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Effects of orexins A and B on expression of orexin receptors and progesterone release in luteal and granulosa ovarian cells

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ABSTRACT

Orexin-A and orexin-B are neuropeptides controlling sleep-wakefulness, feeding and neuroendocrine functions *via* their G protein-coupled receptors, orexin-1R and orexin-2R. They are synthesized in the lateral hypothalamus and project throughout the brain. Orexins and orexin receptors have also been described outside the brain. Previously we demonstrated the presence of both receptors in the ovary, their increased expression during proestrous afternoon and the dependence on the gonadotropins.

Here we studied the effects of orexins on the mRNA expression of both receptors, by quantitative real-time PCR, on luteal cells from superovulated rat ovaries and granulosa cells from diethylstilbestrol-treated rat ovaries. Effects on progesterone secretion were also measured.

In luteal cells, 1 nM of either orexin-A or orexin-B decreased progesterone secretion. Orexin-A treatment increased expression of both orexin-1R and orexin-2R mRNA. The effect on orexin-1R mRNA expression was abolished by an orexin-1R selective receptor antagonist SB-334867 and the effect on orexin-2R mRNA expression was abolished by a selective orexin-2R antagonist JNJ-10397049. Orexin-B did not modify orexin-1R mRNA expression, but increased orexin-2R mRNA expression. The effect of orexin-B on orexin-2R was abolished by a selective orexin-2R antagonist. Neither the expression of orexin receptors nor progesterone secretions by granulosa cells were affected by orexins. FSH, as positive control, increased both steroid hormones secretion, but did not induce the expression of OX receptors in granulosa cells isolated from late preantral/early antral follicles. Finally in ovaries obtained immediately after sacrifice, the expression of orexin-1R and orexin-2R was higher in superovulated rat ovaries compared to control or diethylstilbestrol treated rat ovaries.

A selective presence and function of both orexinergic receptors in luteal and granulosa cells is described, suggesting that the orexinergic system may have a functional role in the ovary.

1. Introduction

The regulatory peptides orexin-A (OXA, also known as hypocretin 1) and orexin-B (OXB, hypocretin 2), acting *via* G protein-coupled receptors orexin-1 receptor (OX1-R) and orexin-2 receptor (OX2-R), control sleep-wakefulness, feeding, and a variety of neuroendocrine functions. The orexins are derived from prepro-orexin (PPO), a 130 amino acid precursor which was isolated from rat hypothalamus, to mature OXA (33 residues) and OXB (28 residues). Both neuropeptides are synthesized by neurons in the lateral hypothalamus and project throughout the brain [1–5]. In addition, a peripheral source of the orexin peptides could be the gut, which expresses PPO [6]. Orexins and orexin receptors have been described outside the CNS. They are expressed in several glands including gonads and genital tract in both sexes [7–16].

In a previous work, we demonstrated the influence of the reproductive state, GnRH and gonadotropins, particularly the hormonal milieu of late proestrus, on the central orexinergic system [17]. We also showed the presence of both receptors, OX1-R and OX2-R in the ovary, their increased expression during the proestrous afternoon and the dependence of this expression on the gonadotropin peaks, but not on the dark–light cycle or food intake [18,19].

Here we studied the *in vitro* effects of both orexinergic neuropeptides, in the presence or absence of selective antagonists, on the expression of OX1-R and OX2-R receptors in luteal and granulosa cells of rats, as well as effect(s) on steroid hormone secretion. In addition, the expression of both receptors was determined in ovaries obtained immediately after sacrifice in controls, superovulated rats and diethylstilbestrol treated rats.

2. Material and methods

2.1. Animals

Female virgin Sprague–Dawley rats from the Instituto de Biología y Medicina Experimental colony were housed in groups in an air-conditioned room (21 °C), with lights on from 07:00 h to 19:00 h. They were given free access to laboratory chow and tap water. At the

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end of experimental procedures, animals were killed by decapitation at 09:00–10:00 h. Trunk blood was collected, ovaries were quickly removed and then tissues and sera were stored at −20 °C for hormone determinations. All procedures were performed according to protocols for animal use, approved by the institutional animal care and use committee (IBYME-CONICET) consistent with NIH guidelines.

Luteal cells, superovulated rat ovaries (SPO): Prepuberal female rats, 23 days old, were injected s.c. with 25 IU of equine chorionic gonadotropin (eCG) (Novormon, Syntex, Buenos Aires, Argentina) and the 25 IU of human chorionic gonadotropin (hCG) (Endocorion, Elea, Buenos Aires, Argentina) 48 h later. These animals were used 5 days after hCG injection. They were quickly decapitated in the morning, between 900 and 1100 h, for sampling. Details of the model are described in references [20,21].

Granulosa cells, diethylstilbestrol treated rat ovaries (DES): Prepuberal female rats, 23 days old, were injected s.c. with 1 mg diethylstilbestrol (Sigma-Aldrich, St. Louis, MO), dissolved in castor oil, daily for 3 days to stimulate the development of early antral follicles. 24 h after the last injection blood and ovaries were collected as described above. Solvent injected animals were used as a control (C) group.

2.2. In vitro procedures

Cells from SPO ovaries, were isolated with collagenase (Life Technologies, Inc., Grand Island, NY), as described previously [20,21]. Each SPO ovary yielded approximately 1×10^6 viable cells/well. Briefly, cells were plated in plastic 24-well culture dishes, precoated with rat tail collagen (Sigma-Aldrich) and incubated in BIC: DMEM-F12 (Life Technologies, Carlsbad, CA) with 2.2 g/l sodium bicarbonate, 10% fetal bovine serum (Life Technologies), 0.01 mg/ml gentamicin (Life Technologies), and 0.01 mg/ml fungizone (Life Technologies). 24 h after plating the media were replaced. After 7 days in culture the cells were washed once with serum-free BIC-BSA: 0.1% BSA-supplemented (Sigma-Aldrich) medium (DMEM-F12 with 2.2 g/l sodium bicarbonate) and the stimuli were added. Stimuli were renewed 24 h later. Samples were collected 24 h thereafter (total incubation time: 48 h) for the corresponding determinations.

Granulosa cell isolation and culture. Granulosa cells from DES-treated rats were isolated, as described previously [22]. Cells were seeded onto plastic 24-well plates (Nunc, Roskilde, Denmark) precoated with rat tail collagen. The initial plating density was 6×10^5 viable cells/well. Ovaries were incubated in Dulbecco Modified Eagle Medium (DMEM), EGTA (6.8 mM), and HEPES (10 mM; 15 min at 37 °C) and then washed twice and incubated in DMEM-FI2 (l: l), sucrose (0.5 M), and HEPES (10 mM; 5 min at 37 °C). Granulosa cells were obtained by pressing ovaries within two pieces of nylon mesh (Nytex 50, Geneva, Switzerland) and were purified by density gradient centrifugation, as described by Magoffin and Erickson [23]. Cells were maintained at 37 °C with 5% CO_2. Androstenedione (Sigma-Aldrich), 0.25 μM was added as an estradiol precursor. After 3 h, the medium was changed to remove

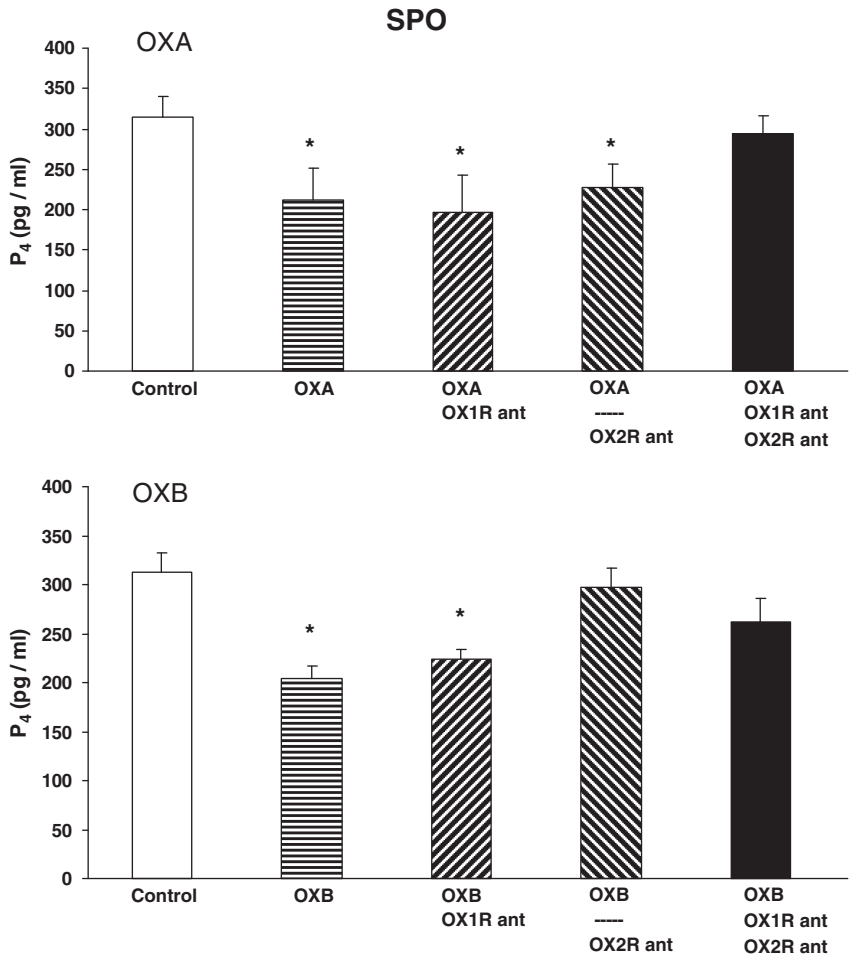

Fig. 1. Progesterone (P_4) secretion into culture media by luteal cells treated with 1 nM OXA (upper panel) or OXB (lower panel), in the presence or absence of 10 μM OX1R antagonist, OX2R antagonist or both orexin antagonists combined. After 7 days in culture media were changed and the stimuli added. Stimuli were renewed 24 h later. Samples were collected 24 h thereafter (total incubation time: 48 h). No effect of OX1R ant, OX2R ant or the OX1R/OX2R antagonist combination was seen in the absence of OXA or OXB stimulation (not shown). Mean ± SEM is shown with * significantly different from control ($p<0.05$) ($n=4$–6). Cultures were repeated 5–7 times; wells by duplicate or triplicate. OXA: orexin A. OXB: orexin B. OX1R ant: OX1R antagonist: OX2R ant: OX2R antagonist.