Both orexin receptors are expressed in rat ovaries and fluctuate with the estrous cycle: effects of orexin receptor antagonists on gonadotropins and ovulation

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Orexins and their receptors have been described outside the central nervous system (CNS). Receptors are expressed in glands such as the pituitary (4, 13, 25, 26, 53), adrenal (25, 29), thyroid (26), and pancreatic islets (41). In human peripheral tissues, immunoreactivity for orexin A was detected in ganglion cells, myenteric plexuses, gastrointestinal endocrine cells, islet cells of the pancreas, placental syncytiotrophoblasts, and decidual cells; mRNA expression for PPO was detected in the kidney, adrenal gland, pancreas, placenta, stomach, and intestinal epithelial cells (39). Immunoreactive orexin A in human plasma was found by radioimmunoassay (RIA) (2). Regarding the gonads, orexin A and B immunoreactivity has been detected in testis, epididymis, penis, and seminal vesicle, whereas PPO was reported only in the epididymis and penis in the human male reproductive system (28). In a screening study comparing several tissues of male and female rats, a very low amount of OX1 mRNA was found in pooled ovaries (26).

Taking in consideration these data and our recent results (53), we decided to study PPO, OX1, and OX2 expression in ovaries of adult rats at different stages of the estrous cycle and correlated them to hormonal status, dark-light cycle, and food consumption. Furthermore, the effects of two potent and selective antagonists of orexin receptors OX1 and OX2 were evaluated on gonadotropin secretion, ovarian histology and ovulation.

MATERIALS AND METHODS

Animals. Adult female virgin Sprague-Dawley rats (200–250 g) from the Instituto de Biología y Medicina Experimental colony were

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housed in groups in an air-conditioned room (21°C), with lights on from 0700 to 1900. They were given free access to laboratory chow and tap water. All studies on animals were performed according to protocols for animal use approved by the Institutional Animal Care and Use Committee (Instituto de Biología y Medicina Experimental, Consejo Nacional de Investigaciones Científicas y Técnicas) and by the National Institutes of Health.

First set of experiments. Regular cycling female rats were killed by decapitation at 1100, 1500, 1700, 1900, and 2300 in all stages of the estrous cycle. The stage of the cycle was determined by vaginal smears for two consecutive weeks. Regular cycles were defined as the occurrence of three consecutive 4-day cycles. Occurrence of ovulation was evaluated by microscopic examination of the oviduct the morning of estrus.

In all cases trunk blood was collected, and sera were stored at −20°C for hormone determinations by RIA. Day and night food intake was also recorded. For this purpose, during the entire experiments rats were kept in individual cages, and food consumption was determined at 0700 and 1900.

Ovaries were rapidly removed and placed on ice for dissection; tissue samples were immediately homogenized in TRIZol reagent (Invitrogen) and kept at −70°C until used. Levels of expression of mRNAs for PPO, OX₁, and OX₂ in the ovaries were determined by quantitative real-time RT-PCR.

Second set of experiments. In a group of proestrous rats, ovulation was blocked, as described (53). Briefly, Cetrorelix, a GnRH receptor antagonist (Serono, Buenos Aires, Argentina), 100 μg/100 μl sterile water ip per rat, or pentobarbital sodium (Nembutal), a barbiturate that blocks ovulation, 30 mg/kg body wt ip, diluted in 1:7:2 parts of absolute ethanol, sterile water, and propylene glycol, respectively, or saline as control, was injected at 1400 of proestrus. Animals were decapitated at 1900, and ovaries and sera collected. Ovarian levels of expression of OX₁ and OX₂ were determined by real-time RT-PCR.

Third set of experiments. Finally, groups of cycling rats were treated at 1400 and 1900 of proestrus with the OX₁ and/or OX₂ blocking drugs.

SB-334867-A, a nonpeptide OX₁-selective receptor antagonist (Tocris Bioscience) (55), was injected intraperitoneally at the dose of 10 mg/kg body wt diluted in 50% DMSO and 50% saline. JNJ-10397049, a selective OX₂ receptor antagonist consisting of a substituted 4-phenyl-[1,3]dioxane-(9H)-2,2-dimethyl-4-phenyl-[1,3]dioxan-5-yl)-urea (37) provided by Johnson and Johnson Pharmaceutical Research and Development, San Diego, CA), was injected intraperitoneally at the dose of 10 mg/kg body wt diluted in 50% DMSO and 50% saline. A third group was injected simultaneously with both blocking drugs: 10 mg/kg of SB-334867-A plus 10 mg/kg of JNJ-10397049. Control ovulating proestrus rats received two injections of vehicle. In addition, two other groups of proestrus rats were injected with Cetrorelix or Nembutal (as in the 2nd set of experiments) at 1400 and with saline at 1900, as controls of nonovulating animals. Blood samples were collected at 1900 from the jugular vein under light ether anesthesia, before the second injection of the blocking drug or saline, according to the respective protocol. Rats were decapitated at 0900 the following morning, and ovaries and sera were collected and kept as above.

Food intake was determined in all groups at 1400 and 1900 of proestrus to evaluate the effectiveness of OX₁ and OX₂ antagonists.

Ovarian morphology. To evaluate changes in the macro- and microstructure, some ovaries of each group were first examined after laparotomy and then immediately fixed in 5% neutral buffered formaldehyde for histological examination. After embedded in paraffin, ovary sections were stained with hematoxylin-eosin to count the number of preovulatory follicles and corpora lutea under a light microscope as described by Parborell et al. (43) and Peluffo et al. (44).

Total RNA preparation and cDNA synthesis. Total RNA was isolated using the TRIZol reagent method from tissue homogenates. 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 with deoxyribonuclease I (Ambion, Austin, TX), first-strand cDNA was synthesized from 1 μg of total RNA in the presence of 10 mM MgCl₂, 50 mM Tris·HCl (pH 8.6), 75 mM KCl, 0.5 mM deoxy-NTPs, 1 mM DTT, 1 U/μl RnaseOUT (Invitrogen), 0.5 μg oligo(dT)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.

Quantitative real-time PCR. Sense and antisense oligonucleotide primers were designed based on the published cDNA PPO, OX₁ and OX₂ receptor, and cyclophilin sequences using the PrimerExpress software (Applied Biosystems, Foster City, CA). Oligonucleotides were obtained from Invitrogen. The sequences of the primers were as follows: PPO sense, GGCCACGAGCAGGAGAGCT; OX₁ sense, GCCGTG-CAGCCCTTGTAGTG; OX₂ sense, CAAGCCTATCGCCGAAGAGAAG; OX₂ sense, GAAAGAATATGAGTGGGTCCTGATC; OX₂ antisense, CAGGACCTCGCCGAGGAGA; and cyclophilin antisense, JAAAATATCAGGCTCTTGAG.

Quantitative measurements of PPO, OX₁, and OX₂ receptor, and cyclophilin cDNA were performed by kinetic PCR using SYBR green I as fluorescent dye (Invitrogen). PCR reaction consisted of 100 ng cDNA, 0.4 μM primers, 10 mM Tris·HCl, 50 mM KCl, 3 mM MgCl₂, 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 30 s, and extension at 72°C for 30 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, meanwhile 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

![Fig. 1. Orexin 1 (OX₁) and orexin 2 receptor (OX₂) mRNA expression in rat ovary at different times of diestrus (diestrus 1 (D1) and diestrus 2 (D2)), proestrus (P), and estrus (E). AU, arbitrary units. * Different for all other times and all other days (P < 0.05) (n = 4–8). Lights on at 0700; lights off at 1900.](http://ajpendo.physiology.org/doi/10.1210/endo-293-10-2477)
mM MgCl₂ and 1 mM K₂HPO₄. Homogenates were centrifuged at 750 g, the pellet was resuspended, and the centrifugation was re-peated. The supernatants were pooled and centrifuged at 18,000 g for 15 min. The pellet was osmotically shocked, centrifuged at 39,000 g, resuspended in 50 mM Tris·HCl, 2.5 mM CaCl₂, pH 7.4 (10 vol/g of original tissue), and washed twice. Membranes were frozen at −70°C.

SDS-10% polyacrylamide gel electrophoresis was then carried out on 50 μg of each of the membrane preparations. Proteins were transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech). Blots were incubated with the polyclonal rabbit antibodies (OX1R1-A, Alpha Diagnostics, 1:1,000 or OX2R2-A, Alpha Diagnostics, 1:800) overnight at 4°C followed with a peroxidase-conju-gated goat anti-rabbit secondary antibody (Santa Cruz, 1:4,000, 1 h at room temperature).

Immunoreactive bands were detected using the Western blotting chemiluminescence Luminol Reagent (Santa Cruz Biotechnology).

Membrane preparation and Western blot analysis. Anterior and mediobasal hypothalamus, frontoparietal cortex, and ovaries from rats were rapidly removed and frozen. The membrane fraction was iso-gated by the method of Olpe et al. (42). Briefly, tissues were homogenized in 10 volumes of ice-cold 0.32 M sucrose, containing 1 mM MgCl₂ and 1 mM K₂HPO₄. Homogenates were centrifuged at 750 g, the pellet was resuspended, and the centrifugation was re-peated. The supernatants were pooled and centrifuged at 18,000 g for 15 min. The pellet was osmotically shocked, centrifuged at 39,000 g, resuspended in 50 mM Tris·HCl, 2.5 mM CaCl₂, pH 7.4 (10 vol/g of original tissue), and washed twice. Membranes were frozen at −70°C.

RESULTS

OX₁, OX₂, and PPO mRNA expression in ovaries of cycling females, and serum hormones and food intake. Both OX₁ and OX₂ mRNA expressions were detected in the ovary by real-
time RT-PCR in diestrus 1 and 2, proestrus, and estrus. OX₁ mRNA expression peaked from 1700 to 2300 only during proestrus, and no changes were found at any other studied time (Fig. 1). A similar pattern was observed for OX₂ mRNA: highest expression in ovary between 1700 and 1900, while no changes were observed in any other stage of the cycle. No PPO mRNA expression was observed in the ovary on any day of the cycle at the studied times (not shown).

As previously described (53), LH, FSH, and PRL peaked during proestrus afternoon; estradiol and progesterone followed the expected patterns of our colony for this day of the cycle. As expected, food intake was always higher at night than during the day on each day of the estrous cycle (Table 1), allowing the expected patterns of our colony for this day of the estrous cycle. As expected, food intake was reduced (Table 2). Interestingly enough, in a preliminary study a single injection of this compound administered at 1400 also significantly reduced ova number at the same dose (10.0 ± 0.6 ova/rat, n = 6, P < 0.02). Food intake was reduced at both studied periods in these animals, indicating the effectiveness of SB-334867-A in this in vivo model (Table 3).

All rats injected at 1400 and 1900 with JNJ-10397049, a selective OX₂ receptor antagonist, ovulated, but a significant reduction in ova number was observed the following morning (Table 2). A significant reduction of LH during proestrus afternoon and of FSH on the following estrous morning was determined. Daytime food intake was also reduced (Table 3).

In the group of cycling female rats injected simultaneously with both orexin receptor blocking drugs, SB-334867-A plus JNJ-10397049, at 1400 and 1900 of proestrus, the blocking effect was more efficacious since four of nine rats did not ovulate at all (44%, P < 0.005), and the remaining five rats only ovulated 3.78 ± 1.31 ova/rat (P < 0.001). In this group, FSH and LH were reduced at all studied times (Table 2). A clear reduction in food intake was observed during the day (measured between 1400 and 1900) and at night (Table 3).

In Cetrorelix- and Nembutal-treated rats, used as controls of ovulation inhibition, ovulation was totally blocked, as expected, and serum FSH, LH, and PRL were significantly reduced (Table 2).

Anatomic examination of ovaries revealed profound differences among groups. In situ bloody ovaries were observed after laparotomy, mainly in rats injected with SB-334867-A (Fig. 4). This observation was confirmed by histology, where great abundance of red blood cells can be observed [Fig. 5, top (A–D) and bottom (E–H)]. Rats pretreated with SB-334867-A

Table 2. Effects of orexin-1-selective (SB-334867-A) and orexin 2-selective (JNJ-10397049) receptor antagonists on ovulation and serum hormones in proestrus rats

<table>
<thead>
<tr>
<th></th>
<th>Ova Number</th>
<th>FSH Proestrus ng/ml</th>
<th>FSH Estrus ng/ml</th>
<th>LH ng/ml</th>
<th>Prolactin ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>15.21 ± 0.50</td>
<td>7.23 ± 0.62</td>
<td>4.06 ± 0.29</td>
<td>12.75 ± 0.85</td>
<td>93.57 ± 10.15</td>
</tr>
<tr>
<td>SB-334867-A</td>
<td>8.50 ± 1.88†</td>
<td>3.84 ± 0.40*</td>
<td>2.34 ± 0.47*</td>
<td>9.14 ± 1.38</td>
<td>77.81 ± 7.92</td>
</tr>
<tr>
<td>JNJ-10397049</td>
<td>9.00 ± 1.26*</td>
<td>4.59 ± 1.82</td>
<td>2.70 ± 0.08*</td>
<td>4.63 ± 1.59*</td>
<td>89.50 ± 10.69</td>
</tr>
<tr>
<td>SB-334867-A + JNJ-10397049</td>
<td>3.78 ± 1.31†</td>
<td>3.14 ± 0.6*</td>
<td>2.97 ± 0.50*</td>
<td>5.59 ± 1.61*</td>
<td>82.92 ± 4.58</td>
</tr>
<tr>
<td>CRX</td>
<td>0.0001</td>
<td>2.96 ± 0.23*</td>
<td>2.15 ± 0.33*</td>
<td>0.36 ± 0.07*</td>
<td>51.53 ± 7.82*</td>
</tr>
<tr>
<td>NEM</td>
<td>0.0001</td>
<td>2.40 ± 0.29*</td>
<td>1.14 ± 0.06*</td>
<td>0.34 ± 0.06*</td>
<td>48.95 ± 8.98†</td>
</tr>
</tbody>
</table>

Values are means ± SE. %Ovulation data were analyzed by the χ² test; ova number and serum hormone data were analyzed by ANOVA. Vehicle, ovulating controls; Cetrorelix (CRX) and Nembutal (NEM), nonovulating controls; FSH, follicle-stimulating hormone; LH, luteinizing hormone; NS, not significant. *P < 0.05, †P < 0.002, ‡P < 0.001.

Table 3. Food consumption in rats pretreated with vehicle, CRX, NEM, and orexin-1-selective (SB-334867-A) and orexin-2-selective (JNJ-10397049) receptor antagonists

<table>
<thead>
<tr>
<th></th>
<th>%Food Intake/Body Weight, g/g</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1400–1900</td>
</tr>
<tr>
<td>Vehicle</td>
<td>8.45 ± 0.082</td>
</tr>
<tr>
<td>SB-334867-A</td>
<td>0.114 ± 0.059*</td>
</tr>
<tr>
<td>JNJ-10397049</td>
<td>0.355 ± 0.150*</td>
</tr>
<tr>
<td>SB-334867-A + JNJ-10397049</td>
<td>0.027 ± 0.014†</td>
</tr>
<tr>
<td>CRX</td>
<td>0.898 ± 0.191</td>
</tr>
<tr>
<td>NEM</td>
<td>0.040 ± 0.018†</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.01, †P < 0.001.
and/or JNJ-10397049 showed more preovulatory follicles and less corpora lutea than controls (Fig. 6).

DISCUSSION

In a previous work, we described a particular relationship between the hormonal milieu of proestrus and the expression of components of the orexinergic system both at the hypothalamus and the pituitary (53). Here PPO, OX₁, and OX₂ expression was determined in ovaries of adult rats at different stages of the estrous cycle and correlated to the endocrine status, the dark-light cycle, and food consumption. Furthermore, the effects of two new potent and selective antagonists of orexin receptors were evaluated on gonadotropin secretion, ovarian histology, and ovulation. To our knowledge, this is the first description of the expression of both orexinergic receptors in this organ on each day of the estrous cycle, with a clear increase in proestrus. Previously, in a screening study, OX₁, but not OX₂, mRNA expression was observed in the gonad of rats using pooled ovaries (26).

Both orexinergic receptors vary along the estrous cycle, with a very marked increase observed in the evening of proestrus (OX₁ expression increases 3-fold, while OX₂ increases 4-fold). The fact that the increase of both orexin receptors’ expression occurred exclusively during the late afternoon and night of proestrus strongly suggests that ovulation is related to this particular hormonal status and probably not related to the sleep-wake cycle or food intake.

To evaluate whether changes in the pituitary control of the gonad modified the expression of orexinergic receptors in the ovary, two of our models were used in proestrous animals (53). As expected, Cetrorelix and Nembutal blocked gonadotropin peaks; and, in addition, they reduced the expression of both receptors OX₁ and OX₂ in the ovary. The fact that the increase in the expression of OX₁ and OX₂ was blunted in the ovary in proestrous animals injected with either agent suggests an important role for gonadotropins as regulators of this expression. In effect, in both models in which the action of the FSH and LH was impaired by different mechanisms, i.e., either GnRH was not properly released as in Nembutal-treated animals, or the effect of GnRH at the GnRH receptor was prevented by Cetrorelix, the proestrous ovarian OX₁ and OX₂ increases were completely abolished. Nevertheless, the participation of other hormones, such as, e.g., PRL or progesterone, in the regulation of ovarian orexin receptor expression cannot be disregarded, as their preovulatory increases were also abolished by Nembutal or Cetrorelix administration in our models (53).

Interestingly enough, no PPO mRNA expression was observed in the ovary, in agreement with data described by Jöhrn et al. (26) in pooled ovaries, even when now analyzed throughout all the estrous cycle. Several hypotheses can be formulated to explain the presence of both receptors in the ovary in the absence of PPO; for example, the orexinergic peptide may originate elsewhere or the receptors may be activated by other molecules present in the gonad. The possibility that orexins may originate from outside the ovary and arrive by circulation is in line with the fact that immunoreactive orexin A has been described in human and rat plasma (2, 36). In this regard, PPO has been shown to be localized in discrete neurons in the lateral, perifornical, and dorsomedial hypothalamus (45). In addition, orexin immunoreactivity was reported in the rat median eminence and pituitary (13).

Furthermore, fluctuations of both orexins in hypothalamus during the estrous cycle were reported (49). Hypothalamic orexin A and B concentrations were reported to be higher in
proestrus than in diestrus in young cycling animals (46), and the greatest orexin A release from hypothalamus was suggested to occur on proestrus (46, 49). The possibility that orexins via general circulation reach the ovary and, alone or in combination with gonadotropins or other factors, may regulate OX1 and OX2 expression is an interesting hypothesis that should be the matter of further research.

Our next objective was to determine whether orexinergic blocking drugs modified serum gonadotropins, the expression of both receptors in the gonad and, as an end point, the occurrence of ovulation. Blocking OX1 and/or OX2 signaling inhibited gonadotropin secretion with particular effects for either agent. A single dose of SB-334867-A administered at 1400 reduced FSH release, without affecting LH, both measured at 1900 of proestrus; in contrast, a single dose of JNJ-10397049 at 1400 significantly diminished LH levels without modifying FSH. Evidently a single dose of one compound was not enough to drastically reduce both hormones. However, the combined effects of both agents decreased FSH and LH at 1900, suggesting that both receptors are involved in

Fig. 5. Top: photomicrographs (×4) of ovaries in vehicle- (A), SB-334867-A- (B), JNJ-10397049- (C), or SB-334867-A plus JNJ-10397049 (D)-injected rats. Bottom: photomicrographs (×40) of corpora lutea in vehicle- (E), SB-334867-A- (F), JNJ-10397049- (G)- or SB-334867-A plus JNJ-10397049 (H)-injected rats. CL, corpus luteum; pF, preovulatory follicle; Bl, red blood cells.
ity and receive contacts from orexinergic axons (8). In vivo

stimulation of GnRH in hypothalamic explants harvested from male rats and from females at proestrus, with no effect at estrus or metestrus; furthermore, orexin A inhibited GnRH-stimulated LH release in dispersed pituitaries from proestrous females (49). Orexin A also induces GnRH gene expression and secretion from GT1–7 neurons (51). In addition, a stimulatory effect of orexin A on LH release may involve direct actions on GnRH neurons (54). In sheep hypothalamus, immunohistochemistry indicated that orexin-containing neurons provide direct input to GnRH neurons (22). In humans, pulsatile LH release is diminished in orexin-deficient narcoleptic men (33). Therefore, our results blocking the orexin receptors in the afternoon of proestrus, together with all the previous data, clearly demonstrate the participation of the orexinergic system in the control of gonadotropin proestrous release. On the other hand, the reverse effect, i.e., the effects of the hormonal milieu on the orexinergic system, has also been studied, and changes in the orexinergic system have been associated with the estrous cycle, pregnancy, and lactation (5, 7, 19, 27, 46, 60).

Recently, we described the effect of the proestrous environment on the expression of PPO and orexin receptors and in the hypothalamus, immunohistochemistry indicated that orexinergic system have been associated with the estrous cycle, pregnancy, and lactation (5, 7, 19, 27, 46, 60).

In vitro, orexin A stimulated the release of GnRH in hypothalamic explants harvested from male rats and from females at proestrus, with no effect at estrus or metestrus. Orexin A is an interesting finding that contrasts with the well-known blocking effect of Cetrorelix and Nembutal on the known blocking effect of Cetrorelix and Nembutal on the PRL was an interesting finding that contrasts with the well-known blocking effect of Cetrorelix and Nembutal on the PRL.

The lack of effect of both orexinergic antagonists on plasma PRL was an interesting finding that contrasts with the well-known blocking effect of Cetrorelix and Nembutal on the PRL. In proestrus, significantly reduced serum FSH in the morning of estrus. This second FSH peak has been reported to be independent of GnRH and due to a decrease in the secretion of ovarian inhibin, a well-known gonadal peptide able to inhibit FSH (11). In the present case, a drug-induced significant decrease in ovulation was observed (see below) that probably left preovulatory follicles secreting inhibin, reducing FSH levels in consequence.

These results using the new OX1 and OX2 blocking agents clarify previous results analyzing the effects of the orexinergic system on gonadotropin secretion. Some works explored the actions of orexins on GnRH and gonadotropin secretions in rodents. GnRH neurons express OX1 receptor immunoreactivity and receive contacts from orexinergic axons (8). In vivo, intracerebroventricular injection of orexin A or orexin B rapidly stimulated LH secretion in a dose- and time-related fashion in estradiol benzoate- and progesterone-pretreated ovarioctomized rats but inhibited LH release in unprimed rats, suggesting a modulation by estrogen of the response of the gonadotropins to orexins (18, 23, 47). Furthermore, orexins injected into the third ventricle suppressed LH pulsatile secretion in ovarioctomized rats (57) and an anti-orexin A antiserum suppressed preovulative LH and PRL surges in the rat (32).

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Blocking orexin receptors early on proestrous afternoon had dramatic effects in the ovary. A significant decrease in the expected late proestrus increase of ovarian OX1 and OX2 expression was observed. Ovulation was markedly affected by these treatments: SB-334867-A administration induced a 25% reduction while the combination of both drugs induced a 44% reduction. In addition, treatments with either drug significantly decreased the number of ova released by the ovary, with the combined treatment being the most effective one. Furthermore, rats pretreated with SB-334867-A or JNJ-10397049 showed more preovulatory follicles and less corpora lutea than controls, in agreement with the decrease in the number of ova in the oviduct, pointing to an impediment in the ovulation process. Whether these effects respond to blockade of local receptors (direct effect) or whether they are due to inhibition of pituitary gonadotropins and/or hypothalamic orexins release (indirect effects), or a combination of both mechanisms, remains to be established.

Macro- and microexamination of ovaries on the morning of estrus revealed bloody ovaries, mainly in rats injected with SB-334867-A or a combination of both antagonists. This observation was confirmed by histology where the presence of large amounts of red blood cells could be observed. The effects of the OX1 antagonist on the ovary were striking, possibly involving an effect on blood vessels. The link between orexins and the control of circulation has been suggested mostly at central levels, interacting with the autonomic system (9, 52) and affecting some local circuits as in the skeletal muscle (63), kidney (58), and brown adipose tissue (61), but the mechanism of action of this antagonist on ovarian rat vasculature and whether these erythrocytes are inside or outside the vasculature should be the matter of further research.

This surprising effect of SB-334867-A and the fact that 25% of rats treated with this drug did not ovulate at all may be indicative of different activities of each compound. Orexin A is
a more selective ligand for OX1 while OX2 binds both orexins A and B. Both receptor genes are widely expressed within the rat brain, but with some differences in distribution, and differential roles for OX1 and OX2 receptors have been suggested (1, 10, 59). Differential distribution of orexin A and orexin B immunoreactivity in the rat CNS has been described (12). Also, orexin A and B may be differentially released or cleared, as demonstrated in hypothalami of lactating rats (7), and orexin A but not orexin B rapidly enters brain from blood (31). Another aspect to take into consideration regarding the differential effects of both orexin receptor antagonists would be the specificity of the compounds. The OX2 antagonist JNJ-10397049 has in vitro ~600-fold selectivity for OX2 over OX1 (37); to our knowledge this is the first in vivo study published using JNJ-10397049. Although data seem to suggest that the effect on ovarian vessels is not OX2 dependent and is either the result of OX1 modulation by SB-334867-A or a consequence of the specificity of SB-334867-A, new studies remain to be done to indicate whether the differences observed between these new compounds depend on the pharmacology of them or to a differential function for each orexin and for each receptor in the ovary.

The feeding data clearly indicate no relationship between food intake and ovarian orexinergic receptors expression. The fact that food consumption was significantly lower in estrus than diestrus 2 is in agreement with previous results in several species, indicating lower intake around the time of ovulation than diestrus 2 is in agreement with previous results in several studies. Food intake and ovarian orexinergic receptors expression. The differential function for each orexin and for each receptor in the ovary may be differentially released or cleared, as demonstrated in hypothalami of lactating rats (7), and orexin A but not orexin B rapidly enters brain from blood (31). Another aspect to take into consideration regarding the differential effects of both orexin receptor antagonists would be the specificity of the compounds. The OX2 antagonist JNJ-10397049 has in vitro ~600-fold selectivity for OX2 over OX1 (37); to our knowledge this is the first in vivo study published using JNJ-10397049. Although data seem to suggest that the effect on ovarian vessels is not OX2 dependent and is either the result of OX1 modulation by SB-334867-A or a consequence of the specificity of SB-334867-A, new studies remain to be done to indicate whether the differences observed between these new compounds depend on the pharmacology of them or to a differential function for each orexin and for each receptor in the ovary.

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REFERENCES

OREXIN RECEPTORS IN OVARY


