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Stimulatory effect of ghrelin on food intake in bullfrog larvae

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ABSTRACT

Ghrelin is a potent orexigenic peptide implicated in appetite regulation in rodents. However, except for teleost fish, the involvement of ghrelin in the regulation of feeding in non-mammalian vertebrates has not been well studied. Anuran amphibian larvae feed and grow during the pre- and prometamorphic stages, but, thereafter they stop feeding as the metamorphic climax approaches. Therefore, orexigenic factors seem to play important roles in growing larvae. In the present study, we examined the effect of intraperitoneal (IP) or intracerebroventricular (ICV) administration of synthetic bullfrog ghrelin (*n*-octanoylated 28-amino acid form) on food intake in larvae at the prometamorphic stages. Cumulative food intake was significantly increased by IP (8 and 16 pmol/g body weight (BW)) or ICV (0.5 and 1 pmol/g BW) administration of ghrelin during a 15-min observation period. The orexigenic action of ghrelin at 8 pmol/g BW (IP) or at 0.5 pmol/g BW (ICV) was blocked by treatment with a growth hormone secretagogue-receptor antagonist, [D-Lys^3]GHRP-6 at 80 pmol/g BW (IP) or at 5 pmol/g BW (ICV). We then investigated the effect of feeding status on expression levels of the ghrelin transcript in the hypothalamus and gastrointestinal tract. Ghrelin mRNA levels in both were decreased 15 and 60 min after feeding. These results indicate that ghrelin acts as an orexigenic factor in bullfrog larvae.

1. Introduction

Ghrelin was first isolated from rat and human stomachs as an endogenous ligand for the growth hormone secretagogue (GHS)-receptor type-1a (GHS-R1a) [33]. In general, ghrelin is a 28-amino acid peptide with an *n*-octanoylated serine residue at the third N-terminal position. Ghrelin is now considered to be a multifunctional peptide that plays an important role in energy preservation by regulating food intake, body weight and glucose homeostasis in mammals [7,47,54]. Ghrelin has also been isolated and characterized from sub-mammalian vertebrates, and its primary structure has been determined in non-mammalian species including the chicken (*Gallus gallus*) [26], red-eared turtle (*Trachemys scripta elegans*) [23], bullfrog (*Rana catesbeiana*) [18], Japanese eel (*Anguilla japonica*) [21], zebrafish (*Danio rerio*) [1], sea bream (*Acanthopagrus schlegeli*) [57], goldfish (*Carassius auratus*) [40], rainbow trout (*Oncorhynchus mykiss*) [19], tilapia (*Oreochromis mossambicus*) [20], channel catfish (*Ictalurus punctatus*) [25], sharks (*Sphyrna lewini* and *Carcharhinus melanopterus*) [29] and stingray (*Dasyatis akajei*) [16]. In the bullfrog, three molecular forms of ghrelin have been purified and identified from the stomach: *n*-octanoylated and *n*-decanoylated ghrelins with 27- or 28-amino acid-residues [18]. Recently, GHS-R1a has been characterized in this and other anuran species, and it has been indicated that bullfrog ghrelin and a GHS-R agonist, GHRP-6, could interact with frog GHS-R1as in vitro [15,18]. However, there is limited information about the control of feeding by ghrelin in anuran amphibians. In a goldfish model, it has been shown that intraperitoneal (IP) and intracerebroventricular (ICV) injections of goldfish ghrelin with an *n*-octanoic acid modification stimulate food intake as well as the release of GH and gonadotropins from the pituitary gland [40,53].

In the larvae of anuran amphibians, it is known that as metamorphosis progresses, the oral and digestive organs are reconstructed [11], resulting in a decline of feeding behavior [34]. Some previous reports have indicated that corticotropin-releasing factor (CRF) reduces feeding or foraging behavior in anurans such as the African clawed toad (*Xenopus laevis*), the Western spadefoot toad (*Spea hammondii*) and bullfrog [4–6,43]. In addition, our recent study has clearly demonstrated that CRF suppresses food intake in bullfrog larvae, suggesting that CRF acts on the central nervous system to suppress food intake during the period of reconstruction of the digestive system [37]. We have also indicated that neuropeptide Y (NPY) stimulates food consumption in bullfrog larvae during pre- and pro-metamorphosis [48]. Therefore, it is likely that anuran

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larvae can feed and grow until the metamorphic climax, and that NPY acts as an orexigenic factor in appetite regulation during these periods. However, there has been no information about the role of ghrelin in the bullfrog larva. The bullfrog has often been used as an animal model in studies on the function of regulatory peptide [3,17,18,31,36,39,43–46,55]. As yet, however, there is no direct evidence that ghrelin affects food consumption in bullfrog larvae.

The present study was conducted to clarify whether ghrelin enhances food intake in bullfrog larvae at the prometamorphic stages, since the larvae grow and gain fat body mass during these stages [30,55]. In this species, ghrelin mRNA is expressed in the gastrointestinal tracts [18]. In the present study, we examined (1) the effect of IP and ICV administration of synthetic bullfrog ghrelin on food intake in bullfrog larvae, and (2) the effect of feeding status on expression of ghrelin mRNA in the hypothalamus and gastrointestinal tract of the larvae.

2. Materials and methods

2.1. Animals

Prometamorphic bullfrog (*R. catesbeiana*) larvae weighing 5–7 g were collected from ponds in the suburbs of Toyama City, Japan. Two hundred fifty larvae at the prometamorphic stages (XI–XIX) were used. The developmental stages of the larvae were determined according to Taylor and Kollros [49]. The animals were kept for 1–2 weeks under controlled light/dark conditions (12L/12D) with the water temperature maintained at 24–28 °C. The larvae were fed every day at noon with a powder diet (Itosui Co., Tokyo, Japan) until used in experiments. Animal experiments were conducted in accordance with the Invasive Alien Species Act of Japan and the University of Toyama's guidelines for the care and use of alien and laboratory animals.

2.2. Chemicals

Synthetic bullfrog ghrelin with 28 amino acid-residues with *n*-octanoyl nodification, GLT(C8:0)FLSPADMQKIAERQSQNKLRHGNMN, was synthesized at ASBIO Pharma Co. Ltd. (Gunma, Japan) [17,18]. Bullfrog ghrelin was dissolved in 0.6% NaCl and 0.02% Na_2CO_3 (saline) at a concentration of 1.0 mM and then stored at −80 °C until use. In order to examine whether the action of ghrelin is mediated by ghrelin receptor signaling, a growth hormone secretagogue (GHS)-receptor antagonist, [D-Lys^3]GHRP-6 (HDWDKWDFK-NH_2; Bachem AG, Bubendorf, Switzerland), was tested. [D-Lys^3]GHRP-6 was also dissolved in saline at a concentration of 50 mM and then stored at −80 °C until use.

2.3. Measurement of food intake in larvae

Details of the methods used for evaluating food consumption in the larvae have been reported elsewhere [39,48]. Two types of powder diet colored green and red, respectively (containing 32–47% proteins, 4–5% dietary fat, 3% dietary fibers, 14–17% minerals, 10–12% water and other components), were obtained from Itosui Co., Tokyo, Japan. First, the test larvae including stages XI–XIX were fed the green-colored diet and kept under laboratory conditions. Then, after a 24-h fast, an adequate amount of the red-colored food was made available at 3% of BW. After 15 min, each animal was decapitated and the gastrointestinal tract was removed. The weight of the red-colored gastrointestinal contents absorbed intestinal juice by tissue paper was measured, and expressed as mg food taken per g BW during the 15-min period. The experiments were conducted around noon.

2.4. Effect of IP and ICV administration of ghrelin on food intake in bullfrog larvae

Each larva that had been fed the green-colored diet was fasted for 24 h. For IP administration, larvae were injected with 20 μl of 4, 8 or 16 pmol/g BW of bullfrog ghrelin. Larvae in the control group were given injections of the same volume of saline. For ICV administration, each animal was placed in a stereotaxic apparatus under anesthesia with MS-222 (3-aminobenzoic acid ethyl ester, Sigma–Aldrich, St. Louis, MO, USA). A small area (approximately 1 mm^2 square) of the parietal skull was carefully removed using a surgical blade (No. 19, Futaba, Tokyo, Japan), and then 0.1 μl/g BW (0.5–0.7 μl) of bullfrog ghrelin dissolved in saline at a concentration of 0.25, 0.5 or 1 pmol/g BW was injected into the third ventricle of the brain using a 10-μl Hamilton syringe with 0.1-μl scale. The gap in the parietal skull was then filled with a surgical bonding agent (Aron Alpha, Sankyo, Japan). The accuracy of the injection site and volume was confirmed after the experiment by examining whether Evans blue dye was present in the ventricle without leakage. Control larvae in each experiment were injected with the same volume of saline in the same way as for the experimental group. Each larva that had received the IP or ICV injection was placed individually in a small experimental tank (diameter 11 cm) containing 700 ml of tap water. After recovery from anesthesia, each larva was supplied with the red-colored food equivalent to 3% of its BW. After 15 min, the weight of the intestinal contents was measured as described earlier.

2.5. Effect of IP and ICV injection of [D-Lys^3]GHRP-6 on the orexigenic action of ghrelin

In order to examine the effect of IP and ICV injection of [D-Lys^3]GHRP-6, a GHS receptor antagonist, on the peripheral and central actions of ghrelin, 20 μl of [D-Lys^3]GHRP-6 at 80 pmol/g BW in addition to bullfrog ghrelin at 8 pmol/g BW was injected into the abdominal cavity, or 0.5–0.7 μl of [D-Lys^3]GHRP-6 at 5 pmol/g BW in addition to bullfrog ghrelin at 0.5 pmol/g BW was injected into the third ventricle of the brain of larvae, as described earlier. The IP- and ICV-injected doses of [D-Lys^3]GHRP-6 had been determined in preliminary experiments using [D-Lys^3]GHRP-6 at 80 and 160 pmol/g BW (IP) or 5 and 10 pmol/g BW (ICV). Larvae in the control group were given injections of the same volume of saline. The feeding experiment was performed according to the procedures described in the above section.

2.6. Effect of feeding status on expression of ghrelin mRNA in the hypothalamus and gastrointestinal tract

The larvae were kept individually under controlled light/dark conditions (12L/12D) in a tank filled with tap water, and fed daily at 3% of their BW. Before feeding, or 15 and 60 min after feeding, the larvae were anesthetised with MS-222 and each hypothalamus and gastrointestinal tract including stomach and intestine was removed. Each part was weighed, placed immediately in liquid nitrogen, and stored at −80 °C until use. Total RNA was extracted from each part with TriPure Isolation reagent (a solution containing phenol and guanidinium thiocyanate; Roche Diagnostics Inc., Basel, Switzerland). For amplification and quantitation of the cDNA fragments encoding ghrelin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the two-step reverse-transcription polymerase chain reaction (RT-PCR) method was carried out. First-stand cDNAs were synthesized from 1 μg of total RNA using a PrimeScript RT reagent kit with gDNA Eraser (Perfect Real Time, Takara, Tokyo, Japan) and the Oligo$(dT)_{15}$ primer. The reaction mixtures consisted of 250 nM each primer and template (100 ng total RNA equivalent) in 1× master mix. Reverse

transcription was carried out at 37 °C for 15 min, and the resulting cDNA was subsequently amplified using 25 cycles for ghrelin and GAPDH of 94 °C for 40 s and 60 °C for 40 s followed by 72 °C for 30 s. To estimate mRNA copy numbers, quantitative real-time PCR (qPCR) samples were run with serially diluted (1 to 10^6 copies) pT7Blue T-Vector (Novagen, San Diego, CA, USA) that consisted of an EcoR I linearized full-length target cDNA. Reactions using SYBR Premix Ex Taq II (Tli RNaseH Plus, Takara) were set up in a 96-well reaction plate and placed in a sequence detection system for cycling (TP850, Takara). cDNA was amplified using 40 cycles of 95 °C for 5 s followed by 60 °C for 30 s. The PCR products from each cycle were monitored using SYBR Green I fluorescent dye. Gene-specific primers for amplification of the ghrelin cDNA fragment were based on the nucleotide sequence of bullfrog preproghrelin (GenBank ID: AB058510.1). PCR with the sense primer (5′-ATC TTT GGG GTT GTC TTG TTC-3′) and antisense primer (5′-CCT GCG TCA TCT TCA TAT CC-3′) yielded a 211-bp product encoding part of the bullfrog preproghrelin cDNA. Bullfrog GAPDH-specific primers were used as the internal control for PCR amplification (sequence determined from a cloned bullfrog GAPDH cDNA, unpublished data). Using these primers (sense primer, 5′-AGA AGT GAA GGC TGA GAA TGG-3′; antisense primer, 5′-CAC GTA GTC GGC ACC AGA AG-3′) a 112-bp product corresponding to a region in the part of the GAPDH cDNA for bullfrog was obtained. The expression levels of ghrelin mRNA were calculated quantitatively as a ratio relative to the expression of GAPDH mRNA.

2.7. Data analysis

All results are expressed as mean ± SEM. Statistical analysis was performed by one- or two-way ANOVA with Bonferroni's method or by Student's *t*-test. Statistical significance was determined at the 5% level.

3. Results

3.1. Effect of IP and ICV administration of ghrelin on food intake in larvae

IP administrations of graded doses of bullfrog ghrelin enhanced food consumption in the larvae. Food intake increased significantly after IP administration of ghrelin at 8 and 16 pmol/g BW (Fig. 1A). The *F* and *P* values between treatments with saline and ghrelin were 3.68 and 0.023, respectively. ICV administrations of graded doses of bullfrog ghrelin also stimulated food consumption in the larvae. Food intake increased significantly after ICV administration of ghrelin at 0.5 and 1 pmol/g BW (Fig. 1B). The *F* and *P* values between treatments with saline and ghrelin were 8.86 and 0.0002, respectively.

3.2. Effect of IP and ICV injection of [D-Lys]GHRP-6 on the orexigenic action of ghrelin

IP injection of [D-Lys^3]GHRP-6 alone at a dose of 80 pmol/g BW did not affect basal food consumption during 15 min after supplying food. The orexigenic activity of bullfrog ghrelin at 8 pmol/g BW was completely blocked by injection with 80 pmol/g BW [D-Lys^3]GHRP-6, and the efficacy of the antagonist was shown to be significant by two-way ANOVA with Bonferroni's method (*F* and *P* values, 15.10 and 0.0004, respectively) (Fig. 2A). ICV injection of [D-Lys^3]GHRP-6 alone at a dose of 5 pmol/g BW did not affect basal food consumption during 15 min after supplying food. The orexigenic activity of bullfrog ghrelin at 0.5 pmol/g BW was completely blocked by preinjection of 5 pmol/g BW [D-Lys^3]GHRP-6, and the efficacy of the antagonist was shown to be significant by two-way ANOVA with Bonferroni's method (*F* and *P* values, 5.64 and 0.023, respectively) (Fig. 2B).

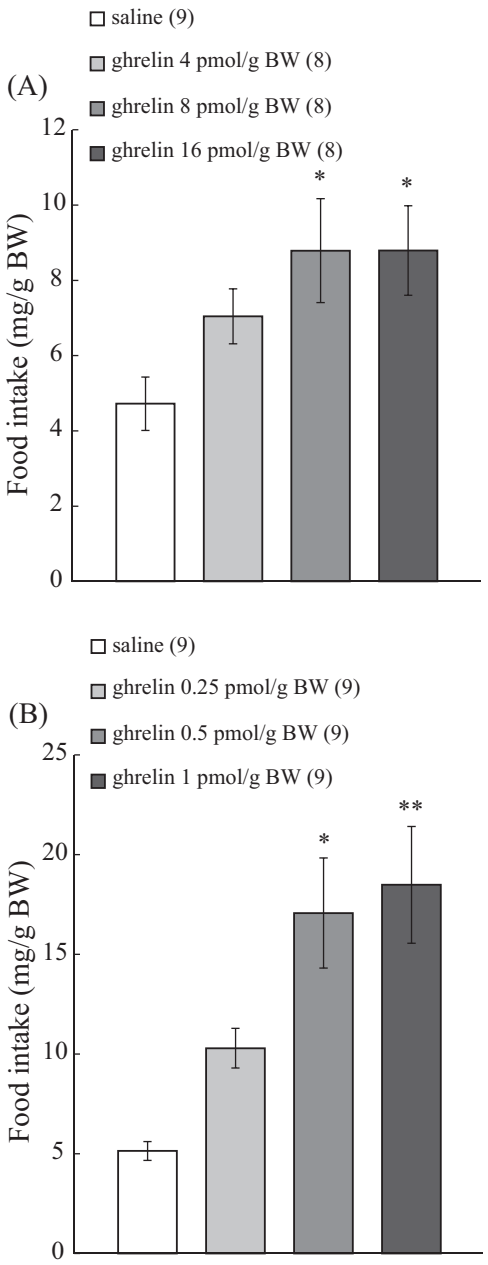

Fig. 1. Effect of IP (A) and ICV (B) administration of *n*-octanoylated ghrelin28 on food intake in bullfrog larvae. The results are expressed as the mean ± SEM, and the number of larvae per group is indicated in parentheses. Significance of differences at each time point was evaluated by one-way ANOVA with Bonferroni's method, as compared with a saline-injected group (*$P < 0.05$; **$P < 0.01$).

3.3. Effect of feeding status on expression of ghrelin mRNA in the hypothalamus and gastrointestinal tract

Fig. 3 shows the levels of expression of ghrelin mRNA in the hypothalamus and gastrointestinal tract collected before feeding, or at 15 and 60 min after feeding. Expression of ghrelin mRNA was estimated quantitatively as a ratio relative to the expression of GAPDH mRNA. The level of ghrelin mRNA expression in the gastrointestinal tract was approximately 10^4 times that of ghrelin mRNA expression in the hypothalamus. Ghrelin mRNA levels in the hypothalamus at 15 and 60 min after feeding were significantly

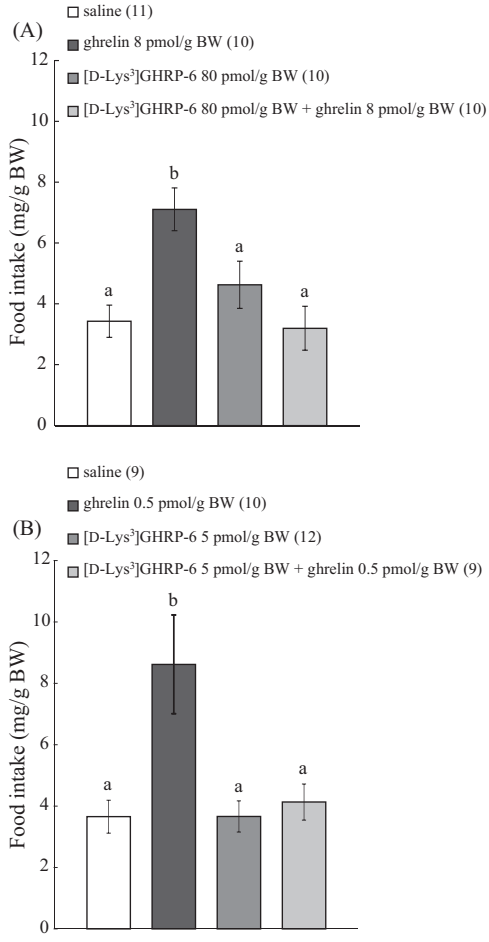

Fig. 2. Effect of IP (A) and ICV (B) injection of the GHS receptor antagonist [D-Lys^3]GHRP-6 on the orexigenic action of *n*-octanoylated ghrelin28. The results are expressed as the mean ± SEM, and the number of larvae per group is indicated in parentheses. Significance of differences was evaluated by two-way ANOVA with Bonferroni's method. Different superscripts indicate statistical significance ($P < 0.05$).

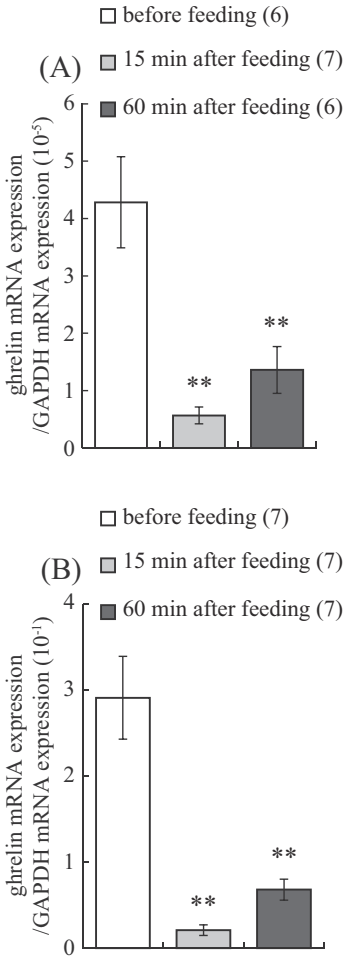

Fig. 3. Effect of feeding status on the expression of ghrelin mRNA in the hypothalamus (A) and gastrointestinal tract (B). The results are expressed as the mean ± SEM, and the number of larvae per group is indicated in parentheses. Statistical significance was evaluated by one-way ANOVA with Bonferroni's method (**$P < 0.01$).

lower than those before feeding (Fig. 3A). The *F* and *P* values before and after feeding were 25.14 and 0.0001, respectively. Levels of ghrelin mRNA in the gastrointestinal tract also showed a similar pattern to that in the hypothalamus (Fig. 3B). The *F* and *P* values before and after feeding were 24.86 and 0.0001, respectively.

4. Discussion

Ghrelin has been purified and identified from the stomach of adult bullfrog [18], and the presence of ghrelin has also been studied in the edible frog (*R. esculenta*, now also known as *Pelophylax esculentus*) [9,12,24]. In both species, transcripts of ghrelin or ghrelin-like immunoreactivity have been observed within the mucosal layer of the stomach, and in the edible frog, neuronal cell bodies and nerve fibers with ghrelin-like immunoreactivity are widely distributed in the brain, suggesting that, in anuran amphibians, ghrelin exerts both neuroendocrine and endocrine activities. In the bullfrog, three molecular forms of ghrelin have been reported: 27 or 28 amino acid-residues with the N-terminal third tyrosine residue modified by *n*-octanoic or decanoic acid (*n*-octanoylated ghrelin-27, *n*-decanoylated ghrelin-27 and *n*-octanoylated ghrelin-28). Recently, it has been revealed that octanoylated and decanoylated peptides have equal potency to interact with the GHS-R1a [17,18]. As *n*-octanoylated ghrelin-28 is considered to be the dominant form, we examined whether ghrelin increases food consumption in bullfrog larvae by using it.

To date, most of the information on the effects of ghrelin on food intake in non-mammals has been obtained in goldfish [27,28,35,37,38,40–42,50–53], and ICV and IP administration of goldfish ghrelin stimulates food intake and affects locomotor activity [27,42,56]. In rainbow trout, IP injection of ghrelin does not affect food consumption, whereas ICV injection of ghrelin decreases food intake [13,14]. In the case of neonatal chicks, ICV administration of ghrelin inhibits food consumption [8]. These suggest diversity of the feeding regulation by ghrelin in vertebrates. However, the situation in other non-mammalian species has remained unclear. Bullfrog ghrelin stimulates the release of GH and prolactin from cultured bullfrog pituitary cells [18]. Ghrelin receptor GHS-R1a has been identified throughout the central and peripheral tissues of the bullfrog [17]. We have recently established the method of measuring food intake in bullfrog larvae after IP or ICV injection of peptides by directly observing and measuring the diets eaten by each individual [39,48]. In the present study, our data provided direct evidence for the involvement of ghrelin in feeding regulation in bullfrog larvae, and we demonstrated for the first time in prometamorphic larvae that ICV and IP administration of bullfrog ghrelin enhanced food intake. Anuran larvae can feed and grow until the metamorphic climax, and orexigenic and anorexigenic factors seem to regulate appetite or satiety and energy balance of larvae during the developmental stages. Indeed, our previous

studies of bullfrog larvae have indicated that ICV administration of NPY stimulates, whereas ICV administration of CRF suppresses food intake, suggesting that NPY is involved in appetite regulation during metamorphosis [48], and that CRF acts as an anorexigenic factor to suppress food intake during the period of reconstruction of the digestive system [39]. It is likely that ghrelin acts centrally and peripherally as an orexigenic peptide, and is involved in growth and energy uptake in bullfrog larvae.

Here we used a GHS-R1a antagonist to characterize the receptor responsible for mediating the orexigenic effect induced by bullfrog ghrelin in bullfrog larvae. Our results revealed that treatment with [D-Lys^3]GHRP-6 completely suppressed the orexigenic action of bullfrog ghrelin, suggesting that, in bullfrog larvae, ghrelin acts pharmacologically as a GHS-R1a agonist to exert its orexigenic effect. Recently, GHS-R1a has been identified in the bullfrog [17], and its transcripts have been observed in the diencephalon, mecencephalon, gastrointestinal tract, kidney, interrenal gland and testis. Furthermore, GHS-R1a has been characterized pharmacologically in this species: bullfrog ghrelin and its agonist GHRP-6 are able to interact with GHS-R1a expressed in HEK 293 [17]. Since our data showed an antagonistic effect of [D-Lys^3]GHRP-6 on the orexigenic action of centrally and peripherally injected bullfrog ghrelin, it is likely that the orexigenic action of bullfrog ghrelin is mediated via the GHS-R1a-signaling pathway in bullfrog larvae. In rodents, ghrelin is a potent orexigenic peptide acting through the GHS-R-signaling pathway [2,10,32,33]. In goldfish, four types of GHS-Rs (GHS-Rs1a-1, 1a-2, 2a-1 and 2a-2) have been characterized, and the orexigenic action of ghrelin or GHRP-6 is mediated via the GHS-R2a-1-signaling pathway [22,56].

We also demonstrated the effect of feeding status on ghrelin mRNA expression in the hypothalamus and gastrointestinal tract of bullfrog larvae. The level of expression of ghrelin mRNA decreased 15 and 60 min after feeding. These results are in good agreement with previous studies of goldfish and zebrafish, which demonstrated an increase of ghrelin mRNA expression in the brain or gut 1–3 h before feeding, and a decrease of ghrelin mRNA expression in the hypothalamus or gut 1 h after feeding [1,41,50]. Our data suggest that ghrelin responds to energy status, and acts on the gastrointestinal tract and the brain as an orexigenic factor in bullfrog larvae. Ghrelin mRNA levels in the hypothalamus and gastrointestinal tract at several hours after feeding were lower than those before feeding (data not shown), suggesting that changes in the levels of ghrelin mRNA are also related to circadian rhythm in addition to appetite. The levels of ghrelin mRNA expression in the gastrointestinal tract were approximately 10^4 times higher compared with those in the hypothalamus, and the present data for bullfrog larvae suggest a degree of functional differentiation between central and peripheral ghrelins. In goldfish, since the effect of IP-injected ghrelin is blocked by treatment with capsaicin, a neurotoxin which destroys primary sensory afferents, the action of peripheral ghrelin seems to be mediated via the vagal or splanchnic afferent pathway [37]. The orexigenic action of IP- and ICV-injected ghrelin is mediated via the NPY-signaling pathway in goldfish [41]. Further study is required to elucidate the signaling pathway underlying the central and peripheral actions of ghrelin on food intake in bullfrog larvae.

The bullfrog larva is considered to be an excellent animal model for studying the neuroendocrine control of feeding behavior by neuropeptides in anuran larvae because it consumes a relatively large amount of food prior to metamorphosis. Moreover, the feeding period (before cessation of feeding and commencement of metamorphosis) is relatively long, and the materials for feeding experiments are available throughout the year. In conclusion, our results indicate that ghrelin has the potential to enhance food intake in bullfrog larvae during the prometamorphic stages, suggesting a role for ghrelin in the feeding behavior of bullfrog larvae.

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