V., Staiano, N., Vittoria, A., 2013. Expression of orexin A and its receptor 1 in the human prostate. J. Anat. 222, 473–480.
Xu, T.R., Yang, Y., Ward, R., Gao, L., Liu, Y., 2013. Orexin receptors: multi-functional therapeutic targets for sleeping disorders, eating disorders, drug addiction, cancers and other physiological disorders. Cell. Signal. 25, 2413–2423.
Zhang, S., Blache, D., Vercoe, P.E., Adam, C.L., Blackberry, M.A., Findlay, P.A., Eidne, K. A., Martin, G.B., 2005. Expression of orexin receptors in the brain and peripheral tissues of the male sheep. Regul. Pept. 124, 81–87.
Zheng, D., Zhao, Y., Shen, Y., Chang, X., Ju, S., Guo, L., 2014. Orexin A-mediated stimulation of 3β-HSD expression and testosterone production through MAPK signaling pathways in primary rat Leydig cells. J. Endocrinol. Invest. 37, 285–292.
Ziolkowska, A., Spinazzi, R., Albertin, G., Nowak, M., Malendowicz, L.K., Tortorella, C., Nussdorfer, G.G., 2005. Orexins stimulate glucocorticoid secretion from cultured rat and human adrenocortical cells, exclusively acting via the OX1 receptor. J. Steroid Biochem. Mol. Biol. 96, 423–429.