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Orexin A and B *in vitro* modify orexins receptors expression and Gonadotropins secretion of anterior pituitary cells of proestrous rats

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ABSTRACT

Aim: Orexin A and orexin B (hypocretins) are neuropeptides synthesized mainly by neurons located in the lateral hypothalamus and projections throughout the brain. They are agonists at both the orexin 1 and orexin 2 G protein-coupled receptors. They have been related to arousal, sleep and feeding, autonomic and neuroendocrine functions. Their role in the brain control of gonadotropins secretion was postulated in rodents and humans. Previously, we demonstrated the participation of the orexinergic system in attaining successful reproduction in *in vivo* studies.
Methods: We studied *in vitro* the effects of both neuropeptides, in the presence or absence of selective antagonists, on the mRNA expression of orexin 1 and orexin 2 receptors in anterior pituitary cells of proestrous rats, as well as the direct effects on FSH and LH secretion.
Results: Both orexin A and orexin B increased FSH and LH secretion; these effects were suppressed by the orexin 1 receptor blocking agent SB-334867 and the orexin 2 receptor antagonists JNJ-10397049. Orexin A and orexin B decreased OX1 receptor mRNA expression and this effect was modified only when both blocking agents were present. Neither orexin A nor the blocking drugs by themselves modified OX2 receptor mRNA expression. Orexin B treatment increased the mRNA expression of OX2 receptor. The effect was abolished only by the OX2 receptor antagonist.
Conclusion: In an *in vitro* model, we demonstrated a direct effect of orexins on gonadotropins release and orexins receptors expression, underlining the hypothesis that orexins participate in the brain control of pituitary functions.

1. Introduction

Orexin A (OXA) and Orexin B (OXB), also referred to as hypocretins A and B, are neuropeptides derived by proteolytic cleavage from a 130 amino acid precursor, prepro-orexin (PPO), which was isolated from the rat hypothalamus [1–3]. They are synthesized mainly by neurons with their soma located in the lateral hypothalamus and projections throughout the brain, including gonadotropin-releasing hormone neurons in the rat and ovine hypothalamus [4–7]. Their effects are mediated by Orexin 1 (OX1-R) and Orexin 2 (OX2-R) G protein-coupled receptors. OX1-R and OX2-R are widely expressed within the rodent brain, with some differences in their distribution; furthermore, differential roles for OX1 and OX2 receptors have been suggested [8–19].

Functionally, orexins have been related to arousal and alertness, regulation of sleep and appetite, food intake and feeding behavior and autonomic and neuroendocrine functions, including reproduction [20–23]. Their participation on the brain control of the pituitary secretion, including gonadotropins secretion, was postulated in rodents and humans [6,14,24–27]. In previous works, we demonstrated the participation of the orexinergic system in the development of the neuroendocrine events necessary for attaining successful reproduction. Specific antagonists of OX1-R and OX2-R, SB-334867-A and JNJ-10397049 respectively, alone or combined, decreased preovulatory serum gonadotropins surges, and reduced ova number the following morning, in addition to inducing ovarian structural changes [28–31].

Following this line of research, here we studied the *in vitro* effects of both OXA and OXB, in the presence or absence of their selective antagonists, on the expression of mRNA OX1-R and OX2-R in anterior pituitary cells of proestrous rats, as well as the direct effects of the neuropeptides on FSH and LH secretion.

2. Material and methods

2.1. Animals

Adult female virgin Sprague–Dawley rats (200–250 g) from the Instituto de Biología y Medicina Experimental colony were housed in groups in an air-conditioned room, with lights on from 07:00 to 19:00 h. They were given free access to laboratory chow and tap water. All studies on animals were performed according to protocols

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for animal use, approved by the Institutional Animal Care and Use Committee which follows the National Institute of Health (NIH) guidelines. The stage of the estrous cycle was determined by vaginal smears for fifteen consecutive days. Regular cycles were defined as the occurrence of three consecutive 4–5 day cycles. Cycling rats were sacrificed by decapitation at 09:00–10:00 on the day of proestrus.

2.2. Anterior pituitary cultures

Cells were obtained as described [32,33]. Briefly, anterior pituitaries were rapidly removed and placed in freshly prepared Krebs–Ringer bicarbonate buffer without Ca^{2+} or Mg^{2+}. Pituitaries were cut into small pieces and incubated in 0.2% trypsin for 30 min. After addition of DNase and lima bean trypsin inhibitor, the fragments were dispersed into individual cells and filtered through Nytex mesh (Nytex 50; Nytex, Geneva, Switzerland). Pituitary cells were plated (700.000 cells/well, in a 24 well plate) in DMEM with low glucose, supplemented with 10% horse serum, 2.5% fetal calf serum, 1% minimum essential medium Eagle nonessential amino acids, fungizone, and gentamicin. Cells were maintained in the incubator for 3 days with medium (control) or OXA (10-9 M, Sigma–Aldrich) or OXB (10-9 M, Sigma–Aldrich), in the absence or presence of the selective antagonists. SB-334867-A (OX1R ant; N-(2-methyl-6-benzoxazolyl)-N″-1, 5-naphtyridin-4-yl urea hydrochloride; 1 M, Tocris Bioscience; MO; USA) is a non-peptide OX1 selective receptor antagonist [34]. Selective OX2 antagonist JNJ-10397049 [35] (OX2R ant; 9,1-(2,4-dibromo-phenyl)-3-((4S, 5S)-2,2-dimethyl-4-phenyl-[1,3]dioxan-5-yl)-urea, 1 M) was provided by Johnson & Johnson Pharmaceutical Research & Development, LLC, S. Diego, USA. Drugs concentrations used were determined by preliminary studies and the literature [7,11,13,16,36–38].

Expression of mRNAs for OX1-R and OX2-R determinations was analyzed in cells, and media were stored (−20 °C) for FSH and LH analysis by RIA. The experiments were performed in quadruplicate and repeated 7–8 times. Incubations were done during 72 h, with renewal of stimuli each 24 h. Expressions of mRNAs for OX1-R and OX2-R were determined by quantitative RT-PCR.

2.3. Total RNA preparation and cDNA synthesis

At the end of the experiment media were collected, cells were washed with PBS and 300 μl/well of TRIzol reagent was added for total RNA isolation as previously described [29]. The RNA concentration was determined based on absorbance at 260 nm and its purity was evaluated by the ratio of absorbance at 260 nm/280 nm (>1.8). RNAs were kept frozen at −70 °C until analyzed. After digestion of genomic DNA by treatment with deoxyribonuclease I (Ambion, Austin, TX), first-strand cDNA was synthesized from 1 μg of total RNA, in the presence of 10 mM $MgCl_2$, 50 mM Tris–HCl (pH 8.6), 75 mM KCl, 0.5 mM deoxy-NTPs, 1 mM DTT, 1 U/μl RnaseOUT (Invitrogen, Buenos Aires, Argentina), 0.5 μg oligo-(deoxythymidine) 15 primer (Biodynamics, Buenos Aires, Argentina), and 20 U MMLV Reverse Transcriptase (Epicentre, Madison, WI). To validate successful deoxyribonuclease I treatment, the reverse transcriptase was omitted in control reactions. The absence of PCR-amplified DNA fragments in these samples indicated the isolation of RNA free of genomic DNA.

2.4. Quantitative real-time PCR

Sense and antisense oligonucleotide primers were designed based on the published cDNA OX1-R and OX2-R, and cyclophilin sequences using the PrimerExpress software (Applied Biosystems, Foster City, CA), as published [9]. Briefly, oligonucleotides were obtained from Invitrogen. The sequences of the primers were as follows: OX1-R sense GCCTGCCAGCCTGTTAGTG, OX1-R antisense CAAGGCATGGCCGA AGAG, OX2-R sense GAAAGAATATGAGTGGGTCCTGATC, OX2-R antisense CAGGACGTTCCCGATGAGA, cyclophilin sense GTGGCAAGATCG AAGTGGAGA AAC, cyclophilin antisense TAAAAATCAGGCCTGTGGAAT GTG.

Quantitative measurements of OX1-R, OX2-R and cyclophilin cDNA were performed by kinetic PCR using SYBR green I as fluorescent dye (Invitrogen). PCR reactions consisted of 100 ng cDNA, 0.4 μM primers, 10 mM Tris–HCl, 50 mM KCl, 3 mM $MgCl_2$, 0.2 mM deoxy-NTPs, and 1.25 U Taq Polymerase (Invitrogen) in a final volume of 25 μl. After denaturizing at 95 °C for 5 min, the cDNA products were amplified with 40 cycles, each cycle consisting of denaturizing at 95 °C for 15 s, annealing at 62 °C for 40 s and extension at 72 °C for 40 s. The accumulating DNA products were monitored by the ABI7500 sequence detection system (Applied Biosystems), and data were stored continuously during the reaction. The results were validated based on the quality of dissociation curves, generated at the end of the PCR runs by ramping the temperature of the samples from 60 °C to 95 °C, while continuously collecting fluorescence data. Product purity was confirmed by polyacrylamide gel electrophoresis. Each sample was analyzed in duplicate along with specific standards and no template controls to monitor contaminating DNA. The calculations of the initial mRNA copy numbers in each sample were made according to the cycle threshold (Ct) method. The CT for each sample was calculated at a fluorescence threshold (Rn) using the ABI7500 sequence detection system software with an automatic baseline setting. For all designed primer sets, linearity of real-time RT-PCR signaling was determined with wide-range serial dilutions of reference cDNA that covered the amount of target mRNA expected in the experimental samples, and clear linear correlations were found between the amount of cDNA and the Ct for the duration of at least 40 real-time RT-PCR rounds.

For the target gene, the relative gene expression was normalized to that of the cyclophilin housekeeping gene by use of the standard curve method, as described by the manufacturer (User bulletin # 2). Results are expressed as arbitrary units (AU) for comparison among samples. AU is defined as the expression level relative to a control sample (calibrator sample).

2.5. Hormone determinations

Medium FSH and LH were determined by RIA using kits obtained through NHPP, NIDDK and Dr. A. Parlow. Results were expressed in terms of RP_3 (reference preparation 3) rat LH and FSH standards. Assay sensitivities were 0.015 ng/ml for LH, and 0.1175 ng/ml for FSH. Intra- and inter-assay coefficients of variation for LH were 7.2% and 11.4%, respectively, for FSH 8.0% and 13.2%, respectively.

2.6. Statistics

Data are presented as mean ± SEM. Cultures were repeated 7–8 times, stimuli in duplicates or quadruplicates. Differences between treatments groups were estimated by one-way variance analysis for repeated measures (ANOVA) followed by Tukey's post-test using the Statistica Software. $P < 0.05$ indicated statistically significant differences.

3. Results

3.1. Gonadotropins secretion into culture media of anterior pituitary cells incubated with OXA and OXB; effects of selective antagonists

OXA increased FSH (Fig. 1, upper) and LH (Fig. 1, lower) secretion. The effects of the neuropeptide on FSH and LH were suppressed by the blocking agents, OX1R antagonist SB-334867 and OX2R antagonist JNJ-10397049, when used alone or combined.

OXB shows a similar effect (Fig. 2). The blocking drugs by themselves had no actions on gonadotropins release into the culture medium (not shown).

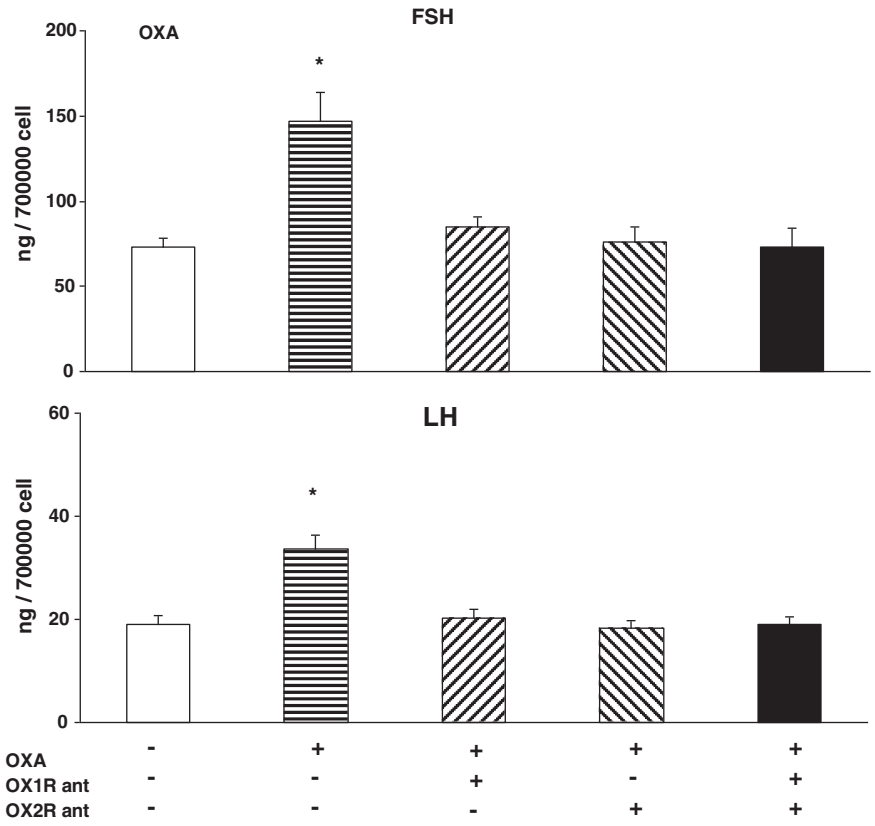

Fig. 1. Effects of OXA on FSH (upper) and LH (lower) secretion into culture media of anterior pituitary cells. OXA increased the release of both gonadotropins; this effect was blunted by the blocking agents. In this and following figures: Mean ± SEM is shown. * $p < 0.05$: different from control. OXA: orexin A. OXB: orexin B. OX1-R: Orexin 1 receptor. OX2-R: Orexin 2 receptor OX1-R ant: OX1-R antagonist. OX2-R ant: OX2-R antagonist. The blocking drugs had no actions by themselves (not shown).

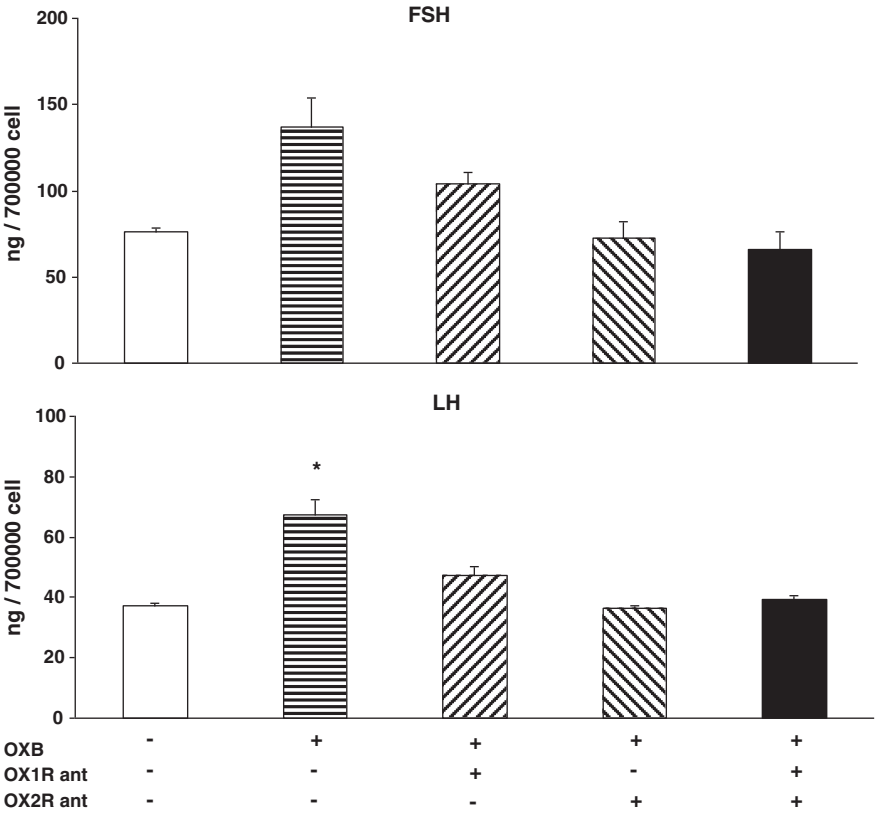

Fig. 2. Effects of OXB on FSH (upper) and LH (lower) secretion into culture media of anterior pituitary cells. OXB increased the release of both gonadotropins; this effect was blunted by the blocking agents.

3.2. OX1-R and OX2-R mRNA expression in anterior pituitary cells treated with OXA and OXB

OXA (Fig. 3 upper panel) and OXB (Fig. 3 lower panel) treatment decreased the mRNA expression of OX1-R. The effect on OX1-R was not modified by either blocking agent when used alone, but it was abolished when both antagonists were present. The blocking drugs alone did not modify expression (not shown).

Neither OXA nor the blocking drugs, alone or combined, modified OX2-R mRNA expression (Fig. 4 upper panel). OXB increased OX2-R mRNA expression and only OX2R antagonist blocked this effects (Fig. 4 lower panel).

4. Discussion

In previous *in vivo* studies, we found that OX1-R and OX2-R expression increased in hypothalamus and anterior pituitary, during the late afternoon and night of proestrus, without variations in other stages of the estrous cycle, or in males. PPO, the precursor of OXA and OXB, also increased in hypothalamus only during proestrus while no PPO was detected in adenohypophysis. Since the changes in OX1-R, OX2-R and PPO observed bared no relationship to the light–dark cycle or to food intake [28,29] we postulated that they were cycle-related events associated to the neuroendocrine status of proestrus and gonadotropins release. Furthermore, we suggested that changes in the reproductive state are able to influence the orexinergic system by different mechanisms in hypothalamus and in anterior pituitary [29]. Previous works explored the actions of orexins on gonadotropin secretions in rodents *in vivo*, but controversy remained regarding the effects observed, probably due to the different and more complex experimental models used [14,25–27,39–42].

In order to clarify this situation, in the present study we used a primary pituitary cell culture in which the neuropeptides OXA and OXB act directly on pituitary cells to determine whether these neuropeptides have effects on FSH and LH release as well as on the mRNA expression of their cognate receptors OX1-R and OX2-R.

We found that both OXA and OXB induced gonadotropins release by a mechanism responding to the receptor blocking agents tested, demonstrating specific effects. In addition, the mRNA expression of OX1-R was decreased by OXA and OXB, and the presence of both, OX1R antagonist and OX2R antagonist, was necessary to suppress this action, suggesting that both receptors are involved indicating an additive effect. In addition, the peptide-induced decrease of the expression of their own receptor suggests a possible inhibitory loop on orexins' actions. In contrast, the mRNA expression of OX2-R was differentially modulated by each orexin. OXA did not alter OX2-R expression while it was significantly increased by OXB, an effect that was abolished only by the OX2R antagonist JNJ-10397049. Thus, both neuropeptides are active directly at the anterior pituitary, increasing FSH and LH release and modifying OX1-R and OX2-R mRNA expression.

The hormonal effects of OXA and OXB, sensitive to both receptor blocking agents, suggest that the increased expression of PPO in the proestrous hypothalamus [28,29] and the increased OXA and OXB presence in the median eminence at this stage of the estrous cycle [14], may have a physiological role by participating in the induction of the preovulatory gonadotropin surges. These results build up on previous information regarding the direct effects of orexins in the pituitary. Chen et al. demonstrated that orexins stimulate GH secretion either alone or in

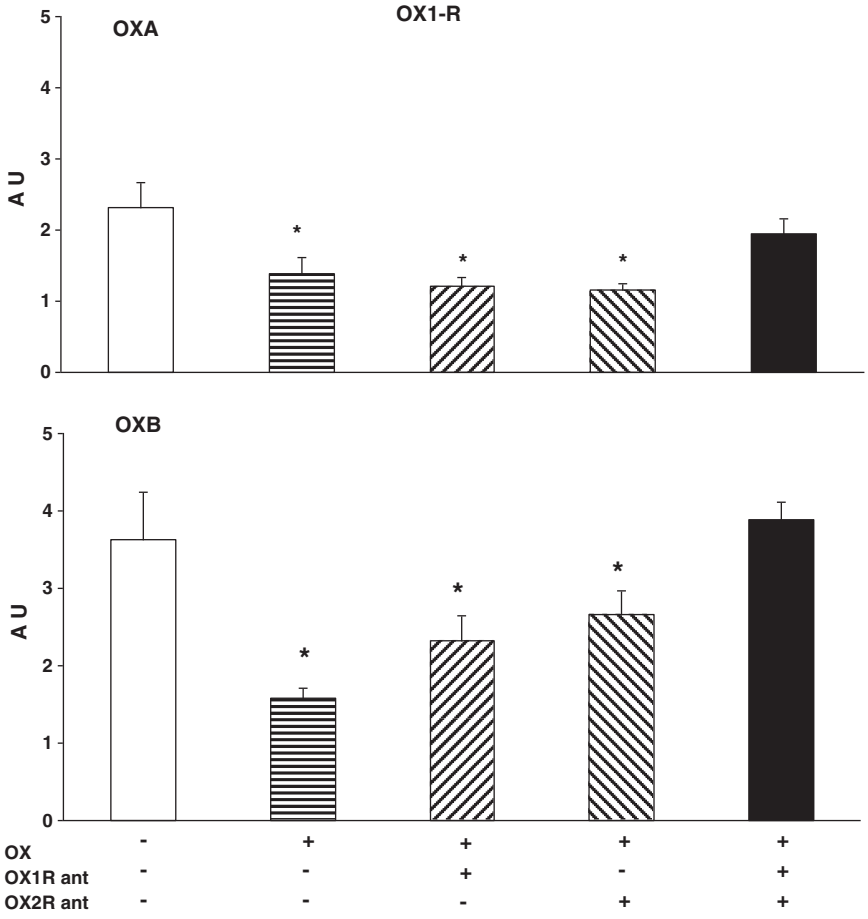

Fig. 3. Effect of OXA (upper) and OXB (lower) on OX1-R mRNA expression in anterior pituitary cells cultures. OXA and OXB decreased the expression of OX1-R. The effect was not modified by the blocking agents when used alone, but it was abolished when both antagonists were present. AU: arbitrary units.

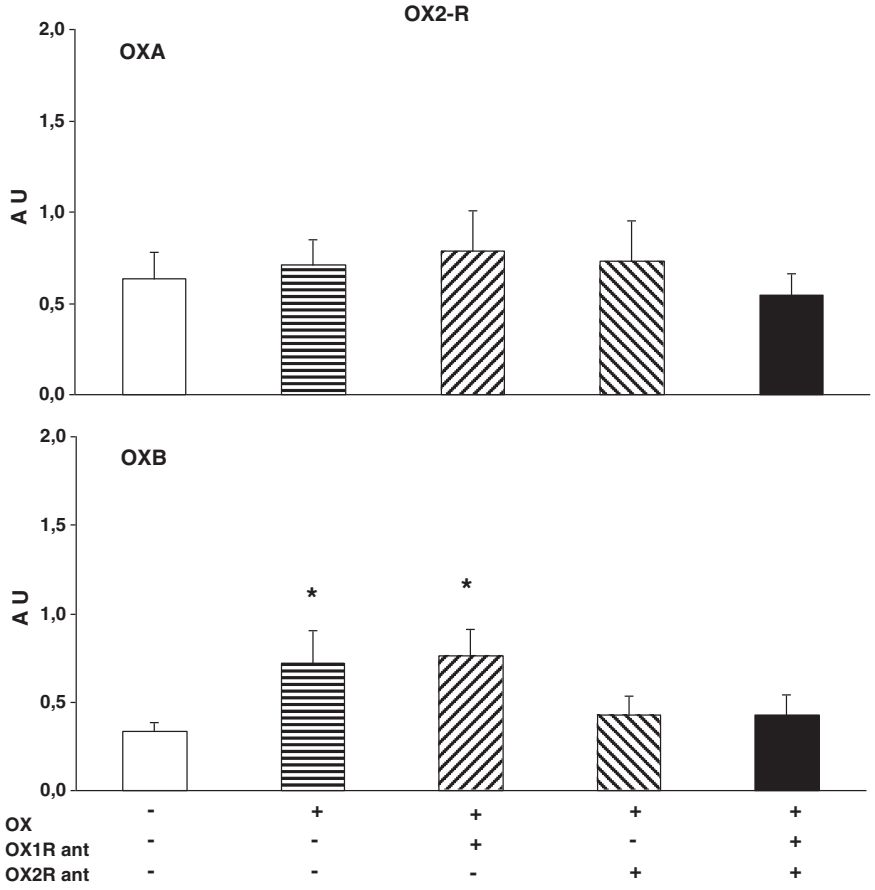

Fig. 4. Effect of OXA (upper) and OXB (lower) on OX2-R mRNA expression in anterior pituitary cells culture. OXA had no effect on OX2-R mRNA expression. OXB increased OX2-R expression and only OX2-R antagonist alone or combined with OX1-R antagonist blocked this affect.

combination with GHRH [43]. Our results on LH and FSH reinforce a role for orexins in the regulation of pituitary physiology. Conversely, orexin-induced changes in the expressions of OX1-R and OX2-R mRNAs seem to be more complex. OXA and OXB decreased OXR-1 expression, an effect that was reversed by the sum of both blocking agents. OXB, but not OXA, increased the expression of OXR-2 and this action could be impaired only by JNJ-10397049. It was postulated that OXA and OXB are agonists at both the OX1-R and OX2-R; OXA is a more selective ligand for OX1-R; OX2-R binds both OXA and OXB [8,13,16,36]. Here we found an effect that was exclusive of OXB by binding to the OX2 receptor: the increase in OX2-R expression. Exclusive effects triggered by the OX2-R have been previously described as is the case of presence of narcolepsy in OX2-R knockout mice [44] vs. absence of this characteristic in OX1-R knock-out mice [45,46]. Whether the effects of orexins on orexin receptor expression are also seen at the protein level remains to be established in order to propose a functional role for this regulation.

Different mechanisms could explain the relationship between the effects of orexins on hormones output (stimulation) and orexin receptors mRNA expression (inhibition). One possibility is that the inhibition of the receptor OXR-1 could be acting by an inhibition of an intracellular mechanism, that in turn, suppresses gonadotropins' release, thus the final results will be an increase in gonadotropins output; new studies are under way to explore this possibility. Furthermore, these neuropeptides could be acting not only on gonadotrophs, since all pituitary cells are present in primary pituitary cultures. In humans, orexins A and B were detected in specific human pituitary cell types by immunofluorescence: orexin A was present mainly in lactotrophs and also, to a lesser extent, in thyrotrophs, somatotrophs, and gonadotrophs, but absent in corticotrophs; conversely orexin B was found in virtually all corticotroph cells of the human anterior pituitary [24]. In rats, the presence of OXA and OXB was described in the median eminence, adenohypophysis, and neurohypophysis [47], though there was no pituitary expression of PPO, as observed by us [28] and others [48]. Therefore, pituitary orexins must originate probably in the hypothalamus and arrive by portal or general circulation [47]. The possibility that orexins may originate from outside the pituitary and arrive by general or portal circulation is in line with the fact that immunoreactive Orexin A has been described in human and rat plasma [49,50]. Furthermore, fluctuations of both orexins in hypothalamus during the estrous cycle were reported; hypothalamic OXA and OXB concentrations were informed to be higher in proestrus than in diestrus in young cycling animals and the greatest OXA release from hypothalamus was suggested to occur on proestrus [14]. In the anterior lobe OXR-1 was more markedly expressed than the OXR-2 [47], in agreement with our previous [28] and other's results [14,18,27,41,42,48].

The possibility that orexins via general or portal circulation reach the gland, and alone or in combination with other factors, may regulate gonadotropins secretion and OX1-R and OX2-R mRNA expression, especially during proestrus, is an interesting hypothesis that should be a matter of further research.

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