Impact of proestrous milieu on expression of orexin receptors and prepro-orexin in rat hypothalamus and hypophysis: actions of Cetrorelix and Nembutal

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Silveyra P, Catalano PN, Lux-Lantos V, Libertun C. Impact of proestrous milieu on expression of orexin receptors and prepro-orexin in rat hypothalamus and hypophysis: actions of Cetrorelix and Nembutal. Am J Physiol Endocrinol Metab 292: E820–E828, 2007; doi:10.1152/ajpendo.00467.2006.—Orexins and their receptors OX1 and OX2 regulate energy balance and the sleep-wake cycle. We studied the expression of prepro-orexin (PPO), OX1, and OX2 in brain and pituitary under the influence of the hormonal status in adult rats. Primarily, PPO, OX1, and OX2 expression was determined in Sprague-Dawley female cycling rats during proestrus and in males. Animals were killed at 2-h intervals. Anterior (AH) and mediobasal (MBH) hypothalami, anterior pituitary (P), and frontoparietal cortex (CC) were homogenized in TRIzol, and mRNAs were obtained for screening of PPO, OX1, OX2 expression by semiquantitative RT-PCR. Main findings were confirmed and extended to all days of the estrous cycle and to males. PPO expression increased in accordance with the proestrous increase, were evaluated on the expression of GnRH receptor, and pentobarbital. OX1 and OX2 expression increased at least threefold in AH, MBH, and P, but not in CC, between 1700 and 2300 of proestrus, without variations in estrus, diestrus, or in males. PPO in AH and MBH showed a fourfold or higher increase only during proestrus afternoon. Cetrorelix or pentobarbital prevented increases of OX1 and OX2 only in the pituitary and blunted gonadotropin surges, but left OX1, OX2, and PPO brain expression unchanged. Reproduction, energy balance, and sleep-wake cycle are integrated. Here, we demonstrate that, in the physiological neuroendocrine condition leading to ovulation, information to the orexinergic system acts in hypothalamus and pituitary by different mechanisms.

adenohypophysis; estrous cycle; gonadotropins; gonadotropin-releasing hormone; prolactin

TWO OREXIN NEUROPEPTIDES (also referred to as hypocretins) have been described, orexin A and orexin B. Orexin A is a 33-amino acid peptide with an NH2-terminal pyroglutamyl residue and two intrachain disulfide bonds. Orexin B is a linear peptide of 28 amino acids. Both are derived by proteolytic cleavage from a 130-amino acid precursor, prepro-orexin (PPO), which was isolated from the rat hypothalamus (13, 40).

Orexins are synthesized mainly by neurons, with their soma located in the lateral hypothalamus and projections throughout the brain (4, 9, 15, 18, 19, 33, 36, 49, 52). These two peptides are potent agonists at both the orexin 1 (OX1) and orexin 2 (OX2) G protein-coupled receptors. Orexin A is a more selective ligand for OX1; OX2 binds both orexins A and B. The structure of orexins and their receptors is highly conserved in mammals, including rodents and humans (40). Both receptor genes are widely expressed within the rat brain but with some differences in the OX1 and OX2 distribution; furthermore, differential roles for OX1 and OX2 receptors have been suggested (2, 11, 43, 49).

The physiological roles described for orexins are as numerous as their central nervous system projections. Also, the widespread distribution of mRNAs encoding OX1 and OX2 in the brain suggests that orexins may be involved in multiple functional pathways (8, 26, 50). They have been related to arousal and alertness, regulation of sleep and appetite, food intake and feeding behavior, and a variety of neuroendocrine and autonomic functions (16, 18, 32, 42). Their participation in metabolism and in the brain control of the pituitary, mainly in ACTH secretion, has been postulated (10, 13, 18, 30, 47, 48). Furthermore, a variety of pathological conditions in which orexins and their receptors are involved has been clearly documented; as for narcolepsy and other sleep disorders (3, 18, 41), their participation in some metabolic and endocrine disorders, such as obesity and stress, has also been suggested (1, 7, 27).

On the other hand, the hormonal secretion of the estrous cycle must be combined with appropriate nutritional and vigilance states for successful reproduction. Although some studies have addressed the impact of orexins on the regulation of pituitary secretion, little is known of the inverse relationship, i.e., the impact of the hormonal milieu on the orexinergic system. To this aim, PPO, OX1, and OX2 expression was evaluated in hypothalamus and pituitary in female rats at different stages of the estrous cycle, and also in males, and correlated to the endocrine status, the dark-light cycle, and food consumption. Furthermore, the effects of two ovulation-blocking agents, Cetrorelix, an antagonist at the gonadotropin-releasing hormone (GnRH) receptor, and pentobarbital sodium (Nembutal), a central anesthetic barbiturate that abolishes the GnRH prostrous increase, were evaluated on the expression of PPO, OX1, and OX2 in the pituitary and in selected brain areas.

MATERIALS AND METHODS

Animals. Adult male and virgin female 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 0700 to 1900. They were given free access to laboratory chow and tap...
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First set of experiments: screening study. Female cycling rats were killed by decapitation at intervals of 2 h on the day of proestrus, starting at 0900 until 2300. The stage of the estrous cycle was determined by vaginal smears for 15 consecutive days. Regular cycles were defined as the occurrence of three consecutive 4-day cycles. Males were killed every 4 h (1100, 1500, 1900, and 2300). In all cases, trunk blood was collected and sera stored at −20°C for hormone determinations by RIA. Day and night food intake was also recorded for male and female rats. For these purposes, during the entire experiments animals were kept in individual cages and food consumption determined at 0700 and 1900.

The brains were rapidly removed and placed on ice for dissection. An area limited anteriorly by the cephalic fissure of the optic chiasm, laterally by the hypothalamic fissures, posteriorly by the fissure caudal to the mamillary bodies, and in depth by the subthalamic sulcus was excised. A transverse section through the insertion of the optic chiasm divided the hypothalamus in two: the medial basal mammillary region (MBH) and the anterior proepic suprachiasmatic area (AH) (5); anterior pituitary (P) and frontoparietal cortex (CC) were also removed. All tissue samples were immediately homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA) and kept at −70°C until used. Levels of expression of mRNAs for PPO, OX1, and OX2 were determined by semiquantitative RT-PCR (n = 6–8).

Second set of experiments. Cycling female rats were killed by decapitation at 1100, 1500, 1700, 1900, and 2300 in all stages of the estrous cycle. Tissues and sera were collected and kept as above. Levels of expression of PPO, OX1, and OX2 were determined by quantitative real-time RT-PCR. Hormones were measured by RIA (n = 4–6).

Third set of experiments. At 1900 of diestrus stage 2 of the estrous cycle, a sample of blood was taken under light ether anesthesia from a third group of 60-day-old rats to record basal estradiol levels. The next day, on proestrus, Cetrorelix Acetate, a synthetic antagonist at GnRH receptors (a gift from Serono, Buenos Aires; 100 H9262

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.

Semiquantitative PCR. Sense and antisense oligonucleotide primers were obtained from Invitrogen, based on the published sequences for PPO, OX1, and OX2 receptors (24) and designed with the DNASTar program for cyclophilin. The PCR consisted of 100 ng of cDNA, 10 mM Tris-HCl, 50 mM KCl, 1,25 mM MgCl2, 0.2 mM deoxy-NTPs, 400 mM primers, and 1.25 U Taq polymerase (Invitrogen) in a final volume of 25 µl. Each cycle consisted of a 5-min hot-start step followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, and extension at 72°C for 30 s, with a final extension for 5 min at 72°C after the last cycle. The reaction products were electrophoresed on 1.5% agarose gels and stained with SYBR Green I. The gels were exposed to UV with a white/UV ultraviolet transilluminator (UVP Laboratory Products, Upland, CA) and the images quantified using the Scion Images NIH software. PPO, OX1, and OX2 mRNA expressions were normalized using cyclophilin mRNA expression, and results are expressed as densitometric units (DU).

Quantitative real-time PCR. Sense and antisense oligonucleotide primers were designed on the basis of the published cDNA PPO, OX1, and OX2, and cyclophilin sequences by use of the PrimerExpress software (Applied Biosystems, Foster City, CA). Oligonucleotides were obtained from Invitrogen. The sequences of the primers were as follows: PPO sense GCTCTACCTCCTTTGGAGATTTTG, PPO antisense GGCAATCGGAAGATGTGT; OX1 sense GCCCTGCCAGCTTGTAAGTG, OX1 antisense CAAGCGATGCGCCGAAAGAG; OX2 sense GAAAGAAATAGTGGTGCGTCTGATGC, OX2 antisense CAGCGTCTCCCGATGAAG; cyclophilin sense GTGCAGAGATGCAGCACG; cyclophilin antisense TAAAATCAGGCCTGTGG.

Quantitative measurements of PPO, OX1, OX2, and cyclophilin cDNA were performed by kinetic PCR using SYBR Green I as fluorescent dye (Invitrogen). The 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 denaturation at 95°C for 5 min, the cDNA products were amplified for 40 cycles, each cycle consisting of denaturation 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 ABI 7500 sequence detection system (Applied Biosystems), and data were stored continuously during the reaction. The results were validated on the basis of the quality of dissociation curves generated at the end of the PCR runs by ramping the temperature of the samples from 60 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 for 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 ABI 7500 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 each 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 no. 2). Results are expressed as arbitrary units (AU) for comparison among samples. AU is defined as the expression level relative to a sample of 11 h of proestrus or saline (calibrator sample).

Hormone determinations. Serum LH, FSH, and prolactin (PRL) were estimated by RIA using kits provided by the National Institute of Diabetes and Digestive and Kidney Diseases. Results were expressed in terms of RP1 rat LH, FSH, and PRL standards. Assay sensitivities were 0.015 ng/ml for LH, 0.175 ng/ml for FSH, and 1.6 ng/ml for PRL. Intra- and interassay coefficients of variation for LH were 7.2 and 11.4%, respectively, for FSH 8.0 and 13.2%, respectively, and 8.1 and 11.4%, respectively, for PRL.

Serum estradiol, progesterone, and testosterone were determined by RIA using specific antisera kindly provided by Dr. G. D. Niswender (Colorado State University, Fort Collins, CO) after ethyl ether extraction. Labeled hormones were purchased from PerkinElmer (Wellesley, MA). Assay sensitivities were for estradiol 11.3 pg, for progesterone 500 pg, and for testosterone 125 pg. Intra- and interassay

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coefficients of variation were 6.8 and 11.7% for estradiol, respectively; 7.1 and 12.15% for progesterone, respectively; and 7.8 and 12.3% for testosterone, respectively.

Statistics. Data are presented as means ± SE. Differences between treatment groups were estimated by one-way ANOVA followed by Tukey’s posttest using the Statistica software. P < 0.05 indicated statistically significant differences.

RESULTS

PPO, OX₁, and OX₂ mRNA expression in hypothalamus and anterior pituitary and serum hormone levels in proestrous female and male rats. As expected, LH, FSH, and PRL peaked during proestrus afternoon; estradiol and progesterone also followed the expected patterns of our colony for that day of the cycle (Fig. 1A). Serum levels of LH, FSH, and testosterone in adults males did not vary at the selected times (Fig. 1B).

OX₁ mRNA expression as measured by RT-PCR peaked from 1700 to 2300 in MBH and AH (Fig. 2A). In adenohypophysis, expression increased significantly as of 1900, and highest significant levels of expression were reached at 2300; on the other hand, no changes were found in CC at any studied time. A similar pattern to OX₁ was observed for OX₂ mRNA expression: highest in MBH between 1700 and 2300, in AH at 2100, 2200, and 2300, and in adenohypophysis at 1900 and 2100, whereas no changes were observed in CC (Fig. 2A).

OX₁ and OX₂ mRNA expression in adult males did not change from 1100 to 2300 in any of the four regions studied (Fig. 2B).

Fig. 1. Serum levels of LH, FSH, prolactin, estradiol, and progesterone in proestrous female rats (A) and serum testosterone (T), LH, and FSH in male rats (B) (n = 6–8).
Fig. 2. Expression of prepro-orexin (PPO), orexin receptor 1 (OX1), and OX2 mRNA in proestrous female (A) and male (B) rat tissues examined by RT-PCR. AH, anterior hypothalamus; MBH, medial basal hypothalamus; P, anterior pituitary; CC, frontoparietal cortex. mRNA expression was higher than at 0900, 1100, 1300, and 1500 (*); 1700 (a); 0900, 1100, and 1500 (b); or 1100 and 1500 (c) \( (n = 6-8; P < 0.05). \) Planned comparisons showed additional results: significantly different from 1100 of proestrus \( ^{a}\text{P}<0.02. \)
PPO mRNA expression increased in AH between 1900 and 2300 and in MBH between 1700 and 2300 of proestrus (Fig. 2A). No PPO expression was observed in CC or P, as expected (13). In males, hypothalamic expression of PPO mRNA was also detected, but without variations throughout the day (Fig. 2B).

Regarding food intake, diurnal consumption was significantly lower than nocturnal in both sexes, as expected, and always higher in the male [food intake (g): diurnal, male: 3.67 ± 0.83; female: 0.74 ± 0.26; nocturnal, male: 21.51 ± 0.53; female: 13.80 ± 0.53; \( P < 0.01 \)].

**Hypothalamic PPO, hypothalamic and pituitary OX expression, and serum hormone levels along the estrous cycle.** As specific increases in the expression of PPO, OX1, and OX2 were determined in the evening and night of proestrus, we then evaluated these parameters on the other days of the estrous cycle by real-time PCR to discriminate between a circadian and a cyclic pattern of expression. Indeed, increases of PPO in hypothalamus and OX1 and OX2 in hypothalamus and hypophysis were specific to proestrus, confirming our results by semiquantitative PCR, as they were absent in all the other stages of the estrous cycle (Fig. 3). Hormonal levels followed the expected cyclic patterns (Fig. 4).

Food intake was always higher at night than during the day on each day of the estrous cycle, marking a difference with

### DISCUSSION

In adult cycling female rats the expression of OX1, OX2, and PPO peaked during the evening of proestrus in hypothalamus and adenohypophysis. No changes were observed in males in any region at any time. The fact that the increase of both orexin receptors’ expression occurred selectively in hypothalamus and P, and not in cortex, and only in females exclusively during the late afternoon of proestrus, strongly suggests that they are...
cycle-related events associated with this particular hormonal status (14, 20, 30, 35, 44). Furthermore, PPO mRNA expression also increased in hypothalamus only during the proestrus afternoon. In males, a decrease at 1900 in PPO expression in AH did not achieve statistical significance, as observed by others with a different experimental methodology (45). Obviously, the increases in OX1, OX2, and PPO observed in females bear no relationship to the sleep-wake cycle or to food intake.

The hypothesis that changes in the hypothalamic-pituitary-ovarian axis modify the orexinergic system was reinforced when Cetrorelix and Nembutal were used in proestrous animals. Both drugs blocked gonadotropin peaks and simultaneously reduced the expression of both receptors, OX1 and OX2, in P. In contrast, in proestrous rats pretreated with Cetrorelix or Nembutal, expressions of PPO, OX1, and OX2 in MBH, AH, and CC were unaffected, suggesting two different mechanisms regulating receptor expression in the brain and in the gland. Interestingly enough, pentobarbital, besides causing extended brain depression, was suggested to impact on orexinergic neurons through mechanisms not involving either GABA_A, OX1, or OX2 receptors (31). The fact that hypothalamic increases in receptor levels persisted in the afternoon of proestrus in Nembutal- or Cetrorelix-treated animals, disregarding the lack of gonadotropin surges, indicates that the expression of OX1 and OX2 in hypothalamus is an event independent of levels of the decapptide and its postulated functions as neurotransmitter/neuromodulator. On the other hand, as the increases in the expressions of OX1 and OX2 were blunted in the adenohypophysis in proestrous animals injected with either agent, an important role for GnRH as regulator of pituitary OX1 and OX2 expression is suggested. In effect, in these two models in which the action of the decapptide is impaired, either the decapptide was not properly released, as in pentobarbital-treated animals, or the effect of GnRH at the GnRH receptor was prevented, as in Cetrorelix-injected rats the proestrous pituitary OX1 and OX2 increases were completely abolished. Nevertheless, the participation of other hormones, such as progesterone or PRL, in the regulation of

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**Fig. 4.** Serum levels of LH, FSH, prolactin, estradiol, and progesterone during the estrous cycle (n = 4–6).

**Fig. 5.** Food intake. Night food consumption (1900 to 0700) was registered in female rats in all stages of the estrous cycle. Results are expressed in grams of food intake. Food consumption was significantly lower in estrus (n = 20–25; *P < 0.05).
pituitary orexin receptor expression cannot be disregarded, as their preovulatory increases were also abolished by pentobarbital or Cetrorelix administration. Thus, our results clearly show that changes in the reproductive state are able to influence the orexinergic system by different mechanisms in hypothalamus and in anterior pituitary.

Some previous studies had investigated a possible relationship between the orexinergic system and the hypothalamic-pituitary-ovarian axis. However, the present study is the first to demonstrate that changes in the reproductive state affect the expression of orexin receptors in both the hypothalamus and the pituitary gland. These findings suggest that the orexinergic system plays a role in the regulation of reproductive functions.

**Fig. 6.** Effect of Cetrorelix and Nembutal on PPO, OX1, and OX2 mRNA expression examined by real-time RT-PCR in tissues of female proestrous rats. SAL, saline; CRX, Cetrorelix; NEM, Nembutal (n = 8). *Significantly different from SAL (P < 0.05).

**Fig. 7.** Serum levels of LH, FSH, prolactin, estradiol, and progesterone at 1900 of proestrus in female rats treated with Nembutal or Cetrorelix (n = 8). *Significantly different from SAL, P < 0.05; aD2 estradiol levels were significantly lower than at proestrus, P < 0.05.
pituitary-gonadal axis. Most works explored the actions of orexins on GnRH and gonadotropin secretions in rodents, although some controversy remained regarding the effects observed (17, 21, 22, 28, 29, 38, 39, 46, 51). It is interesting to add that, in general, the absence of orexins in men has not been associated with a loss of reproductive function (29); to our knowledge, this was not studied in women.

Fewer reports have studied the inverse relationship, i.e., the effects of the endocrine reproductive state on orexinergic neurons, and these have also shown conflicting results. In rats, high hypothalamic concentrations of orexins were described on the day of proestrus and postulated to contribute to the LH and PRL surges (37). In addition, orexin A release after administration of KCl was significantly greater in hypothalamic explants harvested on the morning of proestrus than at estrus or metaestrus, and orexin A release was stimulated by estradiol in explants from males (39). Nevertheless, these same authors showed that orexin A content was lower in hypothalamus and higher in midbrain, medulla, and thalamus at late proestrus compared with other cycle stages. Moreover, hyperestrogenization in female rats reduced orexin A content in hypothalamus and other brain areas, including cortex (39). A sex-dependent regulation of hypothalamic PPO expression, the precursor of orexins, was also suggested (25).

Regarding orexin receptors, OX₁ mRNA was reported to be highly expressed in the brain and at lower levels in the pituitary gland. High levels of OX₂ mRNA were found in adrenal glands of male rats and low amounts in P. Interestingly enough, a sexually dimorphic expression of OX₁ and OX₂ in the hypothalamus, pituitary, and adrenal glands suggested sex-specific roles of orexins in endocrine functions (24). In rat hypothalamus, OX₁ mRNA expression was shown to be significantly higher during late proestrus than at metaestrus using semiquantitative PCR (50), coinciding with our quantitative observations with real-time RT-PCR, in which a tight correlation with the hormonal status of the animal is suggested. In contrast, no differences in the mRNA levels of PPO, OX₁, and OX₂ were observed in hypothalami of control, gonadectomized, and steroid-treated female or male rats (23).

Furthermore, in the rat, the presence of orexin A and B was described in the median eminence, adenohypophysis, and neurohypophysis (12), although there was no pituitary expression of PPO, as observed here and in other works (24). Therefore, pituitary orexins must originate in the hypothalamus and/or arrive by circulation. In the human, orexins A and B were also detected in specific human pituitary cell types (6). OX₁ and OX₂ mRNAs were clearly expressed in the pituitary intermediate lobe and were also found in the posterior lobe in rodents. In the anterior lobe, OX₁ was more markedly expressed than OX₂ (12), in agreement with our and others’ results (24). The pituitary orexin receptor expression was described as regulated by gonadal steroids (23). Here, we show original results suggesting that GnRH may regulate pituitary OX₁ and OX₂ expression. The mechanism by which the decapeptide may exert this effect will be matter of further studies.

In the present work using a physiological model, female rats in different stages of the estrous cycle and normal males, we demonstrated the influence of the reproductive state, particularly the hormonal milieu of late proestrus, on the orexinergic system. Dissociation in the expression of orexin receptors between the hypothalamus and the pituitary when the effects of GnRH are abolished indicates an underlying different mechanism of regulation and suggests a possible direct action of the decapeptide in the pituitary in this event.

Our findings add to the notion that, physiologically, energy balance and reproduction must be tightly interrelated for efficient species conservation. An input from the orexigenic network to the neuroendocrine system has been amply documented, as mentioned, including very recent results with peptide 26RFα (34). In addition, clearly, information from the hormonal milieu of proestrus impacts on the orexergic system with particular regulations for different areas of expression, indicating that the information is bidirectional, as demonstrated by our results. The hormonal status of proestrus does not seem to influence food intake, one of orexin’s best described actions, as this parameter in proestrus does not differ from diestrus. On the other hand, an impact on alertness on this particular night of proestrus, when females are receptive to males, would be of utmost importance for successful reproduction. This hypothesis will be evaluated in future works.

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REFERENCES

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