Analyses of the facilitatory effect of orexin on eating and masticatory muscle activity in rats

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Analyses of the facilitatory effect of orexin on eating and masticatory muscle activity in rats. J Neurophysiol 106: 3129–3135, 2011. First published September 21, 2011; doi:10.1152/jn.01108.2010.—The orexins (orexin-A and orexin-B) are neuropeptides that are secreted from neurons in the lateral hypothalamus and that participate in the regulation of feeding behavior. It remains to be determined, however, how the orexins exert their effects on feeding behavior, including masticatory movements. To this end, we analyzed food intake behavior and masticatory muscle activity using video analysis and electromyography (EMG) recording methods. The results showed that the cumulative food intake over 4 h was larger in rats intraventricularly injected with either orexin-A or orexin-B than in saline-injected control rats. The latency to eating and the feeding time for a fixed amount of pellets were shortened by injections of orexin-A in a dose-dependent manner, with a more potent effect by orexin-A than orexin-B. The shorter feeding time corresponded to a decreased number of chewing cycles. EMG recordings from both the digastric and masseter muscles showed two distinct patterns of bursts corresponding to the gnawing and chewing phases. After the injection of orexin-A, the magnitude of the bursts became larger in both phases in the masseter muscle, the burst duration became longer in the chewing phase in the masseter muscle, and the interburst interval became shortened in the gnawing phase in both muscles. Consequently, the burst frequency in the chewing phase was increased in the digastic muscle and, conversely, reduced in the masseter muscle. These results suggest that the orexin-A-induced facilitatory feeding behavior is characterized by a dynamic jaw-opener activity that opens the mouth rapidly and a powerful jaw-closer activity for crushing the increased amount of food taken into the mouth. The possible involvement of orexin-A in binge eating disorder is discussed.

orexigenic neuropeptide; feeding behavior; mastication; electromyogram

FEEDING IS AN INDISPENSABLE life activity to take in essential nutrients and maintain energy homeostasis. This behavior is known to be regulated by the reciprocal activation of the feeding and satiety centers in the lateral hypothalamic area and ventromedial nucleus of the hypothalamus, respectively (Morgan 1961). Feeding behavior is also controlled by chemical mediators such as blood-borne glucose (Le Magnen 1984) and free fatty acids (Oomura et al. 1975), as well as leptin, which is produced by white adipose tissue (Zhang et al. 1994), and neuropeptide Y (NPY), α-melanin-concentrating hormone (α-MCH), agouti-related peptide (AgRP), dynorphin, and the orexins (orexin-A and orexin-B), which are produced in the hypothalamic nuclei, all of which are known to be orexigenic neuropeptides (Clark et al. 1984; Ollmann et al. 1997; Qu et al. 1996; Sainsbury et al. 2007; Sakurai et al. 1998). Among the orexigenic neuropeptides, α-MCH and the orexins are unique in the sense that they are produced by the neurons in the feeding center, whereas the others exert effects on these neurons but are produced elsewhere.

The orexins were originally identified as the endogenous ligands for two orphan G protein-coupled receptors (Sakurai et al. 1998). The orexin-related neurons, which project throughout the central nervous system, including the hypothalamic nuclei, are known to be important in the control of feeding, sleep and wakefulness, neuroendocrine homeostasis, and autonomic regulation (Willie et al. 2001). Recent studies have demonstrated that the activation of orexin neurons is strongly linked to a preference for cues associated with food reward (Harris et al. 2005). Intracerebroventricular (ICV) injection of orexins into the lateral ventricle facilitates food intake, and orexin-A, rather than orexin-B, predominantly contributes to this effect (Sakurai et al. 1998). Yamada et al. (2000) demonstrated inhibition of food intake with an anti-orexin antibody in fasted rats. Furthermore, orexin-knockout mice exhibit decreased food intake compared with wild-type mice; nevertheless, since the energy expenditure is even lower than this decline in intake in this animal model, obesity developed (Hara et al. 2001, 2005; Willie et al. 2001). Furudono et al. (2006) showed that gustatory stimulation after the intake of water containing saccharin increased the expression level of orexin mRNA compared with straight water intake. They also showed that ICV administration of orexin-A increased saccharin intake and induced phasic contractions in the distal stomach, along with relaxation in the proximal stomach, via the effect on the vagus nerve (Kobashi et al. 2002). These findings suggest that the orexins are one of the potent factors in promoting ingestive behaviors toward food of attractive taste.

Although orexins accelerate feeding behavior, there are no reports about the manner in which orexin-administered animals actually ingest their food, e.g., how they masticate and swallow it. Therefore, the present study aims to elucidate the oral-motor behavior of the increased food intake in orexin-administered rats by analyzing the features of the chewing pattern using video analysis and electromyography (EMG) recording methods. It would be of considerable interest if the results were to closely correlate with the chewing and intake patterns shown by people afflicted with eating disorders.
MATERIALS AND METHODS

Animals and Surgical Treatment

A total of 47 male Wistar rats, weighing 280–300 g, were obtained from the Charles River Breeding Laboratories (Kanagawa, Japan). Rats were separately housed in a controlled environment room (23°C, 60% humidity) under a 12:12-h light-dark cycle with lights on at 8:00 AM and given ad libitum access to food (MF Pellets; Oriental Yeast, Tokyo, Japan) and water before surgery. The experimental protocols we conducted were approved by the Institution Animal Care and Use Committee of Osaka University.

Rats were anesthetized with pentobarbital sodium (50 mg/kg ip) and then fixed in a stereotaxic apparatus (SR-5R; Narishige Group, Tokyo, Japan). A stainless steel guide cannula (23-gauge; Plastics One, Roanoke, VA) was positioned at the left lateral ventricle. The tip location coordinates were 1.0 mm posterior to the bregma, 1.5 mm lateral to the midline, and 3.2 mm ventral to the skull surface. An inner cannula (30-gauge; Plastics One) was inserted into each guide cannula as a dummy. For the EMG recording, a pair of Teflon-coated stainless steel wires (outer diameter: 250 μm) were inserted into the lateral ventricle. Saline (3 μl) was injected as the vehicle control. Microinjections were made through the inner cannula connected to a 10-μl Hamilton microsyringe. The inner cannula was extended 1.0 mm beyond the tip of the guide cannula. Each solution was delivered at 1 μl/min with a microinfusion pump. The inner cannula was left in place for another 60 s to ensure complete dispersal of the solution from the cannula tip. Immediately after the injection, the rat was put into the chamber, and then a fixed amount (2 g) of the pellets was put on the floor of the chamber and simultaneous behavioral and EMG recordings were started. After this amount food had been eaten, more pellets were added. Video recordings were made over a period of 4 h, and the amount of food intake was measured every hour. EMG recordings were made during the eating of the 2 g of pellets. The EMG signals were amplified 1,000-fold, filtered with optimal bandwidths of 100–1,000 Hz, and then digitized at a sampling rate of 1,000 Hz and recorded utilizing the data acquisition program pClamp 10.2 (Molecular Devices) and LabChart 6.0 software (ADInstruments).

Protocol

Experiment 1: effects of orexin-A and orexin-B injection on feeding. A total of 41 rats were allowed to freely eat for 2 days (days 1 and 2). On day 3, each rat was implanted with the guide cannula under general anesthesia, as described above. After surgery, animals were returned to their home cages for a recovery period of 7 days (days 3–9). During the recovery period, each rat was transferred into a recording chamber for 3 days of habituation to the experimental conditions (days 7–9). On day 10, the correct position of the cannula was verified by central administration of angiotensin II (100 ng/3 μl; Sigma-Aldrich, St. Louis, MO) to test for a dipsogenic response. Rats that drank at least 15 ml of water for 30 min after the injection were used for the experiments. The rats were then housed separately for a recovery period of 2 days (days 11 and 12). On day 13, the recording of feeding behavior was performed after the injection of either orexin-A (0.03, 0.3, or 3 nM), orexin-B (0.3 or 3 nM), or saline (n = 5–10 per group). All recordings were performed from 10:00 AM to 2:00 PM to minimize circadian rhythm effects.

Experiment 2: effects of orexin-A injection on masticatory muscle activity. Six rats were allowed to freely eat for 2 days (days 1 and 2). On day 3, they were implanted with the guide cannula for drug administration and electrodes for EMG recording under general anesthesia. Each rat was then treated as in experiment 1. Simultaneous recordings of feeding behavior and EMG activities of the masticatory muscles were performed after the injection of either orexin-A (3 nM) or saline on days 13 and 17 with a 3-day recovery period in between (days 14–16). The injection of orexin-A and saline was counterbalanced.

Recording Procedures

Following the established procedure for the taste reactivity test (Grill and Norgren 1978), each rat was placed in a clear, plastic chamber (30 × 30 × 30 cm³). Two digital video cameras (Victor GZ-HD30 and SONY HDR-XR-520V) were placed on both sides of the chamber to record the movements of the mouth. One was used for recording through the chamber and another through a mirror placed at a 45° angle beneath the transparent Plexiglas floor. Rats were injected with 3 μl of orexin-A (Peptide Institute, Osaka, Japan) dissolved in saline at a dose of 0.03, 0.3, or 3 nM, previously shown to stimulate food intake (Sakurai et al. 1998). Orexin-B (Peptide Institute) dissolved in saline at a dose of 0.3 or 3 nM was administered into the lateral ventricle. Saline (3 μl) was injected as the vehicle control. Microinjections were made through the inner cannula connected to a 10-μl Hamilton microsyringe. The inner cannula was extended 1.0 mm beyond the tip of the guide cannula. Each solution was delivered at 1 μl/min with a microinfusion pump. The inner cannula was left in place for another 60 s to ensure complete dispersal of the solution from the cannula tip. Immediately after the injection, the rat was put into the chamber, and then a fixed amount (2 g) of the pellets was put on the floor of the chamber and simultaneous behavioral and EMG recordings were started. After this amount food had been eaten, more pellets were added. Video recordings were made over a period of 4 h, and the amount of food intake was measured every hour. EMG recordings were made during the eating of the 2 g of pellets. The EMG signals were amplified 1,000-fold, filtered with optimal bandwidths of 100–1,000 Hz, and then digitized at a sampling rate of 1,000 Hz and recorded utilizing the data acquisition program pClamp 10.2 (Molecular Devices) and LabChart 6.0 software (ADInstruments).

Behavioral Analyses

The meal parameters were defined using the following criteria (Cooper et al. 2006): 1) cumulative food intake over 4 h; 2) latency to eating, or the time elapsed from the presentation of the pellet to the initiation of eating; 3) feeding time, or the total time necessary to eat the 2 g of pellets; 4) feeding rate, calculated by dividing the 2 g of pellets by the feeding time; and 5) number of chewing cycles during the eating of the 2 g of pellets.

EMG Analyses

Masticatory EMG recordings are characterized by the number of bursts that correspond to the gnawing and chewing cycles. We analyzed representative bursts during the first 150 s of eating in each rat under the saline-injected and orexin-A (3 nM)-injected conditions. The raw EMG signals were rectified, smoothed using a time constant of 0.1 s, and then processed to determine the EMG burst duration and integrated area of the rectified burst. Each onset and offset of an EMG burst were determined on the basis of the mean ± 2SD of baseline activity (or the noise level calculated in the periods without bursts). The EMG parameters were defined using the following criteria: 1) the integrated EMG, calculated as the integrated area of the rectified burst every 1 s; 2) the burst duration, or the time from onset to offset of a burst; 3) the interval of bursts, or the time from the offset of the previous burst to the onset of the next burst; and 4) the burst frequency, calculated by dividing the number of bursts by the total time from the start to the end of continuous bursts (bursts/s). This is defined by the following equation:

\[ \text{Burst frequency} = n / \left( \sum_{i=1}^{n} BD_i + \sum_{i=1}^{n} BI_i \right) \quad (3 \leq n \leq 5), \]

where \( n \) is the number of bursts, \( BD_i \) is the burst duration, and \( BI_i \) is the burst interval. Data were analyzed with a combination of software packages [Clampfit 10.2 (Molecular Devices), LabChart 6.0 (ADInstruments), and Spike2 (CED, Cambridge, UK)].

Statistical Analysis

The behavioral data from experiment 1 are expressed as means ± SE, the normality of data was assessed using the Mann-Whitney rank sum test, and the data between groups were compared by unpaired t-test. The mean values obtained in the EMG analyses from experiment 2 were compared between the orexin-A-injected and saline-injected conditions in the same rats and statistically examined with the
Wilcoxon signed rank test using StatView (SAS Institute, Cary, NC). Differences were considered significant at \( P < 0.05 \).

**Histology**

After completion of the experiments, the rats received an overdose of pentobarbital sodium (100 mg/kg ip) and were perfused transcardially with 0.02 M phosphate-buffered saline, followed by 10% formalin in 0.1 M phosphate buffer (PB). The brains were soaked in 30% sucrose in 0.1 M PB and stored at 4°C. Coronal sections were cut at 50 \( \mu \)m, stained with cresyl violet, and examined under microscopy for the placement of the cannula.

**RESULTS**

**Effects of Orexin-A or Orexin-B Injection on Feeding Behavior**

Each rat received an ICV injection of orexin-A, orexin-B, or saline on different days. Histology confirmed that the tip of the cannula was successfully positioned within the left side of the lateral ventricle (Fig. 1A). Administration of orexin-A (0.03–3 nM) or orexin-B (0.3–3 nM) enhanced food intake in a dose-dependent manner. As shown in Fig. 1B, the cumulative food intake at every hourly time point up to 4 h was significantly larger \( (P < 0.01) \) in rats receiving 0.3 and 3 nM, but not 0.03 nM, orexin-A injection (orexin-A rats) compared with saline-injected control rats. The total food intake during 4 h in the orexin-A rats at 0.03, 0.3, and 3 nM was 2.2, 4.5, and 8.1 times larger than in control rats, respectively, whereas that in orexin-B rats at 0.3 and 3 nM was 2.4 and 3.0 times larger, respectively (Fig. 1C). The latency to eating of pellets after presentation was dose-dependently shorter in the orexin-A than in the control rats. Orexin-B tended to shorten \( (0.3 \text{ nM}: P = 0.4; 3 \text{ nM}: P = 0.07) \) the latency, but the difference was not statistically significant (Fig. 1D). The feeding time to eat all of the 2 g of pellets were both significantly \( (P < 0.01) \) shorter by 10.22\( \pm \)0.33.5 on November 4, 2016 http://jn.physiology.org/ Downloaded from
After the orexin-A injection, the jaw muscle activities that were typically observed in control rats underwent a change. This rhythmical pattern of brief gnawing alternating with long chewing phases was often collapsed together such that the number of gnawing phases was increased and the chewing phase was frequently and irregularly interrupted (Figs. 2B and 3B). After the orexin-A injection, EMG activity tended to be enhanced in both muscles, but significant enhancement was detected in the masseter muscle as shown in Figs. 2B and 4A and B, and the changes were similar for the two muscles in terms of the burst duration and interval of bursts except the burst frequency (Fig. 5): the frequency increased in digastric muscle but decreased in masseter muscle in the chewing phase (Fig. 5Bc). Figure 5 provides detailed comparisons of each burst parameter of the two muscles between the gnawing and chewing phases, as well as between the control and orexin-A rats. In terms of the digastric bursts in the orexin-A rats, the duration became slightly longer (gnawing: \(P = 0.22\); chewing: \(P = 0.09\)) (Fig. 5, Aa and Ab), and the interval became significantly shortened (gnawing and chewing: \(P < 0.05\)) (Figs. 5, Ba and Bb). Consequently, the frequency became higher in the chewing phase but not in the gnawing phase (chewing: \(P < 0.05\); gnawing: \(P = 0.5\)) (Fig. 5, Ac and Bc). With regard to the masseter bursts in the orexin-A rats, the duration became significantly longer in the chewing phase (Fig. 5Ba), the interval became significantly shorter in the gnawing phase (Fig. 5Ab), and the frequency became significantly lower in the chewing phase (Fig. 5Bc), whereas the frequency in the gnawing phase tended to be higher (\(P = 0.17\)) (Fig. 5Ac).

**DISCUSSION**

In accordance with previous reports (Sakurai et al. 1998), we found an enhanced food intake by ICV administration of orexin-A or orexin-B, as evidenced by a dose-dependent increase in the cumulative food intake over a period of 4 h. The orexin administration was performed in the early part of the light cycle, when the orexin level in the brain is comparatively low compared with that in the nighttime (Yamanaka et al. 1999). The new findings obtained in the present study for the orexin-A and -B rats are that the latency to eating after presentation of food was shortened and also that the feeding time was dose-dependently shortened. It was also demon-
strated that the number of chewing cycles was decreased in the orexin-A (3 nM)-injected rats. These results suggest that the orexin-A rats have an altered pattern of eating that is characterized by the taking of a large amount of food into the mouth and swallowing it quickly without the normal amount of chewing. This altered pattern may help explain not only the rapid eating but also the increased amount of food intake over time (Fig. 1, B, C, E, and F). These facilitatory effects induced by orexin-B were smaller than those by orexin-A, which is consistent with the previous finding by Sakurai et al. (1998).

To further investigate whether orexin-A administration has a facilitatory effect on eating behavior, we analyzed EMG activities of the jaw opening (digastric) and closing (masseter) muscles in the orexin-A and control rats. Our EMG analyses clearly demonstrated that the distinct features of the oral movements during the eating of pellets consisted of two phases: gnawing (or biting) and chewing (or mastication) (Fig. 2). Although the gnawing and long chewing phases generally alternated in the control rats, this pattern was often collapsed into an irregular and frequently occurring gnawing phase with a shorter chewing phase (Fig. 3), indicating a hurried intake of food. More precisely, in the gnawing phase, each activity burst in both muscles corresponding to each bite was increased, as was clearly demonstrated by the integrated EMG (Figs. 3 and 4). The interval of bursts was shortened and the burst frequency tended to increase in the orexin-A rats. These findings suggest that the orexin-A rats bit the pellets rapidly and powerfully with their incisor teeth before chewing with the molar teeth, thus accounting for the observation in the behavioral analysis that the orexin-A rats held a larger amount of food in the mouth before chewing. In the chewing phase, the burst activity corresponding to each chewing stroke was increased, together with an increased duration and shortened burst interval in both muscles. It is interesting to note that in the case of orexin-A, the burst frequency was increased in the digastic muscle, whereas it was reduced in the masseter muscle (Fig. 5Bc). These findings indicate that when the orexin-A rat chewed the pellets with its molars, the jaw-closing phase was extended, with an extended duration and reduced burst frequency in the masseter muscle, whereas the jaw-opening phase was shortened, exhibiting a shortened burst interval in each of the two muscles.

According to previous studies (Morimoto et al. 1985; Thomas and Peyton 1983), the toughness, size, and type of food in the oral cavity affect the masticatory muscle, i.e., the amplitude of the masseter activity was increased and the burst duration extended during the chewing of solid and/or large pellets compared with tender pudding and/or small pellets. Thus the EMG characteristics in the orexin-A rats are similar to those in naive rats chewing bigger and harder pellets, indicating that the orexin-A rats have to crush the increased pellet amount taken into the mouth with more powerful jaw-closing activities. Although we have suggested in the behavioral analysis that orexin-A rats swallow the food without normal molar chewing, the powerful muscle activities observed in fact might be effective enough to compensate for the decreased number of chewing strokes.

One interpretation of our findings is that the effects of orexins on eating are mediated by the orexin receptors in the
trigeminal nuclei. This possibility is supported by the following
evidence. First, orexin neurons project to the brain stem
tegmental mesencephalic trigeminal nucleus (MTN) and the trigeminal
motor nucleus (TMN) (Date et al. 1999; Nambu et al. 1999).
Both orexin receptor-1 and -2 were shown to be expressed at
the protein and mRNA levels in the MTN as well as TMN of
the rat with the use of in situ hybridization and immunohisto-
chemical methods (Greco and Shiromani 2001). MTN neurons
receive orexin-A hypothalamic innervation with a somatotopic
arrangement of the projections in the nucleus (Stoyanova and
Lazarov 2005), and orexin-B immunoreactive fibers and ter-

majority of orexin-A rats exhibited highly accelerated ingestive
behavior, putting pellets hastily into their mouth with rapidly
repeated bites, performing a reduced number of chewing
strokes before swallowing, and eventually ingesting a larger
than normal quantity of food. Although the factors causing
binge eating disorder are thought to be complexly intermingled,
the present study suggests that elevated levels of orexins
in the brain may play an important role, at least in part, in the
pathogenesis of this type of eating disorder.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

T.T., T.Y., and M.K. conception and design of research; T.T. and S.B.
performed experiments; T.T. and S.T. analyzed data; T.T. and S.B. interpreted
results of experiments; T.T. and S.T. prepared figures; T.T. drafted manuscript;
T.T. and T.Y. edited and revised manuscript; T.T., T.Y., S.T., S.B., and M.K.
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