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Impacts of proestrus milieu on expression of orexin receptors and preprotorexins in rat hypothalamus and hypophysis: actions of Cetrorelix and Nembutal

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Submitted 5 September 2006; accepted in final form 14 November 2006

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 been described, orexin A and orexin B. Orexin A is a 33-amino acid peptide with an NH2-terminal pyroglutamyl residue and been described, orexin A and orexin B.

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...
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: 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 chiasma, 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 chiasma divided the hypothalamus in two: the medial basal mammillary region (MBH) and the anterior preoptic 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 semi-quantitative 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 diestrous 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 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 stage of the estrous cycle was determined by vaginal smears starting at 0900 until 2300. The estrous cycle was divided 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 following curve method, as described by the manufacturer (User Bulletin no. 2). 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 following curve method, as described by the manufacturer (User Bulletin no. 2).

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, 0.1175 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 PRL, 8.0 and 13.2%, respectively, and 8.1 and 11.4%, respectively, for FSH. Serum estradiol, progesterone, and testosterone were determined by RIA using specific antiserum 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
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 (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 PPO, OX1, and OX2 expression that increased only in the evening-night of proestrus, as shown above. In addition, food consumption was lower during estrus than on any other day of the cycle (Fig. 5).

Effect of Cetrorelix or pentobarbital administration on OX1, OX2, and PPO expression in proestrus. In proestrus rats, pretreatment with Cetrorelix or Nembutal left the expression of PPO in hypothalamus unchanged (Fig. 6); in addition, the expressions of OX1 or OX2 mRNAs in MBH, AH, and CC were also unaffected. In sharp contrast, the GnRH antagonist as well as the barbiturate significantly reduced the expression of both receptors in P (Fig. 6) and blunted gonadotropin release (Fig. 7). Progesterone and PRL were also inhibited by both treatments. Proestrus serum estradiol was unaffected by either treatment and was significantly higher than at diestrus 2.

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 decapeptide 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 decapeptide is impaired, either the decapeptide 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 proestrus 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 axis. However, the results of these studies were inconsistent, and further research is needed to clarify the role of orexin in reproductive function.

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; #2 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 orexigenic 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 compared with other cycle stages. Furthermore, 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 quantitative 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 neuropehysis (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 orexigenic 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 26RFa (34). In addition, clearly, information from the hormonal milieu of proestrus impacts on the orexigenic 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 in 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.

ACKNOWLEDGMENTS

This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 2231 to V. Lux-Lantos, PIP 5540 to C. Libertun), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, BID 1501/OC-AR PICT 2000 05-08664 to C. Libertun), and Universidad de Buenos Aires (ME 048 to C. Libertun).

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