BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS (2000) 267-2:527-531.

# Inhibition of food intake by central injection of anti-orexin antibody in fasted rats

Yamada, H; Okumura, T; Motomura, W; Kobayashi, Y; Kohgo, Y

**Inhibition of food intake by central injection of anti-orexin antibody in fasted rats**

Hiroto Yamada, Toshikatsu Okumura, Wataru Motomura, Yoshifumi Kobayashi*, Yutaka Kohgo

Third Department of Internal Medicine and Department of Otorhinolaryngology* , Asahikawa Medical College, Asahikawa 078-8510, Japan

All correspondence and reprint address should be:
Toshikatsu Okumura, M.D., Ph.D.
Third department of Internal Medicine
Asahikawa Medical College
Asahikawa 078-8510
Japan
Tel 81-166-68-2462
Fax 81-166-68-2469
E-mail okumurat@asahikawa-med.ac.jp

**Abstract**

It has been shown that intracerebroventricular injection of synthetic orexins stimulated food intake in rats. This pharmacological evidence suggests that orexins may have a role for the central regulation of feeding. In the present study, we investigated the hypothesis whether endogenous orexins indeed play a vital role in feeding behavior. An anti-orexin polyclonal antibody was used throughout the study. First, we examined the specificity of the antibody to orexin by Western blot analysis and immunohistochemistry. Next, the effects of central injection of the orexin antibody on food intake in 24-hr-fasted rats were evaluated. Western blot analysis revealed that the orexin antibody detected synthetic orexin-A. Immunohistochemical study showed that orexin-positive neurons were identified only in the lateral hypothalamic area, being in agreement with previous reports. Neither control antibody nor the orexin-antibody preabsorbed with excess amount of orexin-A detected neurons, indicating the orexin antibody is specific. Intracisternal but not intraperitoneal injection of the orexin antibody dose-dependently suppressed feeding. All these results suggest that immunoneutralization of endogenous orexins in the brain reduced food intake. In other words, we suggest that endogenous brain orexin may have a physiologically relevant action on feeding behavior.

## Introduction

Feeding is a complex behavior that involves multiple neural pathways to and from the hypothalamus (1, 2, 3). So far, a number of neuropeptides have been shown to play a positive or negative role in food intake. For instance, neuropeptide Y, melanin concentrating hormone, galanin and agouti-related protein act in the brain to stimulate food intake while bombesin, cholecystokinin, corticotropin-releasing hormone, melanocortin and leptin suppress feeding (4, 5). Recently, a novel hypothalamic peptide family, orexins, was discovered by Sakurai et al. (6). Orexins are localized in neurons within the lateral hypothalamic area and its adjacent area (6, 7). Because of its striking localization of the peptides in the hypothalamic area, Sakurai et al. have examined the effects of centrally administered synthetic orexins on food intake and clearly demonstrated that intracerebroventricular injection of orexins stimulated food consumption in rats (6). This pharmacological evidence suggests that orexins may play a role in brain regulation of feeding behavior. There is however a question whether endogenous orexins in the brain is indeed involved in feeding behavior. In the present study, we tried to clarify the above question by immunoneutralization of endogenous orexins with specific antibody which selectively blocked orexins effects.

## Materials and Methods

### Animals

Male Sprague-Dawley rats weighing approximately 200 g were housed under controlled light /dark conditions (lights on: 07:00 - 19:00) with the room temperature regulated to 23-25$^{o}$ C. Rats were allowed free access to standard rat chow (Solid rat chow, Oriental Yeast Co., Tokyo,

Japan) and tap water.

Orexin polyclonal antibody

Orexin polyclonal antibody was made by order to the Peptide Institute, Osaka, Japan. The antibody was purified as IgG from anti-orexin antiserum as described previously (8). Anti-orexin antiserum was raised in rabbits by immunization with synthetic orexin-A conjugated with bovine serum albumin. The control IgG was purified from pre-immune serum in the same rabbit. The specificity of the orexin antibody was determined by inhibition of binding of the antibody to orexin-A which was coated on the microplate wells by enzyme-linked immunosorbent assay system. No detectable inhibition was observed with the tested compounds including insulin, glucagon-like peptide-1 (7-36 amide) and beta-endorphin at concentrations of up to 10 µg/well. To further confirm the specificity of the orexin antibody, two different experiments were performed. First, we checked if the antibody indeed detects orexins by Western blot analysis. Next, immunohistochemical detection of orexins in the brain was done by sequential incubations of free-floating sections in the anti-orexin antibody.

Western blotting

Solution of synthetic orexin-A or rat orexin-B (Peptide Institute, Osaka, Japan) dissolved in saline (1 µg/20 µl) was applied on a 16 % Tricine gel (Novex, San Diego, CA). After electrophoresis, the peptide was transferred to nitrocellulose membrane (Amersham Life Science, Inc., Piscataway, NJ), blocked overnight in TBS-Tween (TBS-T) with 10% skim milk at 4$^{o}$C, subsequently reacted with either orexin antibody (diluted 1: 100), control antibody (diluted 1: 100) or orexin antibody preabsorbed

with excess amount of synthetic orexin-A (10 μg/ml), and washed. After reaction with horseradish peroxidase-conjugated anti-rabbit IgG, immune complexes were visualized by using the ECL detection reagents (Amersham, Buckinghamshire, UK).

Immunohistochemistry

Six rats were used in this study. Preparation of brain tissues was performed according to our previous report (9). Rats were deeply anesthetized with intraperitoneal administration of pentobarbital (40 mg/kg) and perfused transcardially with approximately 150 ml of 0.01 M phosphate buffered saline (pH 7.4) followed by approximately 150 ml of 4 % paraformaldehyde, 0.2 % picrate and 0.35 % glutaraldehyde dissolved in 0.1 M phosphate buffer at 4$^{o}$C. The brain was removed and post-fixed for 1 day at 4$^{o}$C in the same fixative without glutaraldehyde. It was then immersed in 0.1 M phosphate buffer containing 15 % sucrose for at least 2 days. Serial frontal sections of the brain were cut with a cryotome at a thickness of 50 μm.

Each brain section was incubated for 2 days with the orexin antibody (diluted 1: 1,000) followed by incubations with biotinylated goat anti-rabbit IgG (2 hr) and avidin-biotin-peroxidase complex (1 hr). Finally, the sections were reacted with 0.02 % 3,3-diaminobenzidine (DAB), 0.3 % nickel ammonium sulfate and 0.005 % $H_2O_2$ in 0.05 M Tris-HCl buffer. Brain tissues obtained from 4 rats were treated with the orexin antibody and those from another two animals were treated with preimmune control IgG (diluted 1: 1,000) or the orexin antibody (diluted 1: 1000) preincubated for 1 hr with excess amount of synthetic orexin-A (10 μg/ml).

Feeding study

Each rat was transferred to a individual metabolic cage one day before the experimental procedures and kept isolated throughout the experiments. This experiment was performed in 36 animals deprived of food for 24 h but with free access to water.

To determine whether an endogenous tone of orexin signaling exists in the rat hypothalamus, we investigated whether immunoneutralization of endogenous orexins in the brain affects feeding behavior. Twenty four-hr-fasted rats received intracisternal injection of anti-orexin antibody (45 µg) or control antibody (45 µg) in a volume of 10 µl at 14:00 - 15:00. Intracisternal injection was performed under brief ether anesthesia with a 10-µL-Hamilton microsyringe after rats were mounted in a stereotaxic apparatus (David Kopf Instruments, Tijunga, CA) as described previously (10). Immediately after the intracisternal injection, rats were returned to their cages with the animals regaining the righting reflex 1-3 min after the injection, and pre-weighted rat chow was given to each rat and the weight of uneaten food was measured every 1 hour for first 8 hours and 24 hours after intracisternal injection. Next, we examined the dose-response effects of anti-orexin antibody on feeding behavior. Rats received intracisternal administration of control antibody or several doses (1.5, 4.5, 15 or 45 µg/10 µl) of anti-orexin antibody and the food intake was monitored. To exclude the possibility that centrally administered orexin-antibody leaked to periphery and thereby suppressed food intake through acting peripheral tissue, we administered orexin-antibody into peritoneal cavity and measured the food consumption.

Statistical analysis

The results are expressed as mean ± SEM. Group comparisons were performed by repeated measures ANOVA and subsequent Fisher's LSD Tests. $P < 0.05$ was considered statistically significant.

Ethical considerations

Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the Public Health Service. The approval of the Research and Development and Animal Care committees at the Asahikawa Medical College was obtained for all studies.

**Results**

Western blot analysis

Figure 1 illustrates the representative result of Western blot analysis. The orexin antibody detected synthetic orexin-A but not orexin-B molecule at the estimated molecular weight. In contrast, neither control IgG purified from pre-immune serum nor orexin antibody preabsorbed with excess amount of synthetic orexin-A detected orexin-A (data not shown).

Orexin immunoreactive neurons

Orexin positive neurons were observed in the lateral hypothalamic area and the perifornical nucleus (Fig. 2A). As shown in Fig. 2B, orexin containing neurons were a variety of shapes with the diameter of 20 to 30 μm. We could not find any orexin positive neurons in other brain sites in the telencephalon, the diencephalon and the hindbrain. Distribution of orexin containing neurons observed in this study was very similar to the previous observations (6, 7, 11, 12), suggesting strongly that the

immunoreactivity indeed indicates cellular orexin. No immunoreactive neurons in the hypothalamus were detected when control antibody or orexin antibody pre-absorbed with excessive orexin-A (data not shown).

Orexin immunoreactive fibers

Apparent positive fibers were present in the frontparietal cortex, central and medial amygdaloid nuclei and the hippocampus. A large amount of positive fibers were seen in the arcuate nucleus (Fig 3A), the periventricular hypothalamic nucleus and the paraventricular nucleus of the hypothalamus (Fig 3B). It was also observed that orexin-rich immunoreactive fibers existed in the lateral and dorsal hypothalamic area. In the thalamus, apparent positive fibers were seen in the midline structures including the reuniens thalamic nucleus, the rhomboid thalamic nucleus, the paraventricular thalamic nucleus (Fig 3C), the medial habenular nucleus and the stria medullaris thalamus. Among these thalamic areas, the paraventricular nucleus of the thalamus contained a large number of positive fibers. Orexin-rich positive fibers were seen in the central gray in the midbrain (Fig. 3D), the locus coeruleus (Fig. 3E) and raphe nuclei including the dorsal raphe, the raphe pontis, the raphe magnus, the raphe pallidus and the raphe obscurus nucleus. Orexin-immunoreactive fibers were also identified in reticular formation in the pons and the medulla, the nucleus ambiguus and the dorsal vagal complex (Fig 3F).

Feeding

Figure 4 illustrates the effect of intracisternal injection of anti-orexin antibody on food intake in 24-hr-fasted rats. Intracisternal administration of anti-orexin antibody at a dose of 45 µg/10 µl potently inhibited food consumption when compared with rats treated with control antibody. The anorectic action of anti-orexin antibody was observed within 1 hour after the injection. During the first 8 hours, food intake was 1.02 ± 0.73 g (mean ± SEM, $p < 0.001$) in rats injected with 45 µg dose of anti-orexin antibody while food consumption was 7.07 ± 0.66 g in control IgG-treated rats. Food intake 24 hr after intracisternal injection of anti-orexin antibody or control antibody was 14.10 ± 2.12 g or 8.07 ± 2.21 g, respectively. During 8-24 hr after the injection, food consumption was approximately 7 g in both groups, suggesting that the anorectic effect of anti-orexin-antibody was diminished 8 hours after the central injection.

We next examined the dose-response effects of anti-orexin antibody on food intake. As shown in Figure 5, intracisternal administration of 1.5 µg dose of anti-orexin antibody did not induce significant inhibition of food consumption for 8 hours in 24-hour fasted rats. However, higher doses (4.5, 15 and 45 µg/10 µl) of anti-orexin antibody significantly suppressed food intake in a dose-dependent manner. As illustrated in Figure 6, intraperitoneal injection of orexin antibody at a dose of 45 µg failed to inhibit feeding.

**Discussion**

In situ hybridization histochemistry revealed that prepro-orexin mRNA expression is found only in the lateral hypothalamic nucleus and its adjacent nuclei (6, 11). Orexin immunoreactive neurons stained with

specific orexin antibody are restricted in the same area of the hypothalamus (7, 11, 12). These results indicated that orexin neurons are highly localized in the lateral hypothalamic area and its adjacent nuclei. The orexin polyclonal antibody used in this study is specific for orexins because immunohistochemical findings of distributions of neurons and fibers were very similar to the previous observations with other specific orexin antibodies (7, 11, 12). We therefore used this antibody in the next experiment to immunoneutralize endogenous orexins in the brain.

Sakurai et al. reported first the novel neuropeptides, orexins (6). The initial report demonstrated the neuropeptides may be involved in the central regulation of feeding because intracerebroventricular injection of synthetic orexins increased rat food consumption. Edwards et al. confirmed that intracerebroventricular administration of orexin-A consistently stimulated food intake in rats, although the appetite-stimulating effect of orexin-A was weaker than that of neuropeptide Y and similar or smaller than that of melanin-concentrating hormone (13). In addition to feeding behavior, we have very recently demonstarted that intracisternal injection of synthetic orexin-A dose-dependently stimulated gastric acid secretion in conscious rats (14). This stimulation was observed after intracisternal administration of orexin-A but not orexin-B. It was furthermore shown that atropine and vagotomy completely blocked the acid stimulation by orexin-A. From these results, we suggested that orexin-A may act in the brain to stimulate acid secretion through modulating the vagal tone. The acid stimulation coupled to the orexigenic action of orexin-A strongly suggest that orexin-A may play a vital role as a central mediator in cephalic phase stimulation as characterized by Pavlov (15). In addition, Ida et al. have shown increases in activity following

intracerebroventricular injection of the orexins (16) and Lubkin and Stricker-Krongrad have demonstrated that intracerebroventricular administration of orexin-A increased the metabolic rate (17). Although the above pharmacological findings suggest that orexins may act as a neurotransmitter to regulate a variety of functions, we do not know whether endogenous orexins in the brain indeed are implicated in functions such as feeding behavior. The major present finding is the dose-dependent inhibition of food intake by centrally administered orexin antibody in 24-hr-fasted rats. Intraperitoneal administration of orexin antibody failed to suppress food consumption, indicating that orexin antibody acted in the central nervous system but not in the peripheral tissue. This evidence strongly indicates that immunoneutralization of endogenous brain orexins decreased food consumption. In other words, endogenous brain orexins indeed plays a key role in the stimulation of feeding in fasted condition. All these results suggest for the first time that orexins in the brain may have a physiological relevance in the central regulation of feeding behavior.

With regard to a role of orexin-B in feeding, the initial report by Sakurai et al. demonstrated that orexin-B as well as orexin-A stimulated rat feeding when injected intracerevroventriculary (6). In contrast, Edwards et al. showed that orexin-A consistently stimulated food intake, but orexin-B only on occasions in rats (13). A lack of stimulation of feeding in rats by orexin-B was also demonstrated by Haynes et al. (18). Thus whether or not orexin-B has a stimulatory effect on feeding behavior is still controversial. The present study demonstrated with the orexin antibody, that highly detected orexin-A but not orexin-B as shown by western blot analysis, potently inhibited food intake in fasted rats. This evidence might

suggest that the orexigenic action of orexins may largely depend upon the action of endogenous orexin-A rather than orexin-B.

Very recently, two independent groups demonstrated that canine narcolepsy is caused by a mutation in the orexin receptor gene (19) and that orexin knockout mice exhibit a phenotype strikingly similar to narcolepsy (20). These results strongly suggest a pathophysiological relevance of orexin in narcolepsy. A further study should be needed to examine a role of orexins in the relationship between the regulation of feeding behavior and the pathophysiology of narcolepsy.

The sites of action of orexins in the brain to initiate feeding remains to be clarified. Immunohistochemical studies including the present findings showed that orexin fibers have been identified in nuclei that are known to be involved in the central regulation of feeding such as the ventromedial hypothalamic nucleus, the arcuate nucleus, the paraventricular nucleus of the hypothalamus and the nucleus of the solitary tract (21-24). These neuroanatomical findings suggest that one or some of the identified nuclei in the brain may be the sites of action of orexin to stimulate feeding process. In addition to the evidence on the distribution of orexin fibers in the brain, Trivedi et al. (25) have demonstrated using *in situ* hybridization that orexin receptor mRNA is expressed in the ventromedial hypothalamus and the paraventricular nucleus of the hypothalamus. This result may furthermore support the speculation that orexins may act in the hypothalamic nuclei such as the ventromedial hypothalamus or the paraventricular nucleus of the hypothalamus to exert its orexigenic action. Based upon these evidence, we speculate that orexin antibody administered into the cerebrospinal fluid immunoneutralized the orexin action in the

feeding related brain regions such as the ventromedial hypothalamus and the paraventricular nucleus of the hypothalamus.

In summary, the present study provides evidence that endogenous brain orexins have a physiologically relevant action on feeding behavior.

**Acknowledgments**

This work was supported in part by grants-in-aid from the Ministry of Education, Science, and Culture of Japan.

**References**


1. Oomura, Y. (1980) In Handbook of the Hypothalamus (Morgane, P.J., and Panksepp, J., Eds.), pp 557-620, New York.

2. Woods, S.C., Seeley, R.J., Porte, D., and Schwartz, M.W. (1998) Science **280**, 1378-1383.

3. Luiten, P.G.M., ter Horst, G.J., and Steffens, A.B. (1987) Prog. Neurobiol. **28**, 1-54.

4. Inui, A. (1999) Trend. Neurosci. **22**, 62-67.

5. Flier, J.S., and Maratos-Flier, E. (1998) Cell **92**, 437-440.

6. Sakurai, T., Amemiya, A., Ishii, M., Matsuzaki, I., Chemelli, R.M., Tanaka, H., Williams, S.C., Richardson, J.A., Kozlowski, G.P., Wilson, S., Arch, J.R.S., Buckingham, R.E., Haynes, A.C., Carr, S.A., Annan, R.S., McNulty, D.E., Liu W-S,Terrett, J.A., Elshourbagy, N.A., Bergsma, D.K., and Yanagisawa, M. (1998) Cell **92**, 573-585.

7. De Lecea, L., Kilduff, T.S., Peyron, C., Gao, X-B., Foye, P.E., Danielson, P.E., Fukuhara, C., Battenberg, E.L.F., Gautvik, V.T., Bartlett II, F.S., Frankel, W.N., Van den Pol, A.N., Bloom, F.E., Gautvik, K.M., and Sutcliffe, J.G. (1998) Proc. Natl. Acad. Sci. USA. **95**, 322-327.

8. Heide K, Schwick HG (1978) In Handbook of Experimental Immunology (Weir DM, Eds.) Oxford.

9. Kobayashi, Y., Matsuyama, K., and Mori, S. (1994) Neurosci. Res. **20**, 43-55.

10. Okumura, T., Fukagawa, K., Tso, P., Taylor, I.L., and Pappas, T.N. (1994) Gastroenterology **107**, 1861-1864.

11. Peyron, C., Tighe, D.K., van den Pol, A.N., de Lecea, L., Heller, H.C., Sutcliffe, J.G., and Kilduff, T.S. (1998) J. Neurosci. **18**, 9996-10015.

12. Date, Y., Ueta, Y., Yamashita, H., Yamaguchi, H., Matsukura, S., Kangawa, K., Sakurai, T., Yanagisawa, M., Nakazato, M. (1999) Proc. Natl. Acad. Sci. USA. **96**, 748-753.

13. Edwards, C.M.B., Abusnana, S., Sunter, D., Murphy, K.G., Ghatei, M.A., and Bloom, S.R. (1999) J. Endocrinol. **160**, R7-R12.

14. Takahashi, N., Okumura, T., Yamada, H., and Kohgo, Y. (1999) Biochem. Biophys. Res. Commun. **254**, 623-627.

15. Pavlov, I. (1910) The work of the Digestive Glands. London: Griffin

16. Ida, T., Nakahara, K., Katayama, T., Murakami, N., and Nakazato M. (1999) Brain Res. **821**, 526-529.

17. Lubkin, M., and Stricker-Krongrad, A. (1998) Biochem. Biophys. Res. Commun. **253**, 241-245.

18. Haynes, A.C., Jackson, B., Overend, P., Buckingham, R.E., Wilson, S., Tadayyon, M., and Arch, J.R. (1999) Peptides **20**, 1099-1105.

19. Lin, L., Faraco, J., Li, R., Kodotani, H., Rogers, W., Lin, X., Qiu, X., de Jong, P.J., Nishino, S., and Mignot, E. (1999) Cell **98**, 365-376.

20. Chemelli, R.M., Willie, J., Sinton, C.M., Elmquist, J.K., Scammell, T., Lee, C., Richardson, J.A., Williams, S.C., Xiong, Y., Kisanuki, Y., Fitch, T.E., Nakazato, M., Hammer, R., Saper, C.B., Yanagisawa, M. (1999) Cell **98**, 437-451.

21. Leibowitz, S.F., and Brown, L.L. (1980) Brain Res. **201**, 289-314.

22. Stanley, B.G., and Leibowitz, S.F. (1984) Life. Sci. **35**, 2635-2642.

23. Akabayashi, A., Wahlestedt, C., Alexander, J.T., and Leibowitz, S.F. (1994) Mol. Brain Res. **21**, 55-61.

24. Shimura, T., Norgren, R., Grigson, P.S., and Norgren, R. (1997) Behav. Neurosci. **111**, 155-168.

25. Trivedi, P., Yu, H., MacNeil, D.J., Van der Ploeg, L.H.T., and Guan, X-M. (1998) FEBS Lett. **438**, 71-75.

**Figure legends**

**Figure 1**

Representative Western blot analysis that shows the detection of orexin-A by an anti-orexin antibody. Solution of 1 µg of synthetic orexin-A (lane 1) or rat orexin-B (lane 2) in a volume of 20 µl was applied on Tricine gel, probed with an anti-orexin antibody and visualized with enhanced chemiluminescence.

**Figure 2**

Orexin-A immunoreactive neurons in the lateral hypothalamic area. Numerous orexin-A positive cells are seen in the lateral hypothalamus and its adjacent area (Figure 1A). Figure 1B shows higher magnification of area around the fornix in Figure 1A. Each bar indicates 200 µm (Figure 1A) and 100 µm (Figure 1B) respectively.

**Figure 3**

Representative orexin-immunoreactive nerve terminals in the brain. Each microphotograph shows orexin-immunoreactive fibers in the arcuate nucleus of the hypothalamus (A), the paraventricular nucleus of the hypothalamus (B), the paraventricular nucleus of the thalamus (C), the central gray (D), the locus coeruleus (E) and the dorsal vagal complex (F). Abbreviations: Third ventricle; 3V, aqueduct; Aq, forth ventricle; 4V and area postrema; AP.

**Figure 4**

Effects of intracisternal injection of orexin-antibody in 24-hr-fasted rats. Rats received intracisternal administration of control (45 µg/10 µl) or orexin IgG (45 µg/10 µl) under brief ether anesthesia. Food consumption was measured every 1 hr after central injection. Each point represents the mean ± SEM of 4 animals. * $p < 0.01$, when compared with control IgG at the corresponding time point.

**Figure 5**

Dose response effects of central orexin-IgG on feeding in 24 hr-fasted rats. Rats received intracisternal injection of orexin-IgG at a dose of 0, 1.5, 4.5, 15 or 45 µg, and food intake was measured 8 hr after the central injection. Each column represents the mean ± SEM of 4 animals. * $p < 0.01$ when compared with 0 µg dose of orexin antibody.

**Figure 6**

Effects of intraperitoneal injection of orexin-antibody in 24-hr-fasted rats. Rats received intraperitoneal administration of control (45 µg/10 µl) or orexin IgG (45 µg/10 µl) under brief ether anesthesia. Food consumption was measured every 1 hr after central injection. Each point represents the mean ± SEM of 4 animals.

Figure 1

Figure 2

Figure 3

Figure 4

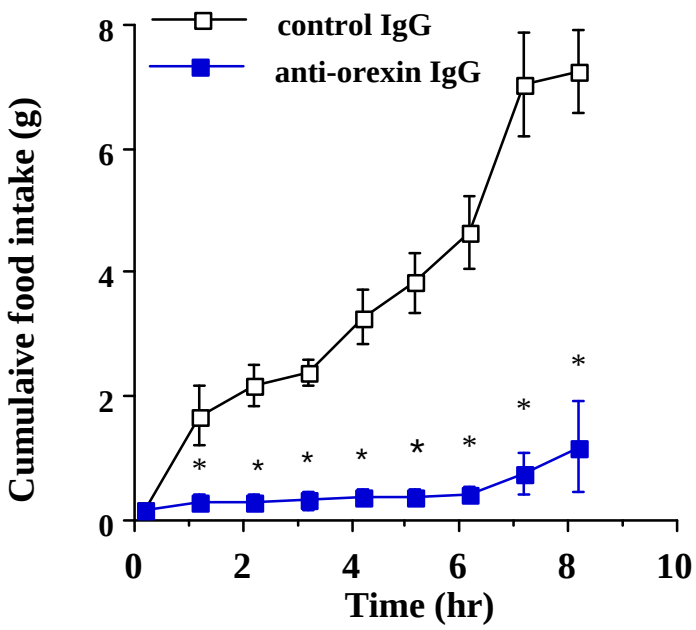

control IgG
anti-orexin IgG
Cumulaive food intake (g)
Time (hr)
0
2
4
6
8
10
*

Figure 5

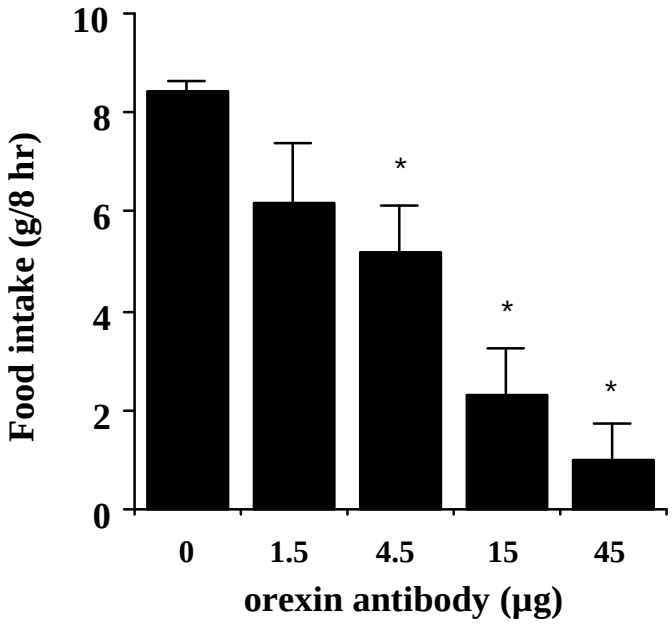
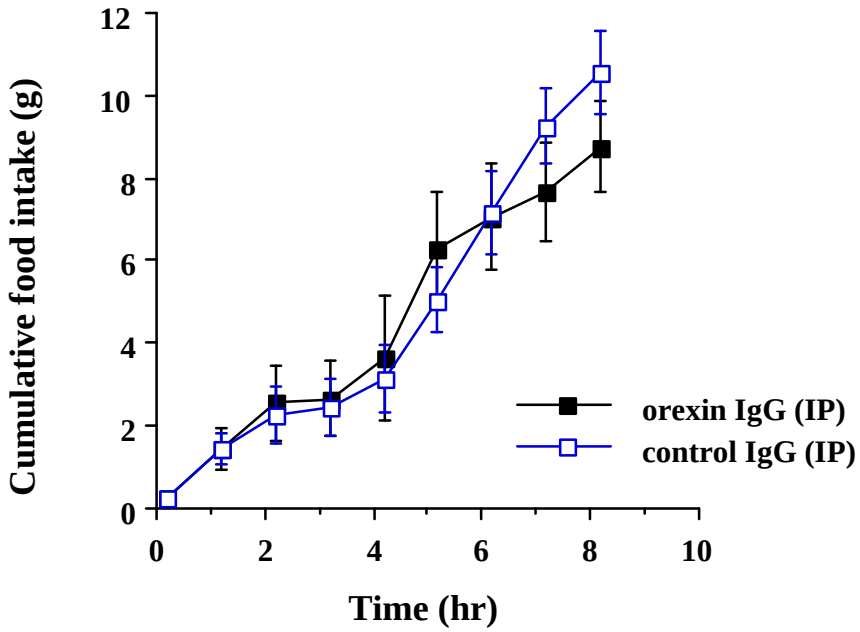


Figure 6


Cumulative food intake (g)
12
10
8
6
4
2
0
0
2
4
6
8
10
Time (hr)
orexin IgG (IP)
control IgG (IP)