Orexin-A does not stimulate food intake in old rats

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Submitted 13 May 2004; accepted in final form 13 July 2004

Orexins are expressed almost exclusively in the lateral hypothalamic area (18), a region classically implicated in the control of mammalian feeding behavior (19). When administered centrally to rats, these peptides stimulate food consumption dose dependently (18). In addition, the preproorexin mRNA level is upregulated on fasting (18). These findings suggested that the orexin neuropeptide system plays a significant role in feeding behavior, and orexins are therefore categorized as orexigenic (appetite-stimulating) factors. Moreover, we have reported (13) that orexin-A stimulates pancreatic exocrine secretion via the vagal efferent nerves and that orexin-A may be an important candidate as a mediator of the cephalic phase of the secretory response to feeding.

In the present study, to determine the mechanism of the age-associated decrease in appetite and food intake, we compared food intake between young and old male rats as an indication of the stimulatory effect of intracerebroventricular administration of orexins. We also examined the protein expression of orexin receptors in the hypothalamus (the most important region of the brain for the regulation of food intake).

MATERIALS AND METHODS

All animal procedures were in accord with the Guiding Principles for the Care and Use of Animals approved by the Physiological Society of Japan. The procedures also were approved by the Ethics Committee for Animal Experimentation of the Tokyo Metropolitan Institute of Gerontology.

Materials. Synthetic orexin-A (a 33-amino-acid peptide) and orexin-B (a 28-amino-acid peptide) were purchased from the Peptide Institute (Osaka, Japan). A stainless steel cannula (22-gauge guide cannula, 28-gauge insert; Plastic One, Roanoke, VA) was used in this study. For Western blot analysis, we used the primary antibody for OX1R (OX1R11-A) and that for OX2R (OX2R11-A) (Alpha Diagnostic International, San Antonio, TX) and the secondary anti-rabbit IgG (horseradish peroxidase-linked whole antibody; Amersham Life Sciences, Piscataway, NJ).

Animals. Young (4-mo-old; 260–335 g body wt) and old (25- to 27-mo-old; 365–440 g body wt) Wistar rats were purchased from Shizuoka Jikken Dobutsu (Shizuoka, Japan) at 4 wk of age and were maintained in a specific-pathogen-free aging farm at our institute. They were given commercial rat chow (CRF-1; Oriental Yeast, Tokyo, Japan) and kept in a room at a controlled temperature (23 ± 1°C) with a 12:12-h light-dark cycle starting at 0800.

Surgical preparations. The rats were anesthetized by intraperitoneal injection of pentobarbital sodium (3–4 mg/100 g body wt). For the intracerebroventricular injection of the orexins, a stainless steel cannula was implanted stereotactically into the left lateral ventricle. After a 7-d recovery period, different doses (0–30 nmol) of orexins were injected into the lateral ventricle without anesthesia. Food and water consumptions were measured at 1, 2, and 4 h after injection. The protein levels of orexin receptors, a specific receptor for orexin-A (OX1R) and a receptor for both orexin-A and -B (OX2R), in the hypothalamus were determined by Western blot analysis and compared between young and old rats. Intracerebroventricular administration of orexin-A stimulated food intake in a dose-dependent manner in young rats. However, no effects were observed at any dose in old rats. The protein level of OX1R in the hypothalamus was significantly lower in old rats than in young rats, although the protein level of OX2R was comparable between groups. Results of the present study indicate that the function of the orexin system is diminished in old rats. The decrease in the OX1R protein level in the hypothalamus could be responsible for orexin-A’s lack of stimulation of food intake in old rats.

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cannula was implanted stereotactically into the left lateral ventricle using the following coordinates from the bregma: anteroposterior, −1.0 mm; lateral, 1.3 mm; and ventral, 4.0 mm (9, 10, 11, 13).

After the operation, the rats were maintained in individual cages (1 rat/cage) under conditions of controlled temperature (24°C) and illumination (12:12-h light-dark cycle) for 7 days. Each rat was provided food and water ad libitum. Each was mock-injected and handled for 10 min daily to minimize injection-induced stress throughout the 7-day recovery period.

Effects of orexins on food and water intake. We measured the food and water consumptions of young and old rats during the nighttime (1730–0930, total 16 h, without treatment) for comparison. Orexins were dissolved in 1% BSA/saline. After the 7-day recovery period, the experiment was conducted without overnight fasting. At 1000, 190–200 g of rat chow (CRF-1) and water were supplied, and orexins were injected intracerebroventricularly. Different doses of orexin-A (0.25, 1.0, or 3.0 nmol/10 μl) were injected subsequently during the respective 7-day recovery period in one rat. In addition, 30.0 nmol/10 μl was administered to old rats to confirm the biological effect. The effect of orexin-B (1.0 or 3.0 nmol/10 μl) was also determined in another animal. Physiological saline was administered as a vehicle. Injection was given for 20 s using a 50-μl Hamilton microsyringe, and injectors were left in place for 10 s after each injection to ensure complete dispersal of the peptide. The remaining chow and water were then weighed at 1, 2, and 4 h after the injection to estimate the food intake and water consumption at each of those time points.

At the end of each experiment, the rat was killed and black ink was injected into the lateral ventricle. The success of the cannulation was verified by visualization.

Determination of orexin receptors by Western blot analysis. Young (n = 3) and old (n = 3) rats were killed by guillotine between 1000 and 1100 without any treatment, and their whole brains were immediately removed. The hypothalamus was quickly dissected and then stored at −80°C for later experiments. The dissection of hypothalamus was carried out according to the method of Gispen et al. (3). Each tissue sample was separately homogenized in lysis buffer. The respective hypothalamus homogenates (100 μg of protein for OX1R and 200 μg of protein for OX2R) were separated on an SDS 10% polyacrylamide gel. The proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, Hercules, CA), and Western blot analysis was performed using antibody specific for orexin receptors (Phoenix, San Diego, CA). Specific bands were visualized with an enhanced chemiluminescence system (Pierce, Rockford, IL) and quantitated by densitometric analysis (ImageQuant; Molecular Dynamics, Sunnyvale, CA).
CA) and then blocked with 5% skim milk for 1 h at room temperature. The membrane was incubated with the polyclonal rabbit antibody (1:500 for OX1R11-A, 1:400 for OX2R21-A; Alpha Diagnostics International, San Antonio, TX) overnight at 4°C. After three washes, the membrane was incubated with a secondary anti-rabbit IgG (1:25,000 for OX1R; 1:30,000 for OX2R) for 1 h at room temperature. After three additional washes, immunoreactive bands were detected using the Enhanced Chemiluminescence Assay System Plus (Amer- sham Biosciences, Little Chalfont, UK) (5). Autoradiograms then underwent semiquantitative densitometric analysis. The data were expressed as means ± SE. The number of rats in each group is indicated in parentheses.

Fig. 3. Effect of intracerebroventricular injection of orexin-A on water intake. No significant effect was observed in any of the results. A: time-course effect of injection (0.25, 1.0, and 3.0 nmol) on water intake in young rats. Water intake was measured at 1, 2, and 4 h after the injection. B: effect on integrated water intake for 4 h in young rats. C: time-course effect of injection (0.25, 1.0, 3.0, and 30 nmol) on water intake in old rats. D: effect on integrated water intake for 4 h in old rats. Water intake is shown as relative to body weight. All values are means ± SE. The number of rats in each group is indicated in parentheses.

Fig. 4. Effect of intracerebroventricular injection of orexin-B on food and water intake. No significant effect was observed for any of the results. A: effect of injection (1.0 and 3.0 nmol) on integrated food intake for 4 h in young rats. B: effect on integrated food intake for 4 h in old rats. C: effect of injection (1.0 and 3.0 nmol) on integrated water intake for 4 h in young rats. D: effect on integrated water intake for 4 h in old rats. Food and water intake is shown as relative to body weight. All values are means ± SE. The number of rats in each group is indicated in parentheses.
of the immunoreactive bands was calculated by using an NIH Image software package.

Statistical analysis. All results were expressed as means ± SE. Results were analyzed by one-way ANOVA or by repeated-measures multiple ANOVA (MANOVA), followed by Fisher’s protected least significant difference test. *P < 0.05 was considered significant.

RESULTS

Food and water consumptions during nighttime in young and old rats. The food intake of old rats was significantly less than that of young rats (10.798 ± 1.080 g, mean ± SE, for old rats vs. 13.716 ± 0.524 g for young rats, *P < 0.05), whereas water consumption was not different between the two groups (18.695 ± 1.472 g, mean ± SE, for old rats vs. 18.484 ± 0.825 g for young rats).

Because the body weight of old rats was significantly higher than in young rats, we indicated values of food and water intake as relative to body weight (g/100 g body wt). Food consumption during nighttime was again significantly less in old rats than in young rats (*P < 0.0001) (Fig. 1A). Similarly, water consumption of old rats was significantly less than that of young rats (*P < 0.05) (Fig. 1B).

Effects of orexin-A on food and water intake. Analysis by the multiple comparison test after repeated-measures MANOVA revealed that the intracerebroventricular injection of orexin-A stimulated food intake in a dose-dependent manner after 1, 2, and 4 h in young rats, although the effect of 0.25 nmol orexin-A was not significant (Fig. 2A). The integrated food intake during the 4-h period is shown in Fig. 2B. Both 1 and 3 nmol orexin-A significantly increased food intake.

In contrast, no significant increase was observed in old rats, even when higher doses (1, 3, and 30 nmol) were applied (Fig. 2, C and D).

The two higher doses of orexin-A tended to increase water intake in young rats, but the difference was not significant (F = 2.843, *P = 0.0522 for 4-h water intake) (Fig. 3, A and B). No effect at any dose was observed in old rats (Fig. 3, C and D).

Effects of orexin-B on food and water intake. Intracerebroventricular injection of orexin-B slightly increased food intake in both young and old rats, but this effect was not significant. The integrated food intake during 4 h is shown in Fig. 4, A and B. Orexin-B did not stimulate water intake at any dose in either young or old rats (Fig. 4, C and D).

Determination of orexin receptors. Western blot analysis using the anti-OX1R antibody (OX1R11-A) detected specific bands corresponding to 54 kDa (Fig. 5A). The intensity of the band in the hypothalamus of the old rats was significantly lower than that of young rats (by ANOVA, *P < 0.05 vs. young).

Western blot analysis using the anti-OX2R antibody (OX2R11-A) detected three bands in the hypothalamus of both young and old rats: at 79, 53, and 43 kDa. Because the OX2R pure protein was 52.5 kDa (1), we compared the intensity of the 53-kDa immunosignal between young and old rats (Fig. 5B). The intensity of the 53-kDa bands was higher with old rats compared with young ones, but the difference was not significant.

DISCUSSION

The present study showed that intracerebroventricular injection of orexin-A (≤3 nmol) stimulated food intake in a dose-dependent manner in young rats as previously reported (4, 18, 20, 22). However, no effect was observed in old rats. To distinguish whether old rats have no response to orexin-A or have a blunted orexin-A response, the effect of a 10-fold higher dose (30 nmol) of orexin-A was determined in old rats. Again, no stimulation was observed. Therefore, it is concluded that old rats have a true insensitivity to the feeding effect of orexin-A.

To examine the mechanism underlying the effect of orexin-A, we measured the protein levels of its receptor in the hypothalamus and compared those levels between young and old rats. The protein level of OX1R was significantly lower in the old rats than in the young. The band intensity of old rats was 45% less than that of young rats. This result indicates that the decrease in the OX1R protein level in the hypothalamus could be responsible for the lack of an orexin-A stimulatory effect on food intake in old rats. However, we cannot answer the question of why an ~50% reduction in the protein level of OX1R would yield a 100% reduction in behavioral response.
Because several neural pathways are involved in the orexigenic action of orexins (16), the aging change of these pathways might be implicated.

Another hypothesis is that the age-related decrease in food intake by orexin-A is a secondary phenomenon due to the decline in the ability of orexin to increase arousal. This problem has been discussed in previous reports (16, 22, 24), although we did not record the awakening time in the present study. Muroya et al. (16) reported that changes in food intake are independent of changes in sleep/wake cycles. Moreover, a selective OX1R antagonist SB-334867 reduced orexin-A-induced food intake (4). Previously, abnormality of OX2R is shown to be a cause of canine narcolepsy (7). Because OX2R expression did not decrease with age in our study, the decrease in food intake in old rats does not seem to depend on the level of arousal.

A previous study (6) reported that the central administration of orexins stimulated water intake significantly. However, we observed that the two higher doses of orexin-A did not significantly increase water intake even in young rats. The differences between the study by Kunii et al. (6) and our present study were the different doses of orexin-A applied and the experimental conditions. Kunii et al. (6) determined the effects of 3- to 30-nmol orexins, whereas we used 0.25–3 nmol; also, they measured water intake without feeding, whereas we measured water and food consumptions simultaneously.

A recent study (17) reported that preproorexin gene expression and contents of orexins in the rat brain decreased in the course of aging. There is also a report (25) that a site-specific decrease in immunostaining for orexin was observed in the locus coeruleus of old cats. These reports indicate deterioration of the orexin system in old animals. We did not measure the orexin-A content in the brain, so whether or not that content decreased with age remains unknown. However, because orexin-A can bind to both OX1R and OX2R and the specificity of OX1R for orexin-A is high (18), the age-associated change in OX1R could be biologically more important than that in orexin-A. Our observation leads us to propose that the age-associated decline in OX1R may be related to the age-associated decline in appetite and food intake.

On the other hand, orexin-B showed no significant effect in either young or old rats. The present observation is compatible with the previous studies (2, 8) in that central orexin-B is a less effective stimulant of food intake than orexin-A. The protein level of OX2R was not significantly different between young and old rats. Terao et al. (23) measured mRNA levels of OX1R and OX2R in eight brain regions of 3-, 12-, 18-, and 24-mo-old C57BL/6 mice. They observed a declining trend of the OX2R mRNA level in the hypothalamus, although there is no age-related change in OX1R mRNA expression. These differences might be due to the species difference, although the precise mechanism is unknown.

In conclusion, the function of the orexin system in stimulating feeding behavior was reduced in old rats compared with young ones. The decrease in the OX1R protein level in the hypothalamus could be responsible for orexin-A’s lack of stimulatory effect on food intake in old rats.

**REFERENCES**


**GRANTS**

This research was supported, in part, by Health Sciences Research Grants (H13–21EBM-018) from the Ministry of Health, Labour and Welfare, Japan.


