Regulation of food intake in the goldfish by interaction between ghrelin and orexin

Tohru Miura a, Keisuke Maruyama a, Sei-Ichi Shimakura a, Hiroyuki Kaiya b, Minoru Uchiyama a, Kenji Kangawa b, Seiji Shioda c, Kouhei Matsuda a, *

a Laboratory of Regulatory Biology, Graduate School of Science and Engineering, University of Toyama, 3190-Gofuku, Toyama 930-8555, Japan
b Department of Biochemistry, National Cardiovascular Center Research Institute, Suita 565-8565, Japan
c Department of Anatomy, Showa University School of Medicine, Tokyo 142-8555, Japan

1. Introduction

Many neuropeptides have been implicated in the regulation of feeding [1]. Ghrelin, which was originally isolated from rat and human stomach, is an endogenous ligand for the growth hormone secretagogue-receptor, and possesses an n-octanoic acid modification at the third N-terminal serine residue (Ser3) [2]. Ghrelin is predominantly found in the stomach, but ghrelin-containing neurons have also been demonstrated in the arcuate nucleus of the hypothalamus [3]. Ghrelin is also now recognized as a multifunctional peptide involved in the regulation of somatic growth, feeding behavior and energy homeostasis in mammals [4]. Orexin is a neuropeptide that was first identified as an orphan receptor ligand, and subsequently as an appetite regulator [5]. There are two molecular forms of orexin derived from the same precursor – one a 33-residue peptide known as orexin-A, and the other a 28-residue peptide known as orexin-B. In the mammalian nervous system, neuronal cell bodies containing orexin are found in the lateral hypothalamus, otherwise referred to as the “orexigenic center”, while orexin-containing nerve fibers are distributed throughout a wide area, including the cerebral cortex, hippocampus, limbic system, and brainstem, suggesting that orexin may be involved in many aspects of brain function [5–9]. In mammals, the orexigenic action of ghrelin is mediated through orexin and neuropeptide Y (NPY)/Agouti-related protein [3,10–16].
In goldfish, ghrelin has been identified by cDNA cloning [17,18]. Ghrelin mRNA is mainly expressed in the intestine (given that goldfish lack a stomach), with some expression also reported in the brain [19]. Goldfish ghrelin with an n-octanoic acid modification at Ser3 stimulates the release of growth hormone and gonadotropin from the pituitary gland, as well as food intake and locomotor activity [17,18,20,21]. In a recent study, we demonstrated that, in the goldfish model, the orexigenic action of ghrelin is mediated by NPY, which itself has been implicated as a powerful orexigenic neuropeptide, capable of regulating feeding behavior in both goldfish and rodents [22–24]. The orexin system is also present in teleost fish [25–28], and orexin has been shown to stimulate food intake in goldfish [28,29]. Moreover, previous reports have also provided evidence that orexin-induced appetite is mediated by NPY, and that co-administration of orexin and NPY results in synergistic orexigenic effect [30–33]. However, the feeding-related interaction of the ghrelin and orexin systems has not yet been clarified in the goldfish model. In the present study, we examined the involvement of orexin in the orexigenic action of ghrelin using a selective orexin receptor-1 antagonist, SB334867. The effect of a growth hormone secretagogue-receptor antagonist, [D-Lys3]-GHRP-6, on orexin-induced feeding was also investigated, as was the interaction between ghrelin and orexin mRNA expression.

2. Materials and methods

2.1. Animals

Young goldfish (Carassius auratus, 3–10 g body weight, BW) of both sexes were obtained commercially, and prior to experimental use, were maintained for 2 weeks under controlled light/dark conditions (12 L/12 D) in a temperature-regulated fish tank (20–24 °C). The fish were fed once a day an amount of food corresponding to 1% of their BW at noon with a commercially available granular diet (Tetragold, Tetra GmbH, Melle, Germany), containing 32% protein, 5% dietary fat, 2% dietary fiber, 6% mineral and 8% water. All animal experiments were conducted in accordance with the University of Toyama guidelines for the care and use of animals.

2.2. ICV injection of test solution and evaluation of feeding behavior in goldfish

Details of the methods for evaluating the feeding behavior of goldfish have been reported elsewhere [20,21,23,28]. Each fish was normally fed before the experiment began at noon, then placed in a stereotaxic apparatus under MS-222 (3-aminobenzoic acid ethyl ester, Sigma Chemical Co., St. Louis, MO, USA) anesthesia. The apparatus was referred to the paper described by Peter and Gill [34] and made by ourselves to be fitted for small fish (3–10 g BW). A small part of the parietal bone was carefully removed using a surgical blade (No. 19, Futaba, Tokyo, Japan). One microliter of test solution containing Evans blue dye was then injected into the third ventricle of the brain using a small Hamilton syringe, according to the brain atlas [34], following which the bone gap was filled with a surgical agent (Aron Alpha, Sankyo, Japan). The accuracy of the injection site was confirmed after the experiment by examining the ventricle for the presence of dye. Control fish in each experiment were injected with the same volume of either vehicle solution (dimethyl sulfoxide diluted with saline containing 0.6% NaCl and 0.02% Na2CO3) or saline. Each fish was then individually placed in a small experimental tank (diameter 24 cm) containing 4.0 L of tap water, and supplied with food equivalent to 3% of its BW. The rest of food (uniform granular diet, 5.6 mg/granule) was measured and food intake was calculated every 15 min for an hour following treatment.

2.3. Effect of ICV injection of SB334867 on the orexigenic action of ghrelin

In order to examine whether ghrelin-induced feeding is mediated by the orexin system, a selective orexin receptor-1 antagonist, SB334867 (N-(2-methyl-6-benzoxazolyl)-N’-1,5-naphthyridin-4-yl urea, Tocris Cookson Ltd., Bristol, UK) was used. This was dissolved in dimethyl sulfoxide and stored at a concentration of 50 mM, prior to being diluted in saline when required for experimental use. Ghrelin cDNA generates two putative ghreliins in goldfish: one is a 19-amino-acid peptide while the other contains 12 amino acids, which may be produced by a different processing mechanism [17]. Although the native mature peptide generated from the ghrelin precursor has not yet been identified, two putative peptides with an octanoic acid modification have been examined and demonstrated to have equally potent orexigenic activity [17]. In the present study, we used the 12-amino-acid form of ghrelin having an octanoic acid modification at Ser3 [20,21,23]. This octanoylated goldfish ghrelin was kindly synthesized at the Biopharma Center of Daiichi Asubio Pharmaceuticals Inc. (Gunma, Japan) as previously described [36]. The peptide was dissolved in saline at a concentration of 1.0 mM and then stored at −80 °C until use. In the subsequent experimental protocol, 1 µL of SB334867 at a concentration of 10 or 100 pmol/g BW was injected ICV, these doses having been selected based on previous reports [35,37]. Two or three minutes later, 1 µL of orexin at 2.8 pmol/g BW or ghrelin at 1 pmol/g BW was similarly injected. Food intake was then measured every 15 min for the next 60 min.

2.4. Effect of ICV injection of [D-Lys3]-GHRP-6 on the orexigenic action of orexin-A

In order to examine whether orexin-A-induced feeding is mediated by the ghrelin system, the growth hormone secretagogue-receptor antagonist [D-Lys3]-GHRP-6 (H-HisD-Trp-D-Lys-Trp-D-Phe-Lys-NH2; Bachem AG, Bubendorf, Switzerland) was used. This was dissolved in saline to a concentration of 100 mM and then stored at −80 °C prior to use. Synthetic orexin-A (American Peptide Co., Sunnyvale, CA, USA) was used in this study as we had previously observed no effect of orexin-B on feeding in goldfish [28]. The peptide was dissolved in saline at a concentration of 1.0 mM and then stored at −80 °C. In the subsequent experimental protocol, 1 µL of [D-Lys3]-GHRP-6 at 10 or 100 pmol/g BW was
injected ICV, these doses being determined from previous reports using fish [18,38,39]. Two or three minutes later, the fish were administrated an ICV injection of 1 μL of either ghrelin at 1 pmol/g BW or orexin-A at 2.8 pmol/g BW, these doses being sufficient to stimulate food intake [20,21,23,28,33]. Food intake was then measured every 15 min for the next 60 min.

2.5. Effect of ghrelin on orexin mRNA expression, and of orexin-A on ghrelin mRNA expression in the diencephalon

Each fish that had received an ICV injection of ghrelin (1 pmol/g BW), orexin A (2.8 pmol/g BW) or saline was kept individually in a small experimental tank. One or two hours after treatment, the fish was anesthetized with MS-222, decapitated, and its brain collected. Each diencephalon was dissected, weighed, immediately placed in liquid nitrogen, before being stored at −80 °C until use. Total RNA was extracted from each diencephalon with Isogen (a solution containing phenol and guanidinium thiocyanate; Nippon Gene, Tokyo, Japan). For amplification and quantitation of the cDNA fragments encoding ghrelin, orexin and β-actin, the one-step reverse-transcription polymerase chain reaction (RT-PCR) method (SYBR Green RT-PCR Reagents Kit, Applied Biosystems, Foster City, CA, USA) was used [20,21,23]. Reactions (including 5 μM primers, 2 × SYBR Green PCR master mix, 6.25 U MultiScribe reverse transcriptase, 10 U RNase inhibitor, RNA template and water) were set up in a 96-well reaction plate and placed in a sequence detection system for cycling (ABI Prism 7000, Applied Biosystems). Reverse transcription was carried out at 48 °C for 30 min and the resulting cDNA subsequently amplified using 40 cycles of 95 °C for 15 s followed by 60 °C for 60 s. The PCR products in each cycle were monitored using SYBR Green I fluorescence dye (Applied Biosystems). Gene-specific primers for amplification of the ghrelin cDNA fragment were based on the nucleotide sequence of goldfish ghrelin [17] (GenBank accession number, AF454390). PCR with the sense primer (5'-TTGATGAGTGCCTCCGTTTTC-3') and the antisense primer (5'-GTCGAATTCAAGTGGCGAATC-3') yielded a 124-bp product encoding goldfish preproghrelin cDNA. The genespecific primers were designed and synthesized from the nucleotide sequence of the cDNA fragment amplified by degenerate primers referred to the primary structure of zebrafish and pufferfish preproorexins [26] and the nucleotide sequence of a genomic zebrafish contig (submitted by Clark, 2004; GenBank accession number, BX005093). The PCR with the sense primer (5'-ACTGCCAGCCAAAGAGATTTG-3') and the antisense primer (5'-GTGGCATTAAAGGCGCCGATATGC-3') yielded a 188-bp product encoding part of the goldfish preproorexin cDNA. Goldfish β-actin-specific primers were used as the internal control for PCR amplification (directly submitted by Matsuba and Murakami, 2000; GenBank accession number, AB039726). Using these primers (sense primer, 5'-GCTATGCCAGCCAAAGAGATTTG-3'; antisense primer, 5'-ATGGACCCCAATCCAGAA-3'), a 112-bp product corresponding to a region in the central part of the β-actin cDNA sequence was obtained. The expression levels of orexin and ghrelin mRNA were calculated quantitatively as a ratio of β-actin mRNA expression.

2.6. Data analysis

All results are expressed as the mean ± S.E.M. Statistical analysis was performed using one-way ANOVA with
Bonferroni’s method or unpaired Student’s t-test. Statistical significance was determined at the 5% level.

3. Results

3.1. Effect of ICV injection of SB334867 on the orexigenic action of ghrelin

SB334867 alone at a dose of 100 pmol/g BW induced a significant decrease in food intake during the first 15 and 30 min following injection, but no effects on food intake were evident at 60 min for doses of either 10 or 100 pmol/g BW (Fig. 1A). The orexigenic activity of orexin-A was completely blocked by preinjection of 10 pmol/g BW SB334867, confirming the efficacy of the antagonist (Fig. 1B), which was then shown to have a similar inhibitory effect on the orexigenic activity of ghrelin at the same dose (Fig. 1C).

3.2. Effect of ICV injection of [D-Lys³]-GHRP-6 on the orexigenic action of orexin-A

ICV injection of [D-Lys³]-GHRP-6 alone at doses of 10 or 100 pmol/g BW had no effect on spontaneous food intake (Fig. 2A). However, the orexigenic activity of ghrelin was completely blocked by preinjection of 10 pmol/g BW [D-Lys³]-GHRP-6 (Fig. 2B). Orexin-A-induced feeding was also attenuated by the same dose of [D-Lys³]-GHRP-6 (Fig. 2C).

3.3. Effect of ghrelin on orexin mRNA expression, and of orexin-A on ghrelin mRNA expression in the diencephalon

Fig. 3 shows changes in the expression levels of orexin mRNA (Fig. 3A) and ghrelin mRNA (Fig. 3B) in the diencephalon 1 and 2 h after ICV injection of 1 pmol/g BW ghrelin and 2.8 pmol/g BW orexin-A, respectively. In each case, mRNA expression was increased significantly at 1 h, but not 2 h.

4. Discussion

In the present study, we have demonstrated that ghrelin-induced feeding is inhibited by the selective orexin receptor-1 antagonist, SB334867, while orexin-induced feeding is inhibited by the growth hormone secretagogue-receptor antagonist, [D-Lys³]-GHRP-6. Furthermore, ICV-injected ghrelin and orexin-A stimulate expression of mRNAs for orexin and ghrelin, respectively, in the goldfish diencephalon. Thus our findings provide evidence for a functional interaction between ghrelin and orexin in terms of both feeding regulation and gene expression in the goldfish. When injected ICV, orexin-A and orexin-B had previously been reported to stimulate food intake in rodents [5]. Subsequently, it was also demonstrated that orexin-induced feeding results in the activation of NPY neurons. This is inhibited by pretreatment with the NPY Y1-receptor antagonists, BIBO-3304 and 1229U91, and orexin-containing axonal terminals originating from the lateral hypothalamus being to make direct synaptic contact with NPY neurons in the arcuate nucleus [40–42]. In the goldfish, a similar functional interaction between orexin-A and NPY in the regulation of feeding has also been demonstrated [30,32,33], although orexin-B has not been reported to have any effect on food intake [28].

![Fig. 2](image-url) – Effect of ICV preinjection of the growth hormone secretagogue-receptor antagonist, [D-Lys³]-GHRP-6, on the orexigenic action of orexin-A. Panel (A) shows the effect of [D-Lys³]-GHRP-6 alone on food intake. Panel (B) shows the effect of [D-Lys³]-GHRP-6 on ghrelin-induced feeding. Panel (C) shows the effect of [D-Lys³]-GHRP-6 on the orexigenic action of ICV-injected orexin-A. The results are expressed as the mean ± S.E.M., and the numbers in parentheses in each panel indicate the number of fish in each group. The significance of differences at each time point was evaluated by one-way ANOVA with the Bonferroni method in comparison with the saline-injected group (*p < 0.05, **p < 0.01).
The discovery of ghrelin in rats and humans has revealed a new relationship in the regulation of food intake by orexin. Toshinai et al. [15] reported that, in rats ghrelin-containing axonal terminals make direct synaptic contacts with orexin-producing neurons in the arcuate nucleus and lateral hypothalamus, and that ICV injection of ghrelin induces c-Fos expression in 23% of orexin-containing neurons. Furthermore, ghrelin-induced feeding is attenuated by pretreatment with anti-orexin-A and anti-orexin-B immunoglobulins. These results indicate that centrally administered ghrelin increases feeding through the activation of the orexin system. In the present study, pretreatment with a selective orexin receptor-1 antagonist inhibited ghrelin-induced feeding, suggesting that, in goldfish, ghrelin could be acting through the same pathway as in rodents. This is supported by the fact that orexin mRNA expression in the diencephalon increased in response to ICV injection of ghrelin. In the present study, we also demonstrated that orexin-A-induced feeding is suppressed by pretreatment with [D-Lys³]-GHRP-6. This provides evidence that orexin-induced feeding is mediated by the ghrelin system, a finding supported by the fact that ICV injection of orexin-A stimulates ghrelin mRNA expression. To our knowledge, this is the first demonstration of a functional interaction between ghrelin and orexin in the regulation of food intake.

We summarize the current state of our knowledge regarding the orexigenic interaction of ghrelin, orexin and NPY in the goldfish. Our recent studies have demonstrated that ICV- or intraperitoneal (IP)-injected ghrelin stimulates food intake in this model [20,21]. In rats, ICV- or IP-injected ghrelin induces expression of c-Fos protein in NPY-containing and orexin-containing neurons in the arcuate nucleus [3,15,43,44], suggesting that similar mechanisms involving central and peripheral ghrelin signals acting through central orexin and NPY may be present in goldfish. Previous reports have indicated that ghrelin-induced feeding is mediated by NPY [23], and that orexin and NPY interact with each other in feeding regulation [33]. Given the present results, which provide evidence of an interaction between ghrelin and orexin, it would be of considerable interest to elucidate the anatomical relationship among orexin-, ghrelin- and NPY-containing neurons in the goldfish brain, as well as undertaking a functional analysis of the peripheral and central interaction of these three orexigenic neuropeptides.

Acknowledgments

We are grateful to the Biopharma Center of Daiichi Asubio Pharmaceuticals, Inc. for synthesizing goldfish ghrelin peptides. This work was supported by Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (K.M.), and by research grants from the University of Toyama and Toyama Marine Biotechnology Association (K.M.).

References


