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Ghrelin receptor in Japanese fire belly newt, *Cynops pyrrhogaster*

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ABSTRACT

We identified cDNA encoding a functional ghrelin receptor (growth hormone secretagogue-receptor 1a (GHS-R1a)) in a urodele amphibian, the Japanese fire belly newt (*Cynops pyrrhogaster*). Two functional receptor proteins, composed of 378- and 362-amino acids, were deduced from the identified cDNA because two candidate initiation methionine sites were found. The long-chain receptor protein shared 80%, 69%, and 59% identities with the bullfrog GHS-R1a, human GHS-R1a and tilapia GHS-R1a-like receptor, respectively. Phylogenetic analysis suggested that the newt receptor is grouped to the clade of the tetrapod homologs, and very closed to anuran amphibians. In functional analyses, homologous newt ghrelin, heterologous bullfrog and rat ghrelin, and a GHS-R1a agonist, GHRP-6 increased intracellular Ca^{2+} concentration in human embryonic kidney (HEK) 293 cells stably expressed newt GHS-R1a. The responsiveness was much greater in the short-chain receptor than in the long-chain receptor. Both receptors preferred to bind Ser^3-ghrelin including newt and rat ghrelin than Thr^3-ghrelin with bullfrog ghrelin. GHRP-6 has a similar affinity to bullfrog ghrelin. GHS-R1a mRNA was expressed in the brain, pituitary, intestine, pancreas, testis and fat body with high level, and eyes, heart, stomach, liver, gall bladder, kidney and dorsal skin with low level. In a fasting experiment, gene expression of GHS-R1a in the brain and pituitary increased 4 days after fasting, and the increased level decreased to the initial level 2 weeks after fasting. These changes are consistent with the change in ghrelin mRNA. In contrast, expression of ghrelin and GHS-R1a mRNA in the stomach decreased on day 4 after fasting, and increased 2 weeks after fasting. These results indicate that ghrelin and its receptor system are present and altered by energy states in this newt.

1. Introduction

In mammals, two isoforms of growth hormone secretagogue-receptor (GHS-R) have been identified: functional receptor, GHS-R1a, and an alternative splice variant, GHS-R1b (Davenport et al., 2005). Ghrelin was discovered from the rat and human stomach as an endogenous ligand for the GHS-R1a (Kojima et al., 1999). Its third serine residue (Ser^3) has an *n*-octanoyl modification at the side chain, and the acyl modification is known to be essential for eliciting ghrelin's activity. The widespread expression of GHS-R1a mRNA and translated protein from the central nervous system to various peripheral tissues supports the multiple actions of ghrelin such as growth hormone (GH) release, appetite regulation, glucose metabolism, cardiovascular function and gastrointestinal functions including motility and gastric acid secretion, and memory (Papotti et al., 2000; Gnanapavan et al, 2002; Kojima and Kangawa, 2005).

Ghrelin is also present in the stomach or intestine of various non-mammalian vertebrates, and shows various functions (Kaiya et al., 2008, 2011b). Recently, we have concentrated on identification and characterization of the ghrelin receptor in non-mammalian vertebrates, and reported ghrelin receptors in two anuran amphibians, the bullfrog (*Lithobates catesbeianus* [*Rana catesbeiana*]) and the Japanese tree frog (*Hyla japonica*) (Kaiya et al., 2011a). In the study, we found interesting features: (i) the two frog GHS-R1a were capable of binding ghrelin, but both Ser^3-ghrelin and Thr^3-ghrelin increased intracellular Ca^{2+} concentrations in a similar dose–response relationship even bullfrog and tree frog have Thr^3-ghrelin and Ser^3-ghrelin, respectively (Kaiya et al., 2001, 2011b); (ii) the identified frog GHS-R1a mRNA was not detected in the pituitary of both species (Kaiya et al., 2011a). This result was quite strange because bullfrog ghrelin is capable of stimulating growth hormone (GH) and prolactin (PRL) secretion from dispersed bullfrog pituitary cells (Kaiya et al., 2001). This result suggests that bullfrog and/or tree frog could have another type of GHS-R1a with different affinity between Ser^3-ghrelin and Thr^3-ghrelin in the pituitary. We recently determined an amphibian ghrelin other than frogs, which is in Japanese fire belly newt, *Cynops pyrrhogaster*, and it was Ser^3-ghrelin (Kaiya et al., 2010). This suggests that a comparative study using newt may provide valuable information on ligand–receptor interaction of the ghrelin system including ligand selectivity of the receptor.

The aim of this study is to isolate cDNA of functional ghrelin receptor in a urodele amphibian, the Japanese fire belly newt. We examined

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functionality of the identified receptor and ligand selectivity, as well as tissue distribution of its mRNA. In addition, we examined possible involvement of the ghrelin system in energy metabolism in this newt by food deprivation experiment for 2 weeks.

2. Materials and methods

All experiments were performed according to the Guideline for Care and Use of Animals approved by the ethical committee of the National Cerebral and Cardiovascular Center.

2.1. Animals

Japanese fire belly newts of both sexes, mean weight 5.6 (5.45–5.76) g, were purchased from a commercial company (Shimizu Jikken Zairyo, Kyoto, Japan). Four newts were bred with a plastic case (W 15 cm × D 25 cm × H 12 cm) in tap water under natural photoperiod and constant room temperature (25 °C), and were provided with commercially available granular feed (3 % body weight) once in 2 days in the morning (10:00–11:00 AM).

2.2. Cloning of ghrelin receptor

Reverse-transcription PCR was performed to amplify approximately 700-bp fragment of GHS-R1a, and then full-length cDNA was determined by 3′ and 5′ RACE PCR using Gene Racer Kit (Invitrogen, Carlsbad, CA).

Total RNA was extracted by Sepasol-RNA I Super regent (Nacalai Tesque, Kyoto, Japan) from frozen whole brain or pituitary. One microgram of total RNA from the pituitary was reverse-transcribed with oligo-dT_{12-18} primer using QuantiTect RT Kit (QIAGEN GmbH, Hilden, Germany). Primary PCR was performed with the template (100 ng total RNA equivalent in the reaction), a primer set (100 pmol/μl GHSR-dSES1 and GHSR-dANT1, Table 1) and ExTaq DNA polymerase (TaKaRa Bio Inc., Shiga, Japan). The reaction conditions were 94 °C for 2 min and subsequent 35 cycles of 94 °C for 1 min, 54 °C for 0.5 min, and 72 °C for 1 min; and final extension was 72 °C for 10 min. The amplified product was purified by a Wizard PCR Preps DNA Purification System (Promega, Madison, WI), and 2 μl (1/25 volume) of the purified product was used as the template of the second-round nested PCR. It was performed under the same conditions as those of primary PCR except another primer set (GHSR-dSES2 and GHSR-dANT1, Table 1) was used. The resultant product was subcloned into the pCRII-TOPO vector (Invitrogen), and the nucleotide sequence of the insert was determined by automated sequencing (Model 3130, Applied Biosystems, Foster City, CA) according to the protocol of the BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems). In this RT-PCR, approximately 840-bp fragments showing high similarity to GHS-R1a were obtained.

For 3′-RACE PCR, brain total RNA (1 μg) was reverse-transcribed with 3′oligo-dT primer using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany). Primary 3′-RACE PCR was performed with the gene-specific primer (GSP) (ntGHSR-s1, Table 1), the 3′-primer supplied in the kit, and HotSTAR Taq Plus DNA polymerase (QIAGEN GmbH). The reaction conditions were 95 °C for 5 min and subsequent 35 cycles of 95 °C for 0.5 min, 57 °C for 0.5 min, and 72 °C for 1.5 min; and final extension was 72 °C for 3 min. After PCR preps of the amplified product, second-round nested PCR was conducted with another GSP (ntGHSR-s2, Table 1), the 3′-nested primer supplied in the kit, and HotSTAR Taq Plus DNA polymerase under the same conditions as those for the primary PCR. In this PCR, approximately 640-bp fragments were obtained.

For 5′-RACE PCR, first-strand cDNAs were synthesized from 2 μg of brain total RNA with an anti-sense GSP (ntGHSR-code AS, Table 1) or oligo-dT_{12-18} primer (Table 1) using Transcriptor High Fidelity cDNA Synthesis Kit. Primary PCR was conducted using anti-sense GSPs (ntGHSR-full-AS or ntGHSR-code-AS, Table 1), the 5′ primer in the kit, HotSTAR DNA polymerase with amplification conditions at 95 °C for 5 min and subsequent 35 cycles of 95 °C for 0.5 min, 57 °C for 0.5 min, and 72 °C for 1.5 min and final extension was 72 °C for 3 min. After PCR preps of the product, second-round nested PCR was performed using other anti-sense GSPs (ntGHSR-AS1, AS2 and AS3, Table 1), and the 5′-nested primer in the kit with only changing the annealing temperature to 53 °C. Specific bands, approximately 750–900 bp in length, were cut out, and the purified cDNAs were further performed the third-round nested PCR in each sample using appropriate GPS set. Finally approximately 750-bp product was isolated.

Full-length cDNA encoding GHS-R1a was amplified with a primer set (ntGHSR-s1 and code-AS) and HotSTAR DNA polymerase using a template for **5′** RACE PCR. The amplified products were performed the second-round nested PCR with a primer set (ntGHSR-s2 and code-AS). Amplification conditions were at 95 °C for 5 min and subsequent 35 cycles of 95 °C for 0.5 min, 55 °C for 0.5 min, and 72 °C for 1.5 min and final extension was 72 °C for 3 min. Two candidate cDNAs of the ghrelin receptor protein, which have 1137 bp (long-chain) and 1089 bp (short-chain), were cloned.

Table 1
Primers used in this study.

Name	Sequence (5′ to 3′)	Purpose
GHSR-dSES1	AAY YTY TAY CTS TSY AGY ATG GC	RT-PCR for cloning
GHSR-dANT1	TTR ATS GCN GCR CTS AGR TAR AA	
GHSR-dSES2	GAY CTS CTS ATY TTY CTS TGY ATG CC	
		3′-RACE
ntGHSR-s1	CAC GAG AAT GGA ACA AAC CCA CTG	
ntGHSR-s2	CCA ATC GGA CCA AAT ACT TCA ATC	
		5′-RACE
ntGHSR-code-AS	TCA TGT GCT GAC GCT GGA CTC TGT	
ntGHSR-full-AS	GAC AGT CAC CCC CAG TCA CAG ATC	
ntGHSR-AS1	TGT TCC ATT CTC GTG TTC TAC CCC	
ntGHSR-AS2	ACA AGT TTC GCT GAT GAA CTG GAA	
ntGHSR-AS3	TTC TAC CCC GAC CAA GAC GAA	
		PCR for coding region
ntGHSR-s1	CAC GAG AAT GGA ACA AAC CCA CTG	
ntGHSR-s2	CCA ATC GGA CCA AAT ACT TCA ATC	
		qPCR
Nt Ghr-QPCR-s	GCA GTA CAC CGA GGC AGG CTC CAG	
Nt Ghr-QPCR-AS	ACA TCT TCA CAC CAA TCT CAA AGG	
ntGHSR-Q-s	TTG GTC GGG GTA GAA CAC GAG AAT	
ntGHSR-Q-AS	CAC AAC AAG CAT TTT TAC AGT CTG	
Nt EF-1a-s	ACG GTA GCT GTC GGA GTT ATA AAG	
Nt EF-1a-AS	GAC CCT AAT GCT CAG TAA CCA GTC	

2.3. Functional analysis of the ghrelin receptor

To examine functional activity of identified receptor protein, we constructed an expression vector for mammalian cell containing the coding region of newt GHS-R1a protein. The cDNA encoding the coding region of GHS-R1a protein was amplified and subcloned into the pcDNA3.1-V5-His-TOPO vector (Invitrogen). The plasmid vector with correct orientation for protein expression was cultured by transformed JM109 (TaKaRa Bio), and was obtained by purification using a Wizard® Plus Minipreps DNA Purification System (Promega Corp., Madison, WI).

Changes in intracellular Ca^{2+} concentrations were measured using $FLIPR^{tetra}$ (Molecular Devices, Menlo Park, CA). Human embryonic kidney 293 (HEK293) cells were cultured in DMEM containing 10% fetal calf serum at a density of 1×10^6 cells in a collagen-coated 10-cm dish for 24 h. An expression vector (2.5 μg) was transfected with ViaFect™ Transfection Reagent (Promega Corp.) according to the manufacturer's protocol. After 24 h of transfection, the cells were plated onto a poly-D-lysine (Sigma Chemical, St. Louis, MO) coated 96-well black plate (Corning Inc., Wilker Barre, PA) at a density of 3×10^4 cells per well. After 20 h of plating, cultured medium was aspirated, and 100 μl fluorescent dye solution containing 4.4 μM Fluo-4AM (Invitrogen) and 1% FCS, 1% Power load concentrate (Invitrogen) in a working buffer (1 × Hanks' BSS [Invitrogen]–20 mM HEPES buffer containing 250 μM probenecid