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

Running title: Effects of orexin on eating behavior

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The orexins (Orexin-A and orexin-B) are neuropeptides which are secreted from neurons in the lateral hypothalamus, which 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 hrs was larger in rats intraventricularly injected with either orexin-A or orexin-B than saline-injected control rats. The latency to eat and the feeding time for a fixed amount of pellets were shortened by injections of orexins 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 inter-burst interval became shortened in the gnawing phase in both muscles. Consequently, the burst frequency in the chewing phase was increased in the digastric 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 which opens the mouth rapidly and 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.

**Keywords:** orexigenic neuropeptide, feeding behavior, mastication, electromyogram
INTRODUCTION

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 (Morgane 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 NPY, α-MCH, 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, while 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 as 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; Hara et al. 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 in comparison 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 towards 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 orexins-administered rats by analyzing the features of the chewing pattern using video-analysis and 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 12-hour light/ dark cycle with lights on at 8:00 A.M., and given ad lib access to food (MF Pellets, Oriental Yeast
Co., LTD, Japan) and water prior to surgery. The experimental protocols were conducted in accordance with the guidelines of the Institution Animal Care and Use Committee of Osaka University.

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), then fixed in a stereotaxic apparatus (SR-5R, NARISHIGE Group, Japan). A stainless steel guide cannula (23-gauge, Plastics One, USA) 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, USA) 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 left masseter and the anterior belly of the digastric muscles with an interpolar distance of approximately 3 mm, and another wire was fixed to an anchor screw to serve as a ground. The wires were subcutaneously passed and soldered to a 5 pin miniature socket. The guide cannula and sockets were fixed with dental acrylic resin and three stainless steel screws were anchored to the skull.

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 1-2). 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). Each rat was then 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 (Sigma-Aldrich Co, USA, 100 ng/3 μl) to test for a dipsogenic response. Rats which drank at least 15 ml of
water for 30 min after the injection were used for the experiments. Then rats were housed separately for a recovery period of 2 days (days 11-12). On day 13, the recording of feeding behavior was performed after the injection of either orexin-A (0.03, 0.3, 3 nM), orexin-B (0.3, 3 nM) or saline (n=5-10 per group). All recordings were performed from 10:00 A.M. to 2:00 P.M. in order 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 1-2). 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 x 30 x 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, Inc., Japan) dissolved in saline at a dose of 0.03, 0.3, or 3 nM, a dose previously shown to stimulate food intake (Sakurai et al. 1998). Orexin-B (Peptide Institute, Inc., Japan) 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, 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 hrs 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 1000-fold, filtered with optimal bandwidths of 100-1000 Hz, then digitized at a sampling rate of 1000 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 hrs, 2) latency to eat 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, 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 which 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, then processed to determine the EMG burst duration and integrated area of the rectified burst. Each onset and offset of an EMG burst was 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 by 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, 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:

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

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), Spike2 (CED, Cambridge, UK)].

**Statistical analysis**

The behavioral data from Experiment 1 are expressed as the means ± SE, the normality of data was assessed using Mann-Whitney Rank Sum test and the data between groups was 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 by 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 sodium
pentobarbital (100 mg/kg, i.p.) and were perfused transcardially with 0.02 M phosphate-buffered saline, followed by 10 % formalin in 0.1 M phosphate buffer. The brains were soaked in 30 % sucrose in 0.1M PB and stored at 4 °C. Coronal sections were cut at 50 μl and stained with cresyl violet and examined under microscopy for the placement of the cannula.

RESULTS

Effects of the orexin-A or orexin-B injection on feeding behavior

Each rat received an intracerebroventricular injection of either 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 -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 timepoint up to 4 hrs 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 hrs in the orexin-A rats at 0.03, 0.3 and 3 nM was 2.2, 4.5 and 8.1 times larger than 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 eat pellets after presentation was dose-dependently shorter in the orexin-A than the control rats. Orexin-B tended to shorten (0.3 nM: P=0.4, 3 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 the treatment with orexin-A at 0.3 and 3 nM (Fig. 1E). Accordingly, the calculated feeding rate (the weight of the pellets/the feeding time) was significantly (P<0.01) higher in the orexin-A rats.
when 0.3 or 3 nM were injected (Fig. 1F). Frame by frame analysis of the video recording showed that the mean number ± SEM of chewing cycles (or rhythmical jaw movements) during the eating of the 2 g of pellets was 965±90 in the orexin-A (3 nM) rats and 1278±116 in the control rats. This difference was statistically significant (P<0.05).

**Effects of orexin-A injection on digastric and masseter muscle activity**

The EMG recordings from both the digastric and masseter muscles during the eating of the pellets exhibited characteristic patterns of bursts corresponding to the gnawing and chewing phases. That is, the simultaneous video and EMG recordings revealed that in the gnawing phase small bursts of the masseter muscle and large bursts of the digastric muscle were evident, reflecting the cutting and intake tasks performed with the front teeth. In the chewing phase, large bursts of the masseter muscle and small bursts of the digastric muscle were evident, reflecting the crushing and grinding performed with the molar teeth. These characteristic EMG patterns were also seen in the control rats (Fig. 2A). More specifically, the amplitude of the burst was typically larger in the gnawing phase than the chewing phase for the digastric muscle (Figs. 2A, 3A and 4A), while the reverse was true for the masseter muscle (Figs. 2A, 3A and 4B). The burst duration was significantly (Dig and Mass: P<0.01) longer and the burst interval was not observably different (Dig: P=0.52, Mass: P=0.10), so consequently, the burst frequency tended to be lower (Dig and Mass: P<0.01) in the gnawing phase compared with the chewing phase (Figs. 2A, 3A and 5). One important finding to be noted here is that the digastric burst was recorded in the jaw-opening as well as the jaw-closing phase. Therefore, the burst frequency of the digastric muscle was twice that of the masseter
muscle (Figs. 5A-c and 5B-c). This finding is consistent with that reported by Thomas and Peyton (1983).

After the orexin-A injection, the jaw muscle activities which were typically observed in control rats underwent a change. This rhythmic 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, 4A and 4B, 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. 5B-c). 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: $P=0.09$) (Figs. 5A-a and 5B-a), and the interval became significantly shortened (gnawing and chewing: $P<0.05$) (Figs. 5B-a and 5B-b). Consequently, the frequency became higher in chewing phase, but not in the gnawing phase (chewing: $P<0.05$; gnawing: $P=0.5$) (Figs. 5A-c and 5B-c). With regard to the masseter bursts in the orexin-A rats, the duration became significantly ($P<0.05$) longer in the chewing phase (Fig. 5B-a), the interval became significantly ($P<0.05$) shortened in the gnawing phase (Fig. 5A-b) and the frequency became significantly ($P<0.05$) lower in the chewing phase (Fig. 5B-c), while the frequency in the gnawing phase tended to be higher ($P=0.17$) (Fig. 5A-c).
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 hrs. 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 eat after presentation of food was shortened and also the feeding time was dose-dependently shortened. It was also demonstrated 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 which 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 (Figs. 1B, 1C, 1E and 1F).

These facilitatory effects induced by orexin-B were less 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 opener ( digastric) and closer (masseter) muscles in the orexin-A and control rats. Our EMG analyses clearly demonstrated 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 digastric muscle, while it was
reduced in the masseter muscle (Fig. 5B-c). These findings indicate that when the
orexin-A rat chews the pellets with its molars, the jaw-closing phase was extended, with
an extended duration and reduced burst frequency in the masseter muscle, while 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 in comparison with tender pudding
and/or small pellets. Thus, the EMG characteristics in the orexin-A rats are similar to
those in naïve 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
closer activities. While 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 deceased 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 brainstem 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 using in situ hybridization and immunohistochemical 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 terminals innervate the sensory and motor neurons controlling masticatory muscles (Zhang et al. 2002). The orexins may therefore exert a direct influence upon rhythmical oral-motor activity, as well as jaw movements at the brainstem level, including the MTN, TMN and the pattern generator. Second, orexin-A and orexin-B microinjections into the TMN increased ipsilateral masseter muscle tone in a dose-dependent manner, and pretreatment with an NMDA antagonist abolished the excitatory response of the masseter muscle to orexin-A (Peever et al. 2003). These findings, together with the finding that orexin modulates presynaptic glutamate release in in vitro studies (Van den Pol et al. 1998), indicate that orexins excite motoneurons by modulating glutamate release and that functional NMDA receptors are important in this response. However, the precise physiological roles of the orexinergic system in the function of the trigeminal system still remain to be clarified.

Another interpretation of the present results is that orexin effect on eating behavior
is associated with muscle tone related to the animal's level of vigilance. This possibility is supported by the report that orexin injection into the cat pedunculopontine tegmental nucleus strongly affects muscle tone (Takakusaki et al. 2005) and that orexin over-expressing mice show abnormal muscle contractions during REM sleep (Willie et al. 2011). In this regard, we also observed abnormal behaviors in rats injected with 3 nM orexin-A, such as running and sniffing as if they were highly excited, as well as abrupt spontaneous jaw movements. Such muscle tone abnormality lasted for a few seconds. The alterations in masticatory muscle activities in the present study suggest that orexins positively regulate muscle function in order to maintain proper behavior, including feeding, during normal wakefulness.

Although feeding behavior is basically controlled by reciprocally alternating activation of the feeding and satiety centers in the hypothalamus, eating disorders quite frequently develop in people who are habitually forced to accept fast and/or binge eating as a consequence of the limited mealtime period in modern life, as well as in patients suffering from anorexia nervosa and bulimia nervosa, as well as atypical conditions such as binge eating disorder. Stunkard (Stunkard et al. 1996) first described binge eating disorder in certain obese individuals that consisted of periodic uncontrolled consumption of unusually large amounts of food and coined the term "night eating syndrome", which is similar to but nevertheless distinct from binge eating disorder. In terms of the signs of binge eating disorder, binge eaters consistently eat an unusually large amount of food at one time even when not really hungry, and they eat much more quickly than normally. These eating characteristics correspond very well with those observed in orexin-A rats, i.e., the 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, at least in part, may play an important role in
the pathogenesis of this type of eating disorder.

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FIGURE LEGENDS

FIGURE 1. Effects of orexin-A or orexin-B injection on feeding behavior. A, the arrow, which is in a coronal section of the rat brain, shows the tract of the cannula ending in the lateral ventricle (LV). B, cumulative food intake during 4-h-long period of free access to pellets. C, food intake at 4 h. D, latency to eating. E, feeding time for 2 g of pellets. F, feeding rate calculated by dividing 2 g of pellets by the elapsed feeding time. Each value is the mean ± SE (n=5-10 per group). The asterisks indicate a significant difference from control (*P<0.05, **P<0.01, Unpaired t-test).

FIGURE 2. Representative sample of the digastric and masseter bursts during the eating of pellets in the saline-injected control and orexin-A injected conditions. A is the actual EMG recordings and the integrated EMG of the digastric (Dig) and masseter (Mass) muscles in the control animals. B is the actual EMG recordings and the integrated EMG of the digastric and masseter muscles under treatment with orexin-A (3 nM). Representative digastric and masseter bursts picked out of the record A and B in the gnawing (solid line square) and chewing phase (broken line square) under the control (A-a and -b) and orexin-A (B-a and -b) conditions are shown, with the time scale shown as an inset record.

FIGURE 3. Sample recordings of the digastric and masseter muscle activity during the eating of the pellets in the saline-injected control and orexin-A injected conditions. A is the actual EMG recordings of the digastric (Dig) and masseter (Mass) muscles in the control animals. B is the actual EMG recordings of the digastric and masseter muscles under orexin-A (3 nM). A-c and B-c show the gnawing and chewing phases in
the above recordings.

**FIGURE 4.** Comparison of the relative integrated EMG of the digastric and masseter muscle for the gnawing and chewing phases between the saline-injected control and orexin-A injected experimental conditions. A is the relative integrated EMG, or the integrated area of the rectified burst, of the digastric muscle when the integrated EMG in the control chewing phase was taken as 1.0. B is the relative integrated EMG of the masseter muscle when the integrated EMG in the control gnawing phase was taken as 1.0. Data are the mean ± SE. The asterisks indicate a significant difference from control (* \( P<0.05 \), ** \( P<0.01 \), Wilcoxon Signed Rank Test, n=6).

**FIGURE 5.** Comparison of the parameters of bursts of the digastric and masseter muscle for the gnawing and chewing phases between the saline-injected control and orexin-A injected experimental conditions. Data are the mean ± SE. The asterisks indicate a significant difference from control (* \( P<0.05 \), ** \( P<0.01 \), Wilcoxon Signed Rank Test, n=6).
Figure 1

A

LV
LV

B

Cumulative food intake (g)

Time (hr)

C

Food intake at 4 h (g)

D

Latency to eat (min)

Con 0.03 0.3 3 0.3 3 Ox-A (nM) Ox-B (nM)

E

Feeding time (s)

Con 0.03 0.3 3 0.3 3 Ox-A (nM) Ox-B (nM)

F

Feeding rate (mg/s)

Con 0.03 0.3 3 0.3 3 Ox-A (nM) Ox-B (nM)
Figure 2

A  Control

Dig
Mass
Dig
Mass

B  Orexin-A

Dig
Mass
Dig
Mass
Figure 3

A
a  Dig  

b  Mass  

c  Phase  

Control

B
a  Dig  

b  Mass  

c  Phase  

Orexin-A

[] : Gnawing phase  [] : Chewing phase
Figure 4
Figure 5

A

Gnawing phase

- Duration (ms)
- Interval (ms)
- Frequency (Hz)

B

Chewing phase

- Duration (ms)
- Interval (ms)
- Frequency (Hz)