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Orexin-A Suppresses the Pulsatile Secretion of Luteinizing Hormone via β-Endorphin

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Orexins, the novel hypothalamic neuropeptides that stimulate feeding behavior, have been shown to suppress the pulsatile secretion of LH in ovariectomized rats. However, the mechanism of this action is still not clear. We examined the effect of naloxone, a specific opioid antagonist, on the suppression of the pulsatile secretion of LH by orexins to determine whether β-endorphin is involved in this suppressive effect. We administered orexins intracerebroventricularly and injected naloxone intravenously in ovariectomized rats, and we measured the serum LH concentration to analyze the pulsatile secretion. Administration of orexin-A significantly reduced the mean LH concentration and the pulse frequency, but coadministration of naloxone significantly restored the mean LH concentration and the pulse frequency. Administration of orexin-B also significantly reduced the mean LH concentration and the pulse frequency, and coadministration of naloxone did not restore them. These results indicate that orexin-A, but not orexin-B, suppresses GnRH secretion via β-endorphin.

Key Words: **orexins; LH; β-endorphin; naloxone; pulse; GnRH.**

Orexins are newly identified hypothalamic neuropeptides that stimulate feeding behavior (1). Orexin neurons are present in the lateral hypothalamus, which has been implicated in the central regulation of feeding behavior and energy homeostasis. Recently, detailed maps of their neuronal projection and their receptors in the brain have been presented (2–6), and it is thought that they are regulatory factors of autonomic or neuroendocrine systems as well as feeding regulatory factors.

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As for regulation of the neuroendocrine system, we have already demonstrated that orexins suppressed the pulsatile secretion of luteinizing hormone (LH) in ovariectomized (OVX) female rats and that the suppressive effect of orexin-A was stronger than that of orexin-B (7). These results indicate that orexins suppress gonadotropin-releasing hormone (GnRH) secretion at the hypothalamic level. However, the mechanism underlying the action of orexins is not clear.

There is general agreement that β-endorphin (β-END), which belongs to the family of endogenous opioid peptides, exerts a suppressive effect on GnRH secretion. Orexin neurons project to the arcuate nucleus (ARC) and innervate pro-opiomelanocortin (POMC) neurons, the precursors of β-endorphin (8). Therefore, it is conceivable that orexins may suppress GnRH secretion via β-END.

This study was undertaken to determine whether β-END is involved in the regulation of GnRH secretion by orexins as one of the factors of indirect pathways. We examined the effect of naloxone, a specific opioid antagonist, on the suppression of pulsatile secretion of LH by orexins to determine whether the suppression can be eliminated by blocking the effect of β-END.

MATERIALS AND METHODS

Animals. All animal experiments were conducted in accordance with the ethical standards of the institutional Animal Care and Use Committee of the University of Tokushima. Eight-week-old female Wistar rats (160–200 g) were purchased from Charles River Japan, Inc. (Yokohama, Japan). They were housed in a room with controlled lighting (lights on between 0800 and 2030 h) and temperature (24°C) and received food and water *ad libitum*. All rats were ovariectomized bilaterally under intraperitoneal (ip) anesthesia with pentobarbital sodium (40 mg/kg body weight) and used for the experiments two weeks later.

Implantation of a brain cannula and atrial cannulation. Two weeks after ovariectomy, a brain cannula was implanted into the third ventricle (3V) under ip pentobarbital anesthesia (50 mg/kg body weight) using the stereotaxic coordinates according to the atlas of Paxinos and Watson (9).

One week after brain cannulation, rats were anesthetized with a mixture of ketamine and xylazine (20:5 mg/kg body weight, ip) and a

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TABLE 1

Baseline Data of Rats

Group	Number of rats	Body weight (g)	Basal LH level (μg/l)*
1 (3V saline + iv saline)	7	284.57 ± 5.50	8.29 ± 1.12
2 (3V saline + iv NAL)	8	268.53 ± 5.63	8.26 ± 0.67
3 (3V orexin-A + iv saline)	7	276.16 ± 5.05	8.21 ± 1.08
4 (3V orexin-A + iv NAL)	7	267.94 ± 6.06	8.37 ± 1.12
5 (3V orexin-B + iv saline)	8	271.39 ± 6.86	9.60 ± 0.59
6 (3V orexin-B + iv NAL)	8	267.46 ± 6.47	8.09 ± 0.73

Note. Values are expressed as means ± SEM.
* Basal LH level denotes the serum LH concentration just before 3V administration at 0 min.

silastic tube was inserted into the external jugular vein and sewn into the skin after confirming its position in the right atrium (10). The tube was rinsed with heparinized saline (1×10^4 U/l saline) and threaded to an exit at the back of the neck.

The procedure has been described in detail previously (7).

Administration of orexins and naloxone. On the day following atrial cannulation, 3 nmol of orexin-A or -B (Peptide Institute, Inc., Osaka, Japan) dissolved in 5 μl of distilled water or the same volume of saline was administered into the 3V using a Hamilton microsyringe. Blood samples were collected through the intra-atrial cannula connected to a long polyethylene tube before and at 6-min intervals for 2 h after the administration without handling the rats.

After each sampling, the blood was replaced by an equal volume of heparinized saline containing naloxone (NAL) (Sigma Chemical Co., St Louis, MO; 0.5 mg/kg/h) or the heparinized saline only. Rats were divided into six experimental groups: group 1, 3V saline + iv saline (control); group 2, 3V saline + iv NAL; group 3, 3V orexin-A + iv saline; group 4, 3V orexin-A + iv NAL; group 5, 3V orexin-B + iv saline; and group 6, 3V orexin-B + iv NAL. The samples were centrifuged and the plasma was stored at −40°C until the measurement of LH concentrations by RIA.

RIA of LH. Serum LH concentration was measured by a double-antibody RIA (11) using a rat LH RIA kit obtained from NIDDK and the National Hormone and Pituitary Program. Values are expressed

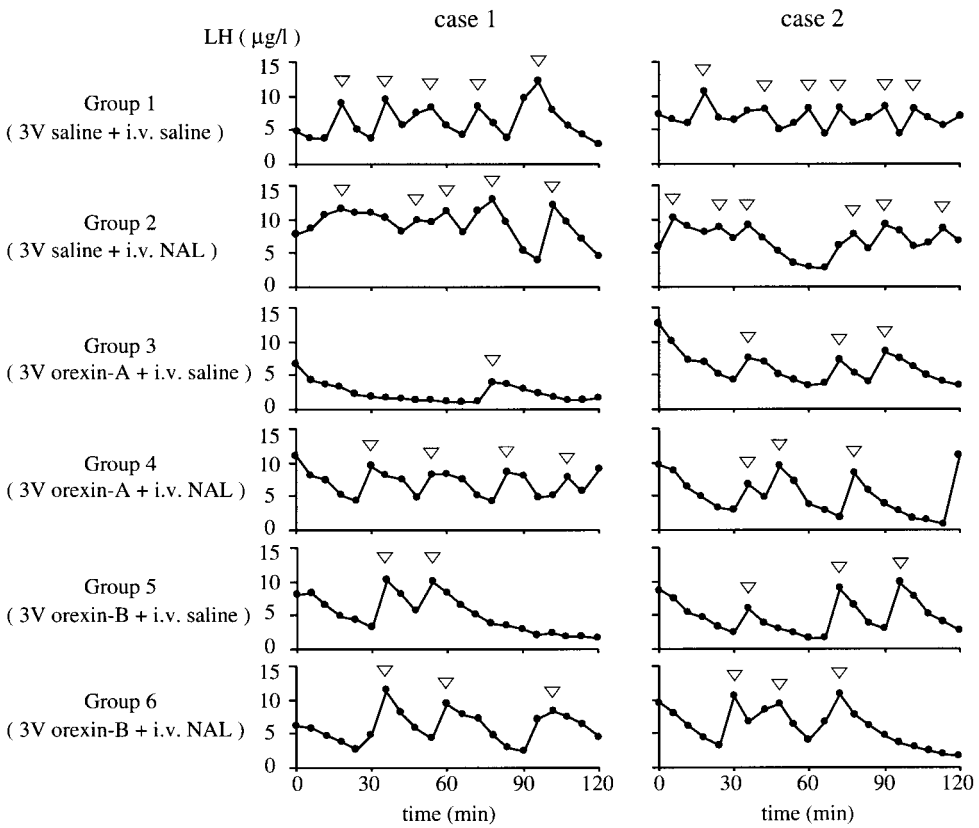

FIG. 1. Effect of third cerebroventricular (3V) administration of orexins and intermittent iv administration of naloxone (NAL) on the pulsatile secretion of LH in ovariectomized (OVX) rats: Representative profiles of two rats each treated with orexin-A (3 nmol/5 μl), orexin-B (3 nmol/5 μl), or saline (5 μl) at 0 min and intermittent injections of NAL (0.5 mg/kg/h) or saline every 6 min for 2 h. ▽ denotes a pulse of LH secretion.

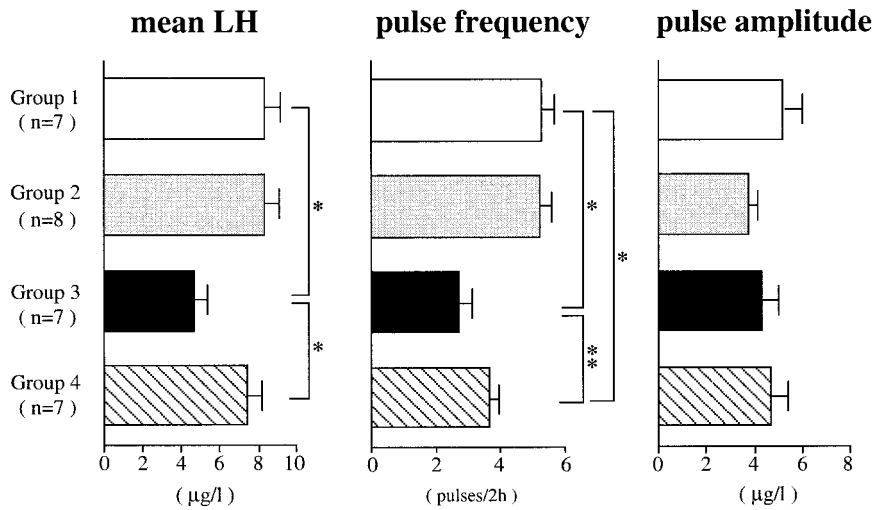

FIG. 2. Effect of third cerebroventricular (3V) administration of orexin-A and intermittent iv administration of naloxone (NAL) on the pulsatile LH secretion in ovariectomized (OVX) rat. Values are expressed as means ± SEM. Group 1, 3V saline + iv saline (control); group 2, 3V saline + iv NAL; group 3, 3V orexin-A + iv saline; group 4, 3V orexin-A + iv NAL: $*P < 0.01$, $**P < 0.05$ (one-way analysis of variance followed by Fisher's protected least significance difference test).

relative to those for the reference preparation (NIDDK-rLH-RP-3). The minimal detectable dose of this assay was 0.02 ng/tube, and the intra- and interassay coefficients of variation (CV) were 4.2 and 5.8%, respectively.

LH pulses were defined and exactly identified using established criteria as described by DePaolo *et al.* (12). Briefly, a CV was calculated from LH concentrations on the ascending and descending phases of a suspected pulse. A pulse was defined if the CV was greater than twice the CV of the LH RIA determined from solutions of LH standards corresponding to the mean LH levels of the suspected pulse. The mean LH concentration, the pulse frequency (number of pulses during a period of 2 h) and the mean pulse amplitude were calculated for each animal.

Statistical analysis. The data were analyzed using one-way analysis of variance followed by Fisher's protected least significance difference test. All results are presented as means ± SEM. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

The profiles of rats in each group just before 3V administration are shown in Table 1. There were no significant differences in body weight or basal LH level among the six groups.

Representative LH secretion profiles of two rats in each group are depicted in Fig. 1. In groups 1 and 2 (orexin-untreated groups), pulses of LH secretion appeared constantly for 2 h. However, in groups 3–6, (orexin-treated groups), LH secretion was suppressed immediately after the administration of orexins and the suppression continued for about 30 min.

The results of statistical analysis of the pulsatile secretion of LH are shown in Figs. 2 and 3. Intermittent administration of NAL by itself did not alter the mean LH concentration or the pulse frequency.

Administration of orexin-A significantly ($P < 0.01$) reduced the mean LH concentration and the pulse frequency. Coadministration of orexin-A and NAL significantly restored the mean LH concentration and the pulse frequency, but the pulse frequency was still significantly ($P < 0.01$) less than in the control group. There were no significant differences in the pulse amplitude among the four groups.

On the other hand, administration of orexin-B also significantly ($P < 0.05$) reduced the mean LH concentration and the pulse frequency. Coadministration of orexin-B and NAL did not alter the mean LH concentration or the pulse frequency. There were also no significant differences in the pulse amplitude among the four groups.

DISCUSSION

We have already demonstrated that 3 nmol of orexin, the dose that was shown to stimulate feeding behavior (1), suppressed the pulsatile secretion of LH in OVX rats (7). Orexin neurons are expressed in the lateral hypothalamic area and project to several regions of the brain, particularly to ARC, the paraventricular nucleus (PVN), and the preoptic area (POA) in the hypothalamus (2–5).

GnRH neurons are mainly expressed in the POA in rodents. Some feeding regulatory neuropeptides, such as β-END (13), neuropeptide Y (NPY) (14, 15) expressed mainly in ARC, and corticotropin-releasing hormone (CRH) (16) in the PVN, have been demonstrated to suppress GnRH secretion. It has also been shown that orexin neurons innervated POMC, the precursor of β-END, and NPY neurons in ARC (8). Therefore, we hypothesized that orexins suppress GnRH secretion directly and/or indirectly via these neuropeptides. In the present study,

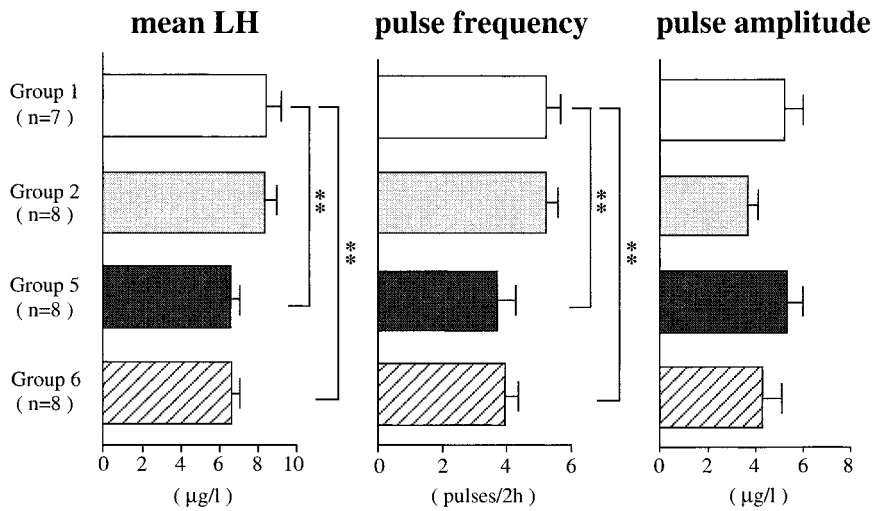

FIG. 3. Effect of third cerebroventricular (3V) administration of orexin-B and intermittent iv administration of naloxone (NAL) on the pulsatile LH secretion in ovariectomized (OVX) rats. Values are expressed as means ± SEM. Group 1, 3V saline + iv saline (control); group 2, 3V saline + iv NAL; group 5, 3V orexin-B + iv saline; group 6, 3V orexin-B + iv NAL: $^{**}P < 0.05$ (one-way analysis of variance followed by Fisher's protected least significance difference test).

we selected β-END among these neuropeptides for investigation of the inhibitory effects of orexins, because β-END mediates many inhibitory signals to GnRH neurons in comparison with other neuropeptides.

It has been reported that intermittent administration of NAL (0.5 mg/kg/h) increased the mean LH concentration (17) and the frequency and the duration of multiunit activity (MUA) volleys (18) in OVX rats. These findings indicate that NAL has a stimulative effect on GnRH secretion. However, in the present study, NAL administered at the same dose as that used in previous studies did not exhibit a stimulative effect on the pulsatile secretion of LH. It is thought that NAL acts in a β-END-activated condition and that β-END might not have been activated without orexin.

Orexin-A significantly reduced the mean LH concentration and the pulse frequency, but coadministration of NAL reversed this effect. The mean LH concentration was restored to almost the same level as that of the control, but the pulse frequency was not completely recovered. The results that NAL partially cancelled the suppressive effect of orexin-A, suggest orexin-A may suppress GnRH secretion via β-END to a considerable degree.

On the other hand, orexin-B significantly reduced the mean LH concentration and the pulse frequency, but NAL did not exhibit a blocking effect. Therefore, it is thought that β-END may not be involved in the suppression of GnRH secretion by orexin-B.

We previously reported that orexin-A more strongly suppressed the pulsatile secretion of LH than did orexin-B, and we proposed two possible reasons for this (7). One is the difference in metabolic speeds related to the molecular structure. The structure of orexin-A is more complex than that of orexin-B and orexin-A may therefore be resistant to inactivating peptidases. The other is the difference in the affinities to their receptors. Orexin-A binds to both OX1R and OX2R, while orexin-B binds mainly to OX2R (1) and orexin-A may therefore suppress GnRH secretion more strongly than does orexin-B. In addition to these possible reasons, the difference in the routes of action may contribute to the difference in their effects, because β-END is one of the most essential regulatory factors of GnRH secretion.

Furthermore, it is still unknown whether factors other than β-END, such as NPY or CRH, are involved in the inhibitory effect of orexins on GnRH secretion and whether orexins directly suppress GnRH secretion.

In conclusion, we demonstrated that orexin-A, but not orexin-B, suppresses the pulsatile secretion of LH via β-END. These results indicate that β-endorphin is involved in the suppression of GnRH secretion by orexin-A as one of the factors of the indirect pathways.

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