Research report

Stimulation of feeding behavior and food consumption in the goldfish, *Carassius auratus*, by orexin-A and orexin-B

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**Abstract**

The neuropeptides, orexin-A and orexin-B, have been demonstrated to have a physiological role in the regulation of food intake in mammals. The effects of human orexin-A and orexin-B intracerebroventricular (i.c.v.) injection on the feeding behavior of goldfish (*Carassius auratus*) were investigated. I.c.v. injection of orexin-A and orexin-B both caused a significant increase in appetite, as indicated by an increased number of feeding acts. Orexin-A and orexin-B both significantly stimulated food consumption, as indicated by increased total food intake during a 60-min observation period; the actions of orexin-A were dose dependent. Orexin-A was more potent than orexin-B in stimulation of both feeding behavior and food intake. These results indicate that orexin peptides are involved in the hypothalamic regulatory pathways of feeding behavior in goldfish. © 1999 Published by Elsevier Science B.V. All rights reserved.

**Keywords:** Orexin; Feeding behavior; Goldfish

1. **Introduction**

Orexin-A and orexin-B (also known as hypocretin-I and hypocretin-II) are hypothalamic neuropeptides, which have recently been discovered and characterized by de Lecea et al. [3] and Sakurai et al. [23]. The amino acid sequences of purified orexin-A and orexin-B exhibit no significant similarities to any known families of regulatory peptides [23]. Orexin-A is a 33-residue peptide and orexin-B has 28 residues and both peptides are encoded by a single mRNA transcript found abundantly in the lateral hypothalamus [23]. These peptide ligands (orexin-A and orexin-B) bind to two closely related orphan G protein-coupled cell surface receptors (OX₁R and OX₂R) localized exclusively in the brain. OX₂R is a non-selective receptor for both orexins while OX₁R is selective for orexin-A [23].

The hypothalamus plays a fundamental role in the regulation of feeding behavior and the control of energy homeostasis. Several hypothalamic sites, such as the ventromedial nucleus, the dorsomedial nucleus, the paraventricular nucleus and the lateral hypothalamic area are associated with this regulation [5,21]. In rodents, orexin-containing neuronal perikarya are localized within and around the lateral hypothalamus, and in the dorsomedial hypothalamus and the perifornical hypothalamic, suggesting a physiological role for orexins in the control of feeding behavior [2,3,20,23]. Both orexin-A and orexin-B stimulate feeding when injected into the lateral cerebral ventricle of the rat [23,24]. Hypothalamic prepro-orexin mRNA levels are up-regulated upon fasting [18,23]. Orexin neurons are stimulated under hypoglycemic conditions [9,19], further indicating involvement in regulation of feeding. A large number of orexin axons are in direct synaptic contact with cells that secrete neuropeptide Y (NPY), the most powerful orexigenic peptide known, suggesting a possible regulation of the NPY system by orexins [13].

In goldfish, there is limited information on the regulatory pathways involved in feeding behavior. However, a few peptides have been shown to influence goldfish feeding behavior, such as NPY (R. Narnaware, personal communication) and galanin [6] which stimulate food intake, and bombesin [11], cholecystokinin [12], serotonin [5], and corticotrophin-releasing factor [4] which inhibit food intake. This is the first study to examine the effects of orexins on the feeding behavior in fish. To explore the regulatory pathways involved in feeding, orexin-A and orexin-B were injected into the brain third ventricle and its effects on feeding behavior and food consumption in goldfish were examined.

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2. Materials and methods

2.1. Experimental animals

Male and female goldfish ranging from 30 to 55 g in weight were purchased from Ozark Fisheries, Stoutland, MO. Fish were kept under a simulated photoperiod of 16L:8D in 65-l tanks which received a constant flow of aerated water at 18°C. Tanks contained a gravel substrate and artificial floating vegetation. The sides of the tanks were opaque to minimize external disturbances. Fish were fed a 2% wet body weight (bw) ration once a day (1400 h), with commercially prepared trout pellets (Trout production 3/32 or 5/32; Rangen, protein 40%, fat 12%, ash 15%, mineral 2%). Fish fed a 2% bw ration exhibit optimal maintenance of growth over 5 weeks of feeding [10]. Fish were acclimated under these standard conditions for approximately 2 weeks before the start of an experiment. One to three days prior to experimentation, two fish were moved into an observation tank. According to Himick [10], cumulative food intake levels remain similar between males and females in either gonadal recrudescent (September–

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**Fig. 1.** Effects of i.c.v. injection of saline (open bar), orexin-A (OXA, black bars), and orexin-B (OXB, shaded bars) on the total number of feeding acts (A) and on the percentage of incomplete feeding acts (B) in goldfish. Fish were untreated (n = 16) or i.c.v. injected with either saline (n = 16), orexin-B (1 ng/g, n = 8; 10 ng/g, n = 8; 100 ng/g, n = 8) or orexin-A (1 ng/g, n = 8; 10 ng/g, n = 10; 100 ng/g, n = 8). Fish received food 15 min post-i.c.v. injection, and were observed for 1 h. Total number of feeding acts includes pellets eaten, pellets engulfed and then spat out, and pellets bumped with a closed mouth. Incomplete feeding acts include pellets engulfed and then spat out, and pellets bumped with a closed mouth. Data are mean ± S.E.M. Letters not joined by underlining indicate groups that differ significantly.
April) or mature (April–May) seasonal stages of reproduction. All females used in the experiments were sexually recrudescent, with a gonadosomatic index (GSI = weight of gonad/total body weight × 100) between 2 and 8; males were recrudescent or mature. Mature male goldfish were identified by the presence of tubercles on the pectoral fins or opercula. Males and females were randomly assigned to treatment groups.

2.2. Intracerebroventricular (i.c.v.) injections

Brain i.c.v. injections were administered following procedures described by Peter and Gill [22]. Briefly, following deep anesthesia, a three-sided flap was cut in the roof of the skull using a dentist drill equipped with a circular saw. The flap was then folded to the side, exposing the brain. Fish were then placed in a specially designed stereotaxic apparatus. The needle of a 5-μl microsyringe was stereotaxically placed in the preoptic region of the brain third ventricle according to coordinates (+1.0, M, D 1.2) taken from the stereotaxic atlas of the goldfish brain [22]. Following injection of 2 μl of test solution, the needle was withdrawn and the space in the cranial cavity filled with teleost physiological saline [1]. The skull flap was put back in place, and secured by surgical thread. Fish were then returned to their tanks, and normally recovered from anesthesia within 2 to 5 min.

2.3. Observational experiments

Fish were tested in random order in terms of treatment and days. For each experiment, two fish were placed in a single observation tank to allow for an accurate observation of feeding behavior and food consumption. An approximate 4% bw ration of pellets per fish was administered at 15-min post-injection. Behavioral observations and measurement of food intake commenced upon entry of pellets into the tank, and ended 1 h later. Observations were divided in 15-min periods. A “feeding act” was defined as being either complete or incomplete. A complete feeding act was when the fish approached a pellet and then consumed it. An incomplete feeding act was when the fish approached a pellet and either engulfed it and then “spat” it out, or “bumped” it with a closed
mouth. The total number of feeding acts (TFA) was calculated by adding the "complete" to the "incomplete" feeding acts. Pellets were either floating or sinking. Occasionally, fish mouthed, picked up and spat gravel. Those acts were not accounted for as a feeding act. Food consumption was converted to milligrams of food consumed/wet body weight/hour feeding based on the mean pellet weight fed to fish.

As a control, a number of fish was tested in the absence of i.c.v. injections.

2.4. Reagents

Human orexin-A (Glp-Pro-Leu-Pro-Asp-Cys-Cys-Arg-Gln-Lys-Thr-Cys-Ser-Cys-Arg-Leu-Tyr-Glu-Leu-Leu-HisGly-Ala-Gly-Asn-His-Ala-Ala-Gly-Ile-Leu-Thr-Leu-NH₂) and orexin-B (Arg-Ser-Gly-Pro-Pro-Gly-Leu-Gln-Gly-Arg-Leu-Gln-Arg-Leu-Leu-Gln-Ala-Ser-Gly-Asn-His-Ala-Ala-Gly-Ile-Leu-Thr-Met-NH₂) were purchased from American Peptide, Sunnyvale, CA. A stock solution of orexin was made, aliquoted, and stored at -20°C. Aliquots were subsequently thawed and diluted in fish physiological saline [1]. Dosages of orexin-A and orexin-B were 1, 10 and 100 ng/g body weight.

2.5. Statistics

Statistical analyses were conducted using ANOVA followed by pairwise Student–Newman–Keuls multiple comparison test. Significance was considered at p < 0.05. All error bars indicate standard error of means (S.E.M.).

3. Results

3.1. Effects of human orexin-A and orexin-B i.c.v. injection on goldfish feeding behavior

There was no significant difference in total number of feeding acts between control untreated fish and saline-injected fish (Fig. 1A). All three treatment doses of both orexin-A and orexin-B caused a significant increase in the total number of feeding acts. There were no significant differences between the three treatment groups for orexin-B. The stimulation of feeding acts by orexin-A was significantly lower at 100 ng/g compared to the two other dosages of peptide tested. The number of feeding acts stimulated by orexin-A was significantly greater than the number stimulated by orexin-B at 1 and 10 ng/g but not at 100 ng/g.

There was no significant difference in the number of incomplete acts between control untreated fish and saline-injected fish (Fig. 1B). All three doses of orexin-A and orexin-B caused a significant increase in the percent incomplete feeding acts. There were no significant differences in the number of incomplete feeding acts between the three treatment groups for orexin-A or orexin-B, and there were no significant differences between the two orexins at any dose.

3.2. Effects of human orexin-A and orexin-B i.c.v. injection on food intake in goldfish

There was no significant difference in the food intake between control untreated fish and saline-injected fish (Fig. 2). At doses of 1 and 10 ng/g of fish, orexin-A caused significant increases in the number of pellets consumed during the 60-min observation period (Fig. 2). Orexin-B induced a significant increase in food intake only at 10 ng/g. There was no significant stimulation of food intake at 100 ng/g for either orexin-A or orexin-B compared to the saline group. There were no significant differences between the three treatment groups of orexin-B. However, orexin-A showed dose-related differences, with the 10 ng/g orexin-A having a greater effect than 1 ng/g orexin-A. At doses of 10 ng/g, the increases in food intake caused by orexin-A were significantly greater than that caused by orexin-B.

Fig. 3 summarizes the cumulative food intake per 15-min intervals for the 60-min observation period. The saline...
control group consumed a constant amount of food in each 15-min time interval. The fish treated with 1 and 10 ng/g orexin-A had significantly greater food intake in all time periods compared to the saline control group. Food intake was similar to saline controls in all time intervals for fish treated with 100 ng/g orexin-A. All three groups of orexin-B treated fish had food intake similar to saline controls 15- and 30-min post-injection, and the 1 and 100 ng/g orexin-B groups were similar to the saline control group 45- and 60-min post-injection. Of the fish treated with orexin-B, only the 10 ng/g orexin-B group had significantly higher food intake 45- and 60-min post-injection compared to the saline controls. Notably, the 100 ng/g orexin-B group had a dramatic decrease in feeding during the third (30–45 min) and fourth (45–60 min) time intervals.

4. Discussion

The injection of both human orexin-A and orexin-B into the brain third ventricle of goldfish stimulated both appetite, as indicated by number of feeding acts, and food consumption, as indicated by number of food pellets consumed. This suggests a role for the orexin peptides in the regulation of feeding behavior in goldfish, similar to that previously described in the rat [8,23]. This also suggests the presence of an orexin-like peptide and orexin receptors in the goldfish brain.

To date, there is limited information on the effect of orexins on food intake. All data available concern mice, rats and pigs. In mice, i.c.v. injected orexin-B has no effect on food intake whereas orexin-A is a poor stimulator of feeding [16,17]. In rats, orexins have been reported to stimulate food consumption in a dose-dependent fashion when injected i.c.v. [8,23]. There is, however, one report of failure of orexin to significantly increase food intake in rats, but having occasional stimulating effects [14]. In pigs, intramuscular injections of orexin-B increase cumulative food intake from 12 to 24 h post-injection when compared with the control animals [7]. In the present study, high dosages of both orexin-A and orexin-B suppressed food intake to levels similar to controls, compared to the increased food intake stimulated by lower dosages.

In rats, orexin-A increases food intake when injected in the lateral hypothalamus, the perifornical hypothalamus [24] and in the paraventricular nucleus, but not to the same extent as i.c.v. injection [8]. Orexin-B stimulates feeding only when injected i.c.v., and has no effect when injected in other brain regions [24], suggesting different sites of action for the two orexins in the brain. Direct visual and histological observations following i.c.v. injection of India ink in goldfish demonstrate that any compound administered in this manner is rapidly distributed throughout the entire brain ventricular system (unpublished observations), thereby broadly exposing the brain to the injected compound. In the present studies on goldfish, orexin-A and orexin-B were found to stimulate both appetite and food intake, although orexin-A was more potent at the lower dosages tested. Orexin-A not only elicited an increase in food intake at lower i.c.v. dosages than orexin-B, but also had a greater stimulatory effect than that of orexin-B at equal dosages. In rats, orexin-A is thought to be more effective than orexin-B; Sakurai et al. [23] report that both orexins increase food intake in a similar fashion, but that the stimulatory effect of orexin-A is longer-lasting than that of orexin-B. Edwards et al. [8] reported that i.c.v. injected orexin-A tended to stimulate food intake more than orexin-B, although this was not significantly different in their study. The longer-lasting action of orexin-A might be attributed to the molecular structure of orexin-A, which renders it more resistant to inactivating peptidases [23].

The higher potency of orexin-A might also be due to the fact that orexin-A binds to both orexin receptors (OX1-R and OX2-R) while orexin-B binds solely to OX1-R [15].

In rats, orexin-A and orexin-B are less effective than NPY in stimulating food intake, but longer-lasting, in the case of orexin-A [23]. Orexins have an orexigenic effect similar to melanin-concentrating hormone (MCH) and galanin [8]. Higher i.c.v. injection dosages of orexin than NPY are needed to elicit significant stimulation of feeding [8,15]. This also appears to be true in goldfish, as galanin i.c.v. injection has been shown to cause a 1.5-fold increase in food intake in goldfish [6] which is similar to the range of increase we report (1.5-fold increase in the 60-min food intake for orexin-B and 2.4-fold for orexin-A). Preliminary studies in our laboratory on the effect of NPY on goldfish feeding behavior also show that a stimulation in food intake equivalent to or higher than the maximal response seen for orexin-A at 10 ng/g is reached for doses of NPY of 2–5 ng/g (Narmaware, personal communication).

Our results suggest that orexins also play a role in regulating goldfish feeding behavior in addition to food intake, as indicated by the increase in the number of total feeding acts. Similar significant increases in feeding and searching behavior have been reported in rats following i.c.v. injection of NPY and orexins [14]. The acute stimulation of appetite and feeding behavior by orexins in goldfish is confirmed by the significant increase in the percentage of incomplete feeding acts. It appears that orexin-treated fish actively searched for food and attempted to consume more pellets than they could ingest. Some fish spat out little flakes of partially digested pellets, indicating that the fish was likely full.

There was little distinctive in the time course response to orexin-A and orexin-B in goldfish. Food intake increased within 15-min post-i.c.v. injection, and decreased thereafter. However, the high dosage of orexin-B caused a pronounced decrease in food intake at 30- and 45-min post-i.c.v. injection, whereas the fish treated with a similar dosage of orexin-A continued to have a similar level of feeding acts as saline controls. On the other hand, the high...
dosage of orexin-A caused a significantly lower number of feeding acts and lower food intake compared to the lower dosages tested, whereas there was no difference between the response to the three dosages of orexin-B in terms of total number of feeding acts and total food intake. The overall decrease in feeding activities might be indicative of an overdose effect and a down regulation of orexin receptors. High doses of orexin (10 nmol) have been reported to cause behavioral seizures in rats [14]. This type of behavior was not seen in our study.

In rodents, orexin-containing neuronal perikarya are localized within and around the lateral hypothalamus, in the dorsomedial hypothalamus and the posterior hypothalamic area, whereas orexin nerve fibers project widely into the cerebral cortex, thalamus, hypothalamus, brainstem [2,3,20,23] and into the spinal cord [25]. The widespread distribution of orexin-immunoreactive neurons, along with their small effect on food intake compared with other orexigenic peptides suggests that orexins may also be involved in the regulation of other behavioral activities in rats, besides feeding. Orexins have been reported to induce vigilance and locomotor behaviors [2] as well as grooming and burrowing [14] in rats. While grooming behavior is difficult to assess in goldfish, actions on locomotor and social behaviors need to be assessed.

To our knowledge, this is the first report on the effects of orexins on the feeding behavior in a fish or any lower vertebrate. I.c.v. injection of both orexin-A and orexin-B were effective in stimulating appetite and food consumption, with orexin-A being more potent. These results suggest a role for orexins in the regulation of feeding behavior and food intake in goldfish.

References
