



Ghrelin receptor in two species of anuran amphibian, bullfrog (*Rana catesbeiana*), and Japanese tree frog (*Hyla japonica*)

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We have identified cDNA encoding a functional growth hormone secretagogue-receptor 1a (GHS-R1a, ghrelin receptor) in two species of anuran amphibian, the bullfrog (*Rana catesbeiana*), and the Japanese tree frog (*Hyla japonica*). Deduced receptor protein for bullfrog and Japanese tree frog (tree frog) was comprised of 374- and 371-amino acids, respectively. The two receptors shared 86% identity, and are grouped to the clade of the tetrapod homologs by phylogenetic analysis. In functional analyses, ghrelin and GHS-R1a agonists increased intracellular Ca^{2+} concentration in GHS-R1a-transfected-HEK293 cell, but ligand selectivity of ghrelin with Ser³ and Thr³ was not observed between the two receptors. Bullfrog GHS-R1a mRNA was mainly expressed in the brain, stomach, and testis. In the brain, the gene expression was detected in the diencephalon and mesencephalon, but not in the pituitary. Tree frog GHS-R1a mRNA was predominantly expressed in the gastrointestinal tract and ovary, but not detected in the pituitary. In bullfrog stomach but not the brain, GHS-R1a mRNA expression increased after 10 days of fasting. For tree frog, GHS-R1a mRNA expression was increased in the brain, stomach and ventral skin by 10 days of fasting, and in the stomach and ventral skin by a dehydration treatment. Intracerebroventricular injection of ghrelin in dehydrated tree frog did not affect water absorption from the ventral skin. These results suggest that ghrelin is involved in energy homeostasis and possibly in osmoregulation in frogs.

Keywords: bullfrog, characterization, cloning, ghrelin, growth hormone secretagogue-receptor 1a, Japanese tree frog

INTRODUCTION

Ghrelin, a 28-amino-acid peptide in which the third serine residue (Ser³) has an *n*-octanoyl modification, is an endogenous ligand for the growth hormone (GH) secretagogue-receptor 1a (GHS-R1a), and was first identified in the stomach of rats and humans (Kojima et al., 1999). Ghrelin was identified as a GH-releasing peptide, but accumulating evidences have demonstrated that ghrelin plays important roles in appetite regulation, glucose metabolism, cardiovascular function and gastrointestinal functions including motility and gastric acid secretion, and memory (Kojima and Kangawa, 2005). The multiple roles of ghrelin are supported by the wide expression of mRNA and protein for GHS-R1a from the central nervous system to several peripheral tissues (Papotti et al., 2000; Gnanapavan et al., 2002; Kojima and Kangawa, 2005). In mammals, two isoforms of GHS-R, a functional receptor, GHS-R1a, and an alternative splice variant, GHS-R1b, have been known (Davenport et al., 2005).

Ghrelin is also present in non-mammalian vertebrates and shows various species-specific actions (Kaiya et al., 2007, 2008). In amphibians, ghrelin has been identified in bullfrog (*Rana catesbeiana*; Kaiya et al., 2001). The mature-peptide length consists of 28 amino acids, although having the same length as the

mammalian counterparts, it only shares 30% sequence identity to human ghrelin. Recently, partial sequence of prepro-ghrelin of an edible frog (*Pelophylax esculentus*, previously: *Rana esculenta*) has been reported (Izzo et al., 2011). The amino acid sequence of mature-ghrelin is almost identical; only the 13th amino acid is changed from arginine (Arg) to glutamine (Gln), while the third amino acid, threonine (Thr³), is conserved as in bullfrog. Particularly, the third acylated amino acid was changed to threonine instead of serine as seen in mammals. This substitution of amino acid affects ghrelin's biological activity: bullfrog ghrelin is 100-times stronger in stimulating both GH and prolactin (PRL) secretion from dispersed bullfrog pituitary cells, when compared to rat ghrelin. On the other hand, a weak effect is observed in GH secretion when bullfrog ghrelin was injected intravenously into rats (Kaiya et al., 2001). This suggests that bullfrog ghrelin binds to rat GHS-R1a with different affinity to rat ghrelin, and the reversed phenomenon could be seen for the bullfrog ghrelin receptor.

Since bullfrog ghrelin has Thr³, it is speculated that ghrelin in all amphibians including anuran and urodele have Thr³. Recently, we found that only frog ghrelin of the genus, *Rana*, has Thr³, but other frogs such as *Xenopus laevis*, *Hyla japonica*, and *Bufo japonicus* and newts such as *Cynops pyrrhogaster* and *Cynops ensicauda*

have Ser³ (Kaiya et al., 2010). This suggests that comparative study of the two species of frogs, which has Thr³ or Ser³, 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 (GHS-Ra) in two species of frogs, bullfrog, and Japanese tree frog (tree frog, *H. japonica*), which have Thr³ and Ser³ in the ghrelin, respectively, for the comparison of the differences in the structure and tissue distribution, as well as functional activity of the receptor. In addition, we examined possible involvement of the ghrelin system in energy metabolism in these frogs by food deprivation experiment. Furthermore, since tree frog is often used for the study of osmoregulation (Tokuda et al., 1995; Kohno et al., 2003; Yamada et al., 2007; Maejima et al., 2010) and ghrelin has been reported to inhibit water intake in rats, chickens and eels (Kozaka et al., 2003; Tachibana et al., 2006; Hashimoto et al., 2007; Hashimoto and Ueta, 2011), we have also examined the effect of ghrelin on water absorption in tree frog.

MATERIALS AND METHODS

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

CLONING OF GHRELIN RECEPTOR

Total RNA was extracted by TRIzol (Invitrogen, Grand Island, NY, USA) from frozen whole brain. Total RNA (1 µg) was reverse-transcribed with 3'-oligo-dT primer of the Gene Racer Kit (Invitrogen) using QuantiTect RT Kit (QIAGEN GmbH, Hilden, Germany). Primary PCR was performed with the template, a primer set (100 pmol/µl GHSR-dSES1 and GHSR-dANT1, **Table 1**) and ExTaq DNA polymerase (TaKaRa Bio, Shiga, Japan). The reaction conditions were 94°C for 2 min and subsequent 35 cycles of 94°C for 1 min, 53°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, USA), and 2 µl (1/25 volume) of the purified product was used as template of the second-round nested PCR, which was performed under the same conditions as those of primary PCR except for using another primer set (GHSR-dSES2 and GHSR-dANT1, **Table 1**), PCR-prepsed template. 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, USA) according to the protocol of the BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems).

For 3'-RACE PCR, primary PCR was performed with the gene-specific primer (GSP), bfGHSR-RACE-s1 for bullfrog and hylaGHSR 3R-SES1 for tree frog (**Table 1**), and the 3'-primer in the kit and ExTaq DNA polymerase. The reaction conditions were 95°C for 2 min and subsequent 35 cycles of 95°C for 0.5 min, 57°C for 0.5 min, and 72°C for 1 min; and final extension was 72°C for 3 min. After PCR preps of the amplified product, nested PCR was performed with another GSP, bfGHSR-RACE-s3 for bullfrog and hylaGHSR 3R-SES2 for tree frog (**Table 1**), and 3'-nested primer

Table 1 | Primers used in this study.

Name	Sequence (5' to 3')
GHSR-dSES1	AAY YTY TAY CTS TSY AGY ATG GC
GHSR-dSES2	GAY CTS CTS ATY TTY CTS TGY ATG CC
GHSR-dANT1	TTR ATS GCN GCR CTS AGR TAR AA
bfGHSR-RACE-s1	CTC TTG TGT AAG CTC TTC CAG
hylaGHSR 3R-SES1	CAC AGG GAC AAG AAT AAC AAA CAG
bfGHSR-RACE-s3	GAG AAT GGT ACC AAT CCT TTT GAG
hylaGHSR 3R-SES2	GCT ATC TGT TCT CTA AGT CCT TCG
bfGHSR-AS1	GTG GGA GGC ACA TGG TCC TAT AGT
hylaGHSR 5R-ANT-X	TCC TTG GTT ATC TTG TCT GAA TC
bfGHSR-AS3	GTA GGA AGA AGA AGA CAC TGG ATG
hylaGHSR 5R-ANT-Y	GTA GGT CTG AGA ATG CCA TGC
bfGHSR-AS2	TTT ACA TTC ATT TGT CTC AAA AGG
bfGHSR-G-s1	CGT TGT TTA CAA TCA CAT ACA GTG
bfGHSR-AS4	AAG ATG AGC AGG TCT GAA AAA GCC
bfGHSR-code-s1	ATG GAG GCA AGA CTG TCT CAA TAT
bfGHSR-code-s2	ATG GAT TAT TAC TAC TAC AAC CAC
bfGHSR-code-AS	TCA TGT GCT GAC ATT AGA CTC TGT
hjGHSR-code-s	ATG GTG AGA ACT TGC CAG GAC TTT
hjGHSR-code-AS	TCA TGA GCT CAC ATA GGA TTC TGT
bfGHSR-Q-s	AGA ATG GTA CCA ATC CTT TTG AGA
bfGHSR-Q-AS	CAG CTA GCA TTT TTA CAG TCT GTC
bfB-actin-s	CTC TGC TAC GTC GCC CTG GAC TTC
bfB-actin-AS	ACA GAC CAC AGT GTT GGC ATA CAG
Frog GRLs2	GCT TAG CCT TCC TGA GTC CAG
Frog GRLAS2	TAT CCT GCA CTG ACC CGA GAG
hjGHSR-Q-s	CAA GTCTGG GCT CCT CAC CAT CAT
hjGHSR-Q-AS	AAC ACC ACC ACA GCT AGC ATT TTG
hjB-actin-s	ATC TGG CAT CAC ACC TTC TAC
hjB-actin-AS	GTC ACA CAA TCA CCA GAG TCC
hyGHSR-spANT	GAA TAA TAT GGC TTG AGT GTT GTC
frogGAPDH-SES	CTG GCT CCT CTT GCA AAG GT
frogGAPDH-ANT	GTG TAT CCC AGG ATT CCC TTC

in the kit and ExTaq DNA polymerase under the same conditions as those for the primary PCR.

For 5'-RACE PCR, first-strand cDNAs were synthesized from 5 µg of total RNA with an anti-sense GSP, bfGHSR-AS1 for bullfrog and hylaGHSR 5R-ANT-X (**Table 1**). Primary PCR was conducted using anti-sense GSP, bfGHSR-AS3 for bullfrog or hylaGHSR 5R-ANT-Y for tree frog (**Table 1**), 5' primer in the kit, ExTaq DNA polymerase, and 2.5% DMSO with amplification conditions at 95°C for 2 min and subsequent 35 cycles of 95°C for 0.5 min, 55°C for 0.5 min, and 72°C for 1 min and final extension was 72°C for 3 min. After PCR preps of the product, nested PCR was performed using another anti-sense GSP, bfGHSR-AS2 for bullfrog and hylaGHSR 5R-ANT-Y for tree frog (**Table 1**), and 5'-nested primer in the kit only changing annealing temperature to 58°C. For bullfrog, a 1715-bp product that contains an unexpected stop codon within a candidate nucleotide sequence was isolated. Hence, we designed a sense primer within the sequence at the 5'-end of the product (bfGHSR-G-s1, **Table 1**), and genomic PCR was performed with bfGHSR-G-s1, bfGHSR-AS4,

ExTaq DNA polymerase and 2.5% DMSO under the amplification conditions at 95°C for 2 min and subsequent 35 cycles of 95°C for 0.5 min, 56°C for 0.5 min, and 72°C for 1 min; and final extension was 72°C for 3 min. As a result, a 1.4-kb product was isolated, and two initiating ATG codon were found in the candidate sequence. Therefore, we designed two sense primers for cloning the coding region of bfGHS-Ra (bfGHSR-code-s1 and -s2, **Table 1**).

FUNCTIONAL ANALYSIS OF GHRELIN RECEPTOR

To examine functional activity of identified receptor protein, we constructed expression vector for mammalian cells containing the coding region of frog GHS-Ra proteins. The cDNA that encodes coding region of frog GHS-Ra proteins was amplified and subcloned into the pcDNA3.1-V5-His-TOPO vector (Invitrogen) after overhang reaction if necessary. RT-PCR for bfGHS-Ra was performed using whole brain cDNA template, a primer set, bfGHSR-code-s1 and bfGHSR-code-AS, pyrobest DNA polymerase (TaKaRa Bio) under the following reaction conditions: 95°C for 2 min and subsequent 35 cycles of 95°C for 0.5 min, 57°C for 0.5 min and 72°C for 1 min, and final extension was 72°C for 3 min. hjGHS-Ra cDNA was amplified using a primer set, hjGHSR-code-s and hjGHSR-code-AS, HotSTAR Taq Plus mix (QIAGEN GmbH) with reaction conditions 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 at 72°C for 3 min. The amplified cDNA was subcloned, and sequenced for correct orientation, and subsequently cultured at 50-ml scale. The plasmid vector was then isolated using a HiSpeed Plasmid Midi Kit (QIAGEN GmbH) and was diluted to 1 µg/µl for transfection experiment.

Changes in intracellular Ca²⁺ concentrations were measured using FLIPR^{tetra} (Molecular Devices, Menlo Park, CA, USA). Human embryonic kidney 293 (HEK293) cells were cultured in DMEM containing 10% fetal calf serum at a density of 1 × 10⁶ cells in a collagen-coated 10-cm dish for 24 h. An expression vector (2.5 µg) was transfected with FuGENE6 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. After twenty-four hours of transfection, the cells were plated onto a poly-D-lysine- (Sigma Chemical, St. Louis, MO, USA) coated 96-well black plate (Corning Inc., Wilkes Barre, PA, USA) at a density of 3 × 10⁴ cells per well. After twenty hours 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 (Sigma Chemical)] was loaded into each well. The plate was incubated for 1 h at room temperature, and the plate was washed three times with a working buffer by an automatic washing machine, followed by the addition of 100 µl synthetic octanoylated bullfrog ghrelin 28-C8, decanoylated bullfrog ghrelin 28-C10 (custom synthesis at ASBIO Pharma Co. Ltd., Gunma, Japan), rat ghrelin (Peptide Institute Inc., Osaka, Japan), GHRP-6 (Bachem AG, Bubendorf, Switzerland), or hexarelin (Phoenix Pharmaceutical Inc., Belmont, CA, USA) at doses of 0, 0.1, 1, 10, 100, and 1000 nM in a working buffer containing 0.001% Triton X-100 using the automated FLIPR system. Changes in intracellular

Ca²⁺ concentrations were measured by excitation at 488 nm and emission at 500–560 nm.

QUANTITATIVE REAL-TIME PCR

Quantitative real-time PCR (qPCR) was used for quantification of gastric and brain mRNA of GHS-Ra in the starvation experiment for bullfrog. First-strand DNA was synthesized from 1 µg of total RNA using QuantiTect RT Kit (QIAGEN GmbH) with oligo-dT primer. qPCR was performed for GHS-Ra and β-actin with two primer sets, bfGHSR-Q-s and bfGHSR-Q-AS, and bfB-actin-s and bfB-actin-AS, respectively (**Table 1**). The expected amplicon size was 240 bp and 255 bp, respectively.

Quantitative real-time PCR was performed using a LightCycler 480 (Roche Applied Science, Mannheim, Germany) and a QuantiFAST SYBR Green PCR Kit (QIAGEN GmbH). The amplification conditions were 95°C for 5 min, and subsequent 40 cycles at 95°C for 10 s and 60°C for 30 s. The reaction mixture consisted of 1 × master mix, 250 nM of each primer and template (100 ng total RNA equivalent). For quantification of GHS-Ra mRNA copy number, a linear regression line was generated using a serially diluted pCRII vector containing each desired cloned cDNA that was linearized by restriction with *Xba*I.

TISSUE DISTRIBUTION OF GHS-Ra mRNA

For bullfrog tissues, first-strand DNA was synthesized from 1 µg of total RNA using QuantiTect RT Kit (QIAGEN GmbH) with oligo-dT primer. PCR was performed with HotSTAR Taq Plus mix (QIAGEN GmbH) using bfGHSR-code-s1 and bfGHSR-AS4 for GHS-Ra, bfB-actin-s and bfB-actin-AS for β-actin, and Frog GRL-s2 and Frog GRL-AS2 for ghrelin (**Table 1**). The expected amplicon size was 240, 255, and 237 bp, respectively. 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 0.5 min.

For tree frog, first-strand DNA was synthesized from 1 µg of total RNA using QuantiTect RT Kit (QIAGEN GmbH) with oligo-dT primer. PCR was performed for GHS-Ra and β-actin with hjGHSR-Q-s and hjGHSR-Q-AS, and hjB-actin-s and hjB-actin-AS were used for the analyses (**Table 1**). The expected amplicon size was 195 and 227 bp, respectively. PCR was performed using a LightCycler 480 and a QuantiFAST SYBR Green PCR Kit under condition described above.

STARVATION EXPERIMENT IN BULLFROG

Juvenile bullfrogs of both sexes, weighing 22–25 g, were randomly divided into four control and four experimental groups. Animals were kept in eight plastic containers (73 cm × 42 cm × 20 cm) under artificial illumination (12:12h dark–light cycle) and constant temperature (23°C). All frogs were provided with food (approximately 0.3 g of bovine liver) once daily at early evening (5:00–6:30 P.M.) for the 3 days before experiment. Control animals were provided with food throughout the experimental period. Frogs in food-deprived group were sacrificed 0, 5, 10, and 20 days after scheduled food deprivation. Frogs in the control groups were sacrificed according to the same schedule as the experimental groups. After pithing each animal, stomach and whole brain were excised from each frog, frozen in liquid nitrogen and stored at –80°C until use. The mRNA expression level of GHS-Ra was quantified by qPCR as described above.

STARVATION OR DEHYDRATION EXPERIMENT IN TREE FROGS

Adult tree frogs of both sexes, weighing 1.3–2.3 g, were bred in breeding cages under artificial illumination (12:12h dark–light cycle) and temperature (23–28°C) for at least more than 1 week before experiment. They were allowed free access to water, and were fed with mealworm or cricket twice a week.

Frogs were kept at constant temperature (23°C) when moved to the experimental condition, and were divided into three groups: (a) dehydration group, which decreased their body weight (BW) to 20% from the initial weight with feeding after 3 to 4 days (Maejima et al., 2008), (b) fasting group, which were food deprived for 10 days with free access to water, and (c) intact group, which were kept in free access to water, and feed was given at once every 2 days. The 10-days of fasting period was referred in the result of bullfrog (section Starvation experiment in bullfrog). A 10-days fasting is considered to be enough to induce negative energy balance because of the smaller body size of tree frogs when compared to bullfrog. Tissues (brain, heart, stomach, intestine, kidney, urinary bladder, and ventral skin) were collected from each group and stored at –80°C. Total RNA was isolated using ISOGEN (Nippon gene, Tokyo, Japan), and DNase-I-treated total RNA (1 µg) was reverse-transcribed with the PrimeScript first-strand cDNA Synthesis Kit (TaKaRa Bio). RT-PCR for GHS-Ra (311 bp) was performed using hylaGHSR 3R-SES2 and hyGHSR-spANT for 28 cycles, while for GAPDH (362 bp), frog GAPDH-SES and ANT were used with 25 cycles of amplification (Table 1). The PCR products were electrophoresed by 2% agarose gel containing 0.005% ethidium bromide, and the band densities were analyzed using Scion Image software (Scion Corporation, MD, USA). The mRNA expression level of GHS-Ra was normalized by that of GAPDH. Three to four RNA samples were used for the analysis, and repeated three times to examine the reproducibility of expression patterns.

EFFECT OF INTRACEREBROVENTRICULAR (ICV) INJECTION OF GHRELIN ON WATER ABSORPTION IN TREE FROGS

This experiment was carried out in the autumn (October and November), and was based on the methods previously described, except dehydration treatment after fully hydration (Maejima et al., 2008, 2010). Frogs of both sexes were continuously kept in tap water for 24 h in order to maintain a fully hydrated condition. Dehydration treatment was then done until their BW lost approximately 13% from the initial level. For ICV injection, a small part of the parietal bone was carefully removed under anesthesia with 0.1% MS-222 (3-aminobenzoic acid ethyl ester, Sigma, St. Louis, MO, USA). Under a dissecting microscope, 0.2 µl/g BW of rat ghrelin solution [82.5 ng (25 pmol)/g BW] containing Evans blue dye was injected into the third ventricle (a middle portion between the telencephalon and mesencephalon with 0.8 mm depth) by a manipulator and 10-µl Hamilton syringe. Injected dose of ghrelin referred the previous study for arginine vasotocin (Maejima et al., 2008). Frogs in the control group were injected with frog Ringer's solution which contained (mM): NaCl, 111; CaCl₂, 2.70; KCl, 3.35; NaHCO₃, 2.38; glucose, 5.50; pH 7.4. In addition, intact group without injection treatment was also prepared. After allowing recovery time for 15 min, the treated frog was put into the water dish (5 cm × 5 cm) containing

tap water in the plastic cage (L20 cm × W30 cm × H20 cm). The behavior was recorded for 360 min with a video-camcorder since the frog tended to stay for longer time than previous experiments even in the hydrated condition (Maejima et al., 2008, 2010). After recording, the time staying in the water dish, which is called residence time, was analyzed. This residence time was defined as water absorption through the pelvic skin, and was compared among ghrelin-injected, frog Ringer's solution-injected and intact groups. After termination of the experiment, position of Evans blue dye was confirmed to ensure that the injected solution was accurately administered into the designated position.

STATISTICAL ANALYSIS

The results are expressed as means ± SEM. The significance of differences between the values was determined at $P < 0.05$ using unpaired Student's *t*-test for single comparisons or one-way ANOVA followed by Bonferroni/Dunn test for multiple comparisons.

RESULTS

IDENTIFICATION OF GHRELIN RECEPTOR-LIKE RECEPTOR cDNA

We identified cDNA encoding GHS-Ra-like protein from each frog. The open reading frame (ORF) of bullfrog cDNA was 1125 bp, which encodes a 374-amino acid protein (Figure 1, acc# AB626731), while ORF of tree frog cDNA was 1116 bp, which encodes a 371-amino acid protein (Figure 2, acc# AB626732). Comparison of the deduced amino acid sequences to other GHS-Ra sequences showed that numerous consensus sequences for GHS-Ra were found in the identified proteins (Figure 3). Frog GHS-Ra-like proteins shared 86% identity with each other (Figure 4A). Chicken GHS-R1a and quail GHS-R1aS showed the highest identity (74%) to the frog proteins (Figure 4B). Phylogenetic analysis showed that bullfrog and tree frog GHS-Ra-like proteins belong to the same branch with clawed frog (*X. laevis*), and other tetrapods such as birds and mammals were contained in the same sub-clade (Figure 4B). Interestingly, a branch including frog GHS-Ra-like proteins belongs to a sub-clade of fish GHS-R2a proteins.

FUNCTIONAL ANALYSES OF IDENTIFIED GHRELIN RECEPTOR-LIKE PROTEINS

The two identified GHS-Ra-like receptor cDNAs were transiently expressed in HEK 293 cells, and the cells were treated with bullfrog ghrelin with *n*-octanoyl (C8) or *n*-decanoyl (C10) modification and rat ghrelin (Figure 5A,B), and two different GHSs, GHRP-6 or hexarelin (Figure 5C,D). In both bullfrog (Figure 5A) and tree frog (Figure 5B), GHS-Ra-like receptors showed the same degree of responses to bullfrog and rat ghrelin at concentrations from 0 to 1000 nM. No differences in the response pattern were seen among the three ghrelin species. Both GHRP-6 and hexarelin showed 1/10 less potency for inducing intracellular Ca²⁺ signaling in bullfrog (Figure 5C) and tree frog (Figure 5D) GHS-Ra-like receptors, when compared with bullfrog ghrelin 28-C8, although the pattern of the dose response was similar between bullfrog and tree frog receptors. Substantiated by these results, we can confirmed that the presently identified

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1  ATGGAGGCAAGACTGTCTCAATATCATATACACAGCCAGGTATGGTCTCAGCCAATAAG 60
   M E A R L S Q Y H I H S Q V W S S A N K
61  ACTTACACCTCAGAGAACAAAACAAATGGATTATTACTACTACAATCACTCCTGGCCCGAA 120
   T Y T S E N K T M D Y Y Y Y N H S W P E
121 GATCCAGGGTATCTCTTCCCAGTGCCAGTCCTCACAGGGATAACTGTTACCTGTGTATTC 180
   D P G Y L F P V P V L T G I T V T C V F
181 CTGTTTCATCATCGGGATATTTGGGAACATCATGACTATGTTGGTGGTGTACAAGTACAAA 240
   L F I I G I F G N I M T M L V V Y K Y K
241 GATATGAGGACTACAATACTCTCTATCTGTCTAGTATGGCTTTTTCAGACCTGCTCATC 300
   D M R T T T N L Y L S S M A F S D L L I
301 TTTGTGTGCATGCCACTTGACCTGTACAGACTATGGCAGTACCGACCTGGAATTTTGGG 360
   F V C M P L D L Y R L W Q Y R P W N F G
361 AGCCTCTGTGTAAGCTCTTCCAGTTTATCAGTGAAAGTTGCACTTACTCCACAATCCTC 420
   S L L C K L F Q F I S E S C T Y S T I L
421 AATATCACTGCCCTTAGTGTGGAGAGATATTTTGCATCTGTTTTCCTCTGAAGCCAAAG 480
   N I T A L S V E R Y F A I C F P L K A K
481 GTGGTCATCACCAAGGGCAGAGTAAAGCTGGTCATCTTTGTGCTGTGGGCGTGTCTTTT 540
   V V I T K G R V K L V I F V L W A V S F
541 GTCAGTGTGGACCCATCTTGTCTTGGTTGGGGTTGAACATGAGAATGGTACCAATCCT 600
   V S A G P I F V L V G V E H E N G T N P
601 TTTGAGACAAATGAATGTAAGCTACAGAGTATGCTGTGAAGTCTGGTCTACTTACTATC 660
   F E T N E C K A T E Y A V K S G L L T I
661 ATGGTCTGGACATCCAGTGTCTTCTTCTTCCCTACCAGTCTTCTGCCTCACAGTTCTCTAC 720
   M V W T S S V F F F L P V F C L T V L Y
721 AGTCTTATTGGAAGAAAACGTGGAGGAGGAAGCGGGAGACTATAGGACCATGTGCCTCC 780
   S L I G R K L W R R K R E T I G P C A S
781 CACAGGGACAAGAATAACAGACAGACTGTAATAAGCTAGCTGTCGTTGGTATTTGCCTTC 840
   H R D K N N R Q T V K M L A V V V F A F
841 GTCCTGTGTTGGTTGCCCTTCCACGTGGCTCGCTACTTGTTTTCCAAGTCTTTTGAAGCC 900
   V L C W L P F H V A R Y L F S K S F E A
901 GGATCCTTGGAATCGCTCTGATAAGTCAATACTGTAACCTGGTTTCCTTTGTGCTTTTC 960
   G S L E I A L I S Q Y C N L V S F V L F
961 TACCTGAGTGTGCCATCAACCCGATTTTATATAACATTATGTCAAAAAAATACCGCGTG 1020
   Y L S A A I N P I L Y N I M S K K Y R V
1021 GCCGCTGTAGACTATTCAAATAAGAGGATCTCCCGTAAAGCACCTTACACCACAAAT 1080
   A A C R L F K L K R I S R K A P Y T T N
1081 GATGAGAGTTCTCCTGCCTGGACAGAGTCTAATGTCAGCACATGA 1125
   D E S S P A W T E S N V S T *

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FIGURE 1 | Nucleotide and deduced amino acid sequences of growth hormone secretagogue-receptor (GHS-R) a-like receptor for bullfrog. Asterisk indicates the termination codon (TGA).

receptors in bullfrog and tree frog were functional ghrelin receptor (GHS-Ra).

EXPRESSION OF GHRELIN RECEPTOR mRNA IN BULLFROG AND TREE FROG

In bullfrog, RT-PCR revealed that GHS-Ra mRNA was highly expressed in the brain, stomach and testis, and to a lesser extent in the small and large intestines, adrenal gland, and kidney (Figure 6A), while ghrelin mRNA was only detected in the stomach. We examined detail distribution of GHS-Ra mRNA in bullfrog brain (Figure 6B). mRNA expression was detected in the diencephalon and mesencephalon, but not in the telencephalon and pituitary in both male and female frogs.

In tree frog, RT-PCR revealed that GHS-Ra mRNA was expressed in almost all tissues examined, and relatively high expression was seen in the intestines such as duodenum, small and large intestines in both male and female (Figure 7). In female frogs, a high expression of GHS-Ra mRNA was detected in the ovary.

EFFECTS OF FASTING ON THE GHRELIN RECEPTOR mRNA EXPRESSION IN BULLFROG STOMACH AND BRAIN

Expression of GHS-Ra mRNA in the stomach for fed frogs did not show significant changes during feed deprivation period for 20 days (Figure 8A). Expression of GHS-Ra mRNA in the stomach for fasted frogs significantly increased after 10 days of fasting when compared with the respective control, although it was not statistically significant when compared with the level at day 0. The increased levels returned to the control level after 20 days of fasting. Expression of GHS-Ra mRNA in the whole brain did not change for 20 days of fasting period (Figure 8B).

EFFECTS OF FASTING AND DEHYDRATION ON THE GHRELIN RECEPTOR mRNA EXPRESSION IN TREE FROG

Ten-days of food deprivation significantly increased GHS-Ra mRNA expression in the stomach and ventral skin of tree frog, when compared with the respective control (Figure 9A). Dehydration by decreased BW to 20% significantly increased


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1  ATGGTGAGAACTTCGCAGGACTTTCTTTATGGCCAGGTGAGAATCATGTCCAGTAAAATT 60
   M V R T S Q D F L Y G Q V R I M S S K I
61  GATTGACACAACATAACCAAGGATTACTACTATAATTTTACCAGCCAAGAAGAGCCCATC 120
   D S D N I T K D Y Y Y N F T S Q E E P I
121 TATCTTTTCCCCCTGCCAGTCCTTACAGGAATAACTGTGGTCTGTGTTCTCCTGTTTATT 180
   Y L F P L P V L T G I T V V C V L L F I
181 ATTGGTATATTTGGTAATATCATGACTATGCTAGTGGTGTCCAAGTACAAGGACATGAGG 240
   I G I F G N I M T M L V V S K Y K D M R
241 ACTACAACCAACCTCTATCTCTCCAGCATGGCATTCTCAGACCTTCTCATCTTTCTCTGC 300
   T T T N L Y L S S M A F S D L L I F L C
301 ATGCCACTTGACTTGTACAAGCTTTGGCAATATAGACCCTGGAATTTTGGGAAGCCTCCTC 360
   M P L D L Y K L W Q Y R P W N F G S L L
361 TGCAAGTGTTTTCAGTTTCGTGAGTAAAGTTGCACTTACTCCACTATCTGAACATCACA 420
   C K L F Q F V S E S C T Y S T I L N I T
421 GCCCTGAGTGTGGAGAGGTATTTGCCATCTGTTTCCATTGAAAGCTAAGGTGGTCATC 480
   A L S V E R Y F A I C F P L K A K V V I
481 ACCAAGGCAGGGTGAAACTGGTCATCTTTGTTCTTTGGACCCTGTCTTTGTTAGCGCT 540
   T K G R V K L V I F V L W T L S F V S A
541 GGACCTATCTTTGCTTGGTGGTGTGAACACGAAAATGGGACCAATCCCCTAGTAACA 600
   G P I F V L V G V E H E N G T N P L V T
601 AATGAATGTAAAGCTACTAAGTATGCAGTCAAGTCTGGGCTCCTCACCATCATGGCGTGG 660
   N E C K A T K Y A V K S G L L T I M A W
661 ACATCAAGTGTCTTCTTCTTCTTGCCTGTCTTTCGCTCACTGTCTGTACAGCCTTATT 720
   T S S V F F F L P V F C L T V L Y S L I
721 GGAAGGAACTGTGGAGAAGGAAGATGGACTCTATAGACCAAGTATCTCTCACAGGGAC 780
   G R K L W R R K M D S I G P S I S H R D
781 AAGAATAACAAACAGACTGTCAAATGCTAGCTGTGGTGGTGTGTTGCCTTCATCCTGTGC 840
   K N N K Q T V K M L A V V V F A F I L C
841 TGGTTACCATTCCACGTGGCCGCTATCTGTTCTCTAAGTCCTTTCGAAGCAGGATCCTGG 900
   W L P F H V A R Y L F S K S F E A G S W
901 GAGATCGCACTGATCAGCCAGTACTGTAAGTGGTCTCTTTTGTGCTTTTCTACCTGAGT 960
   E I A L I S Q Y C N L V S F V L F Y L S
961 GCTGCCATCAACCCATTCTATACAACATCATGTCCAAAAAATACCGCGTGGCCGCTTGC 1020
   A A I N P I L Y N I M S K K Y R V A A C
1021 AGACTATTCCGACTAAAGAAGGCGTGCAGAAGAGCACCTTACACGACGAATGATGAAAGT 1080
   R L F R L K K A C R R A P Y T T N D E S
1081 TCACCTGCCTGGACAGAATCTATGTGAGCTCATGA 1116
   S P A W T E S Y V S S *

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FIGURE 2 | Nucleotide and deduced amino acid sequences of growth hormone secretagogue-receptor (GHS-R) a-like receptor for Japanese tree frog. Asterisk indicates the termination codon (TGA).

mRNA expression of GHS-Ra in the whole brain, stomach and ventral skin, compared with the respective control (**Figure 9B**).

EFFECTS OF ICV INJECTION OF GHRELIN ON WATER ABSORPTION FROM THE VENTRAL SKIN IN TREE FROG

Dehydration increased GHS-Ra mRNA expression in the ventral skin (**Figure 9B**). Ghrelin may affect water absorption in dehydrated frog. We injected rat ghrelin (Ser³-ghrelin) into the lateral ventricle of the brain in 13% dehydrated tree frogs, and examined the effect on water absorption. There was no difference in the residence time among ghrelin-injected, frog Ringer's-injected and intact frogs (**Figure 10**).

DISCUSSION

This is the first report to have identified ghrelin receptor in amphibian. cDNAs from two species of frogs that encoded functional GHS-Ra protein as demonstrated by functional assay were isolated.

Sequence alignment and phylogenetic analyses of ghrelin receptor proteins including GHS-Ra-like receptor (GHSRa-LR) showed that the two frog proteins were grouped to the same branch with clawed frog, *X. laevis*. However, the branch was found to belong to the sub-clade that also included the fish GHS-R2a as well as GHS-R1a of birds and mammals. GHS-R2a is a relatively newly ghrelin receptor that was recently found in some teleost fish such as zebrafish, channel catfish and goldfish, and the sequence identities between GHS-R1a and 2a are 67–74% (Kaiya et al., 2008, 2010; Small et al., 2009). It is considered to be a paralog generated by the second-round whole genome duplication (2R WGD) or fish-specific 3R WGD (Meyer and Van de Peer, 2005). Therefore, fish GHS-R1a has created another sub-group from GHS-R1a for birds and mammals. Furthermore, GHSRa-LR is considered to be another sub-group. Receptors that are not functionally confirmed by signal transduction study with ghrelin or GHSs, or that needs a high dose of ghrelin or GHSs to induce intracellular Ca²⁺ signaling but are sharing high sequence identity

Bullfrog	1	MEARLSQ-YHHSQVNSA-NK-TYT---S-ENKTMDDYY---YNHSPW-EDGVL--	65
Tree frog	1	M-VRTSGDF-LYGVKRMSS-SL-D-D---S-DYKRYV-LPFT-F-Q-EDFTL	62
Clawed frog	1	-----MS-SE-IYI-----QNRMDYYSSNNY-SWP-EDPVLV-PP	50
Human-1a	1	-----MWNATPSEEPFNLT-LDLDWDASPGNDSLGDLEQLL-EPAP	57
Rat-1a	1	-----MWNATPSEEPFNVL-DLDWDASPGNDSLDELLEF-EPAP	56
Dog-1a	1	-----MNRATAREG-PGSAGW-DLLEF-EPAP	49
Catfish-1a	1	-----MTNPTN-SNCS---SNCSMDANAMV-----TSLP-----PIT	40
Goldfish-1a-1	1	-----MPTWNR-SNCSFN---SMDNAT-Y-----WGIQFVNI-PP	51
Zebrafish-1a	1	-----MTNRTNA-SSC-----LCD-GDA-PD---STHTDLDWDFYVQV	55
Catfish-2a	1	-----MTNRTNA-SSC-----LCD-GDA-PD---STHTDLDWDFYVQV	51
Goldfish-2a-1	1	-----MTNRTNA-SSC-----LCD-GDA-PD---STHTDLDWDFYVQV	55
Zebrafish-2a	1	-----MTNRTNA-SSC-----LCD-GDA-PD---STHTDLDWDFYVQV	55
Chicken-1a	1	-----MREGSSENRTGG-ESPLRI-EPAP	38
Quail-1aS	1	-----MREGSSENRTGG-ESPLRI-EPAP	38
Rainbow trout-DQTA	1	-----MRSWPNR-TDC-LSPVNSWEDNYVYFNFS-YQ-G-PVP-FENL-PP	60
Rainbow trout-ERAT	1	-----MRSWPNR-TDC-LSPVNSWEDNYVYFNFS-YQ-G-PVP-FENL-PP	60
Seabream-1a	1	-----MRSWPNR-TDC-LSPVNSWEDNYVYFNFS-YQ-G-PVP-FENL-PP	60
Tilapia	1	-----MPSWPN-EC-LH-RNCWTEET-NN---TISKADFS-P-P-E-LNYS	56
Bullfrog	66	IFSNGLLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	145
Tree frog	63	IFSNGLLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	142
Clawed frog	51	ISGNGLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	130
Human-1a.seq	58	IAGNGLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	137
Rat-1a	57	ISGNGLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	136
Dog-1a	41	VAGNGLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	120
Catfish-1a	52	VAGNGLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	129
Goldfish-1a-1	50	VAGNGLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	131
Zebrafish-1a	52	VAGNGLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	131
Catfish-2a	56	ISGNGLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	135
Goldfish-2a-1	58	IAGNGLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	137
Zebrafish-2a	57	IAGNGLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	136
Chicken-1a	39	VLAGNGLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	118
Quail-1aS	39	VLAGNGLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	118
Rainbow trout-DQTA	61	VAGNGLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	140
Rainbow trout-ERAT	61	VAGNGLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	140
Seabream-1a	57	VAGNGLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	136
Tilapia	57	VAGNGLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	136
Bullfrog	146	SVRYVAICFPHARVWVGRK-RKVI-LVAVAVSAGPFWVGVGHEH-ET	211
Tree frog	143	SVRYVAICFPHARVWVGRK-RKVI-FVAVAVSAGPFWVGVGHEH-ET	208
Clawed frog	131	SVRYVAICFPHARVWVGRK-RKVI-SVAVAVSAGPFWVGVGHEH-ET	196
Human-1a	138	SVRYVAICFPHARVWVGRK-RKVI-FVAVAVSAGPFWVGVGHEH-ET	203
Rat-1a	137	SVRYVAICFPHARVWVGRK-RKVI-LVAVAVSAGPFWVGVGHEH-ET	202
Dog-1a	121	SVRYVAICFPHARVWVGRK-RKVI-LVAVAVSAGPFWVGVGHEH-ET	186
Catfish-1a	130	SVRYVAICFPHARVWVGRK-RKVI-LVAVAVSAGPFWVGVGHEH-ET	195
Goldfish-1a-1	132	SVRYVAICFPHARVWVGRK-RKVI-LVAVAVSAGPFWVGVGHEH-ET	197
Zebrafish-1a	132	SVRYVAICFPHARVWVGRK-RKVI-LVAVAVSAGPFWVGVGHEH-ET	197
Catfish-2a	136	SVRYVAICFPHARVWVGRK-RKVI-LVAVAVSAGPFWVGVGHEH-ET	201
Goldfish-2a-1	138	SVRYVAICFPHARVWVGRK-RKVI-LVAVAVSAGPFWVGVGHEH-ET	203
Zebrafish-2a	137	SVRYVAICFPHARVWVGRK-RKVI-LVAVAVSAGPFWVGVGHEH-ET	202
Chicken-1a	119	SVRYVAICFPHARVWVGRK-RKVI-LVAVAVSAGPFWVGVGHEH-ET	184
Quail-1aS	119	SVRYVAICFPHARVWVGRK-RKVI-LVAVAVSAGPFWVGVGHEH-ET	184
Rainbow trout-DQTA	141	SVRYVAICFPHARVWVGRK-RKVI-LVAVAVSAGPFWVGVGHEH-ET	210
Rainbow trout-ERAT	141	SVRYVAICFPHARVWVGRK-RKVI-LVAVAVSAGPFWVGVGHEH-ET	210
Seabream-1a	141	SVRYVAICFPHARVWVGRK-RKVI-LVAVAVSAGPFWVGVGHEH-ET	208
Tilapia	137	SVRYVAICFPHARVWVGRK-RKVI-LVAVAVSAGPFWVGVGHEH-ET	207
Bullfrog	212	AVKSGLLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	282
Tree frog	209	AVKSGLLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	279
Clawed frog	197	AIRSGLLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	267
Human-1a	204	AVKSGLLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	274
Rat-1a	203	AVKSGLLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	272
Dog-1a	187	AVKSGLLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	257
Catfish-1a	196	AVKSGLLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	265
Goldfish-1a-1	198	AIRSGLLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	268
Zebrafish-1a	198	AIRSGLLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	268
Catfish-2a	202	AIRSGLLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	271
Goldfish-2a-1	204	AIRSGLLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	273
Zebrafish-2a	203	AIRSGLLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	272
Chicken-1a	185	AIRSGLLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	255
Quail-1aS	185	AIRSGLLIMVWYKXDRRTTNVFLSASGDLIFIQCPDLYRMYRYPANFQD	255
Rainbow trout-DQTA	211	DTSECKPQYA-VESGL-LAAMA-IVSFFPVPVCLTVVY-----SLIGRL	269
Rainbow trout-ERAT	211	DTSECKPQYA-VESGL-LAAMA-IVSFFPVPVCLTVVY-----SLIGRL	269
Seabream-1a	209	DTSECKPQYA-VESGL-LAAMA-IVSFFPVPVCLTVVY-----SLIGRL	266
Tilapia	208	DTSECKPQYA-VESGL-LAAMA-IVSFFPVPVCLTVVY-----SLIGRL	267
Bullfrog	283	CWLPBWRVLYSKSFEA-GSLEI-ALIS-QY---CWVSVFVLE-VI---SA	350
Tree frog	280	CWLPBWRVLYSKSFEA-GSWEI-ALIS-QY---CWVSVFVLE-VI---SA	347
Clawed frog	268	CWLPBWRVLYSKSFEA-GSLEI-ALIS-QY---CWVSVFVLE-VI---SA	335
Human-1a	275	CWLPBWRVLYSKSFEA-GSLEI-ALIS-QY---CWVSVFVLE-VI---SA	342
Rat-1a	273	CWLPBWRVLYSKSFEA-GSLEI-ALIS-QY---CWVSVFVLE-VI---SA	340
Dog-1a	258	CWLPBWRVLYSKSFEA-GSLEI-ALIS-QY---CWVSVFVLE-VI---SA	325
Catfish-1a	266	CWLPBWRVLYSKSFEA-GSLEI-ALIS-QY---CWVSVFVLE-VI---SA	335
Goldfish-1a-1	269	CWLPBWRVLYSKSFEA-GSLEI-ALIS-QY---CWVSVFVLE-VI---SA	336
Zebrafish-1a	269	CWLPBWRVLYSKSFEA-GSLEI-ALIS-QY---CWVSVFVLE-VI---SA	336
Catfish-2a	274	CWLPBWRVLYSKSFEA-GSLEI-ALIS-QY---CWVSVFVLE-VI---SA	338
Goldfish-2a-1	274	CWLPBWRVLYSKSFEA-GSLEI-ALIS-QY---CWVSVFVLE-VI---SA	341
Zebrafish-2a	273	CWLPBWRVLYSKSFEA-GSLEI-ALIS-QY---CWVSVFVLE-VI---SA	340
Chicken-1a	256	CWLPBWRVLYSKSFEA-GSLEI-ALIS-QY---CWVSVFVLE-VI---SA	323
Quail-1aS	256	CWLPBWRVLYSKSFEA-GSLEI-ALIS-QY---CWVSVFVLE-VI---SA	323
Rainbow trout-DQTA	270	---A---R---DKS-NR-QTKMLAVVVFVFLQW-PFHHRIMVMSBSSGSS	335
Rainbow trout-ERAT	270	---A---R---DKS-NR-QTKMLAVVVFVFLQW-PFHHRIMVMSBSSGSS	335
Seabream-1a	268	---A---R---DKS-NR-QTKMLAVVVFVFLQW-PFHHRIMVMSBSSGSS	335
Tilapia	267	---S---R---DKS-NR-QTKMLAVVVFVFLQW-PFHHRIMVMSBSSGSS	332
Bullfrog	351	ISRK--APYTTNDESSPAWTESVST-----	374
Tree frog	348	ACRR--APYTTNDESSPAWTESVSS-----	371
Clawed frog	336	VSRK--APYTTNDESSPAWTESVNSI-----	359
Human-1a	343	FSQRKLSL--KDESSPAWTESVSI-----	366
Rat-1a	341	FSQRKLSL--KDESSPAWTKSSINI-----	364
Dog-1a	326	FSQRKLSL--KDESSPAWTESVSI-----	349
Catfish-1a	336	SFTSSLSI-----	344
Goldfish-1a-1	337	SPRISTSA--VKGTSPCWTESTASL-----	360
Zebrafish-1a	337	IPRRSTSA--VKGTSPCWTESTASL-----	360
Catfish-2a	340	TQRRK--KSL--NSKCPVMS--SQCP-----	362
Goldfish-2a-1	342	APGRSQIV--NAESVVMNYSNST-----	367
Zebrafish-2a	341	APGRSQIV--NAESVVMNYSNST-----	365
Chicken-1a	324	LPKRLSS--TQDSSRVWTEPTVA-----	347
Quail-1aS	324	LPKRLSS--TQDSSRVWTEPTVA-----	347
Rainbow trout-DQTA	336	INPIYNTMSRYGAARLFLGLDPSPPRRGTASTVKGDGSSNGWTESTISF	387
Rainbow trout-ERAT	336	INPIYNTMSRYGAARLFLGLDPSPPRRGTASTVKGDGSSNGWTESTISF	387
Seabream-1a	334	INPIYNTMSRYGAARLFLGLDPSPPRRGTASTVKGDGSSNGWTESTISF	385
Tilapia	333	INPIYNTMSRYGAARLFLGLDPSPPRRGTASTVKGDGSSNGWTESTISF	384

FIGURE 3 | Comparison of the amino acid sequence of frog GHS-Ra-like receptor with other GHS-Ra and GHSR1a-like receptors. The boxed letters indicate identical amino acids across all species, and the shaded letters show identical amino acids in more than half of the examined species. Amino acid sequences are available from the DDBJ/EMBL/GenBank databases: clawed frog (XM_002931572), human-1a (NM_198407), rat-1a (NM_032075), dog-1a

(NM_001099945), catfish-1a (FJ707319), goldfish-1a-1 (AB504275), zebrafish-1a (XM001335981), catfish-2a (FJ707320), goldfish-2a-1 (AB504277), zebrafish-2a (XM001340372), chicken-1a (AB095995), quail-1aS (AB469019), rainbow trout GHSR1a-like receptor (GHSR1a-LR)-DQTA (AB362479), rainbow trout GHSR1a-LR-ERAT (AB362480), seabream-1a (AY151040), tilapia GHSR1a-LR (AB361053).

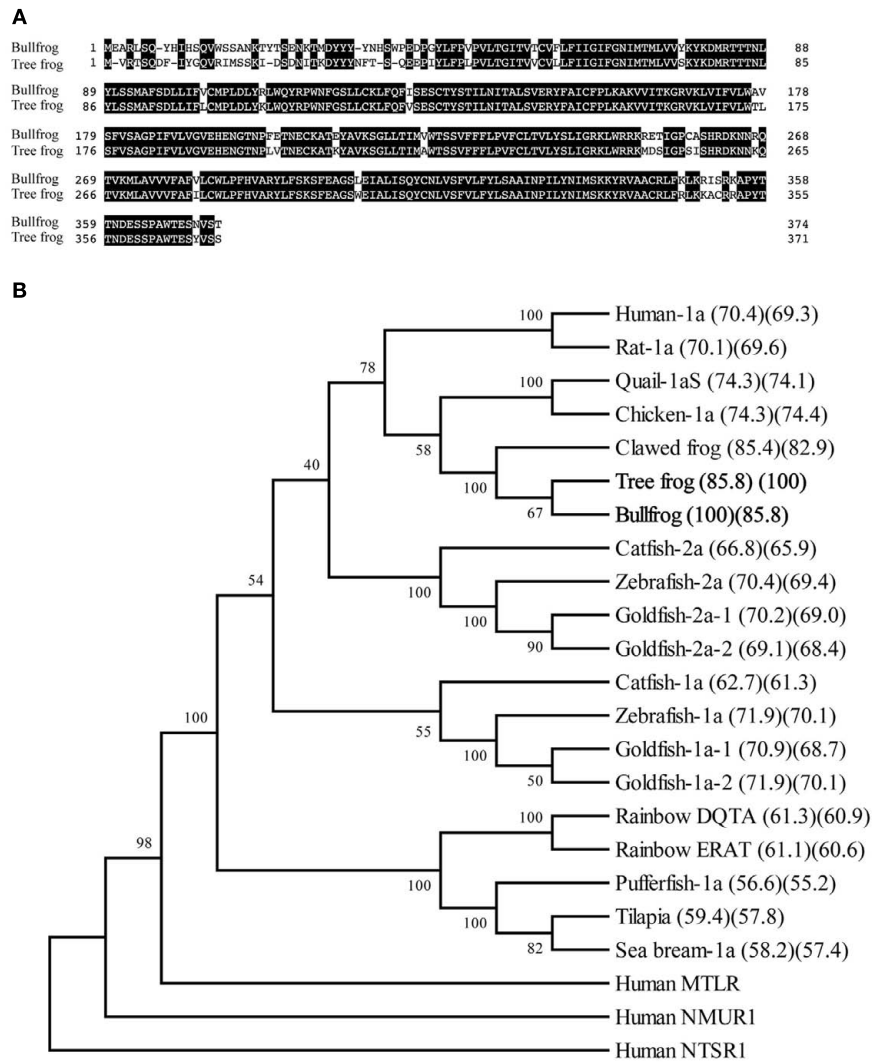


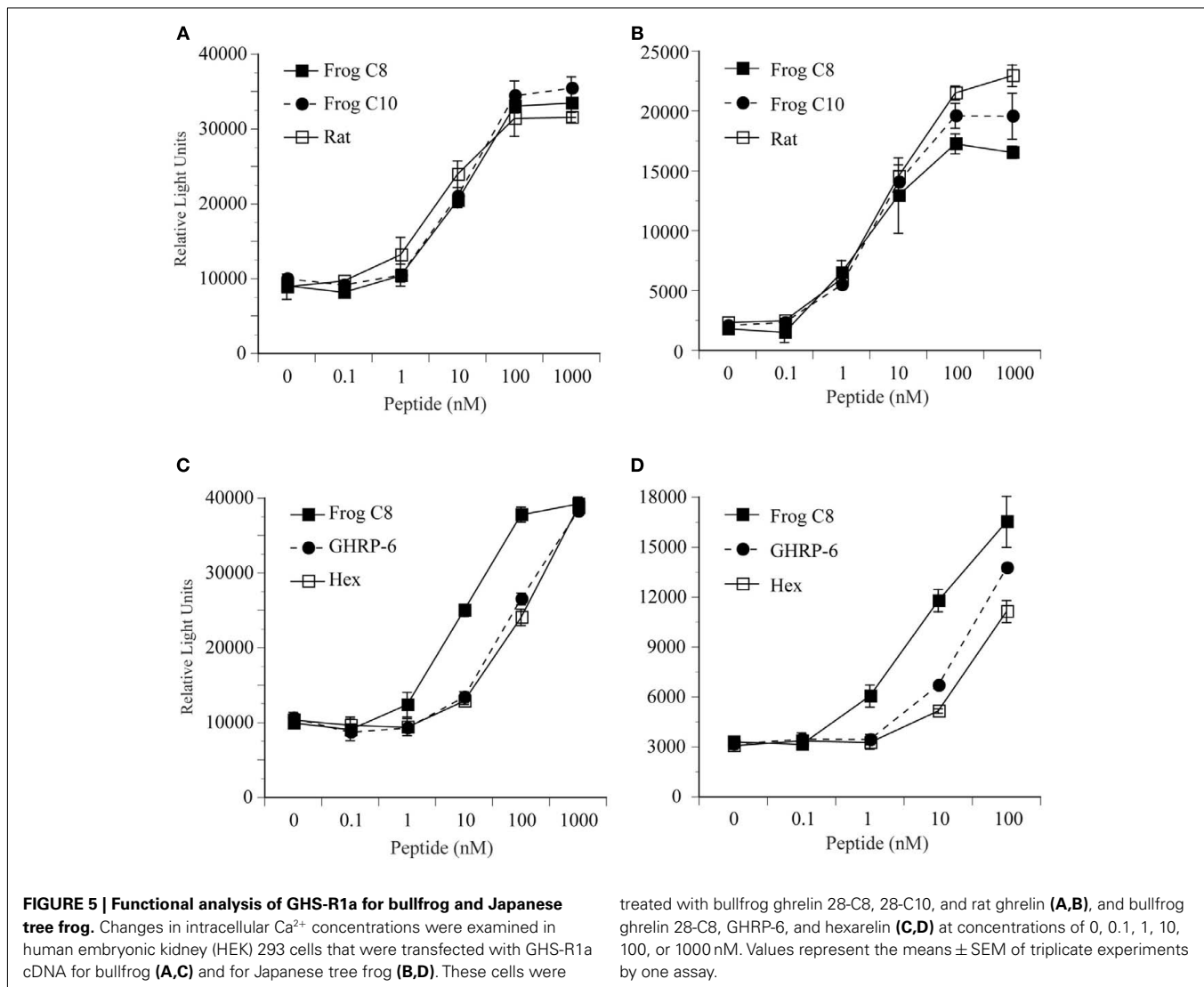
FIGURE 4 | Comparison of the amino acid sequence of GHS-Ra-like receptor between bullfrog and Japanese tree frog (A), and phylogenetic analysis of GHS-Ra and related receptors (B). (A) The boxed letters indicate identical amino acids between the two species. The sequence identity is 86%. (B) A phylogenetic tree was generated by the NJ method of MEGA4 (<http://www.megasoftware.net/>). Human motilin receptor (MTLR), neuromedin U receptor-1 (NMUR1), and neurotensin

receptor-1 (NTSR1) were included as an out-group. The numbers at branch points represent bootstrap values (1000 repetitions). The value in parenthesis indicates amino acid sequence identity to bullfrog (left) and tree frog (right). Amino acid sequences except those indicated in **Figure 3** are available in the DDBJ/EMBL/GenBank databases: human MTLR (NM_001507), human NMUR1 (NM_006056), human NTSR1 (NM_002531).

with GHS-Ra (Palyha et al., 2000; Chen and Cheng, 2004; Kaiya et al., 2009a,b) are regarded as GHS-Ra-LR. When the amino acid sequence of fish GHS-Ra are compared to human GHS-R1a, fish GHS-R2a shows higher identity than fish GHS-R1a [catfish-2a (65.7%) > catfish-1a (62.1%); goldfish-1a-1 (68.2%) > goldfish-2a-1 (66.8%); goldfish 1a-2 (68.1%) > goldfish 2a-2 (65.6%); zebrafish-1a (68.1%) > zebrafish-2a (67.4%)]. If the identity toward human GHS-R1a takes precedence over in terms of naming of GHS-R, it should call (re-name) present fish GHS-R2a as GHS-R1a. Based on these facts, we designate our identified receptor GHS-R1a for frogs.

Expressed frog GHS-R1a proteins were found to be functional, which were able to bind bullfrog and rat ghrelin, and two GHS-R1a

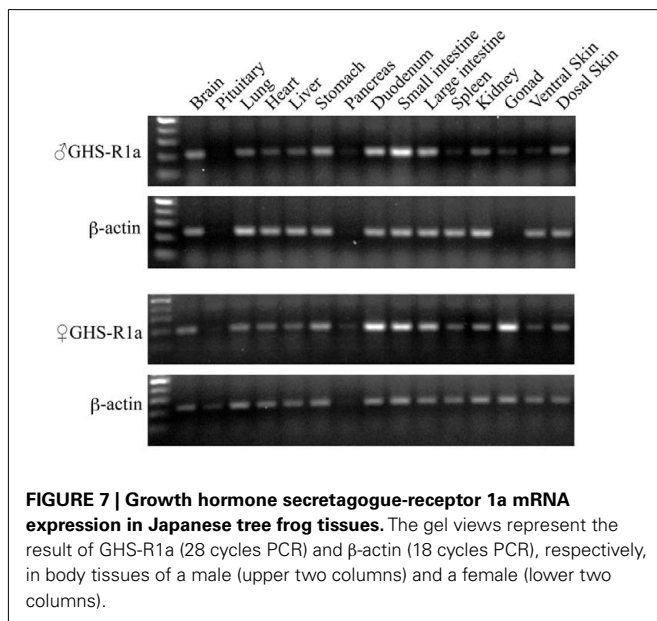
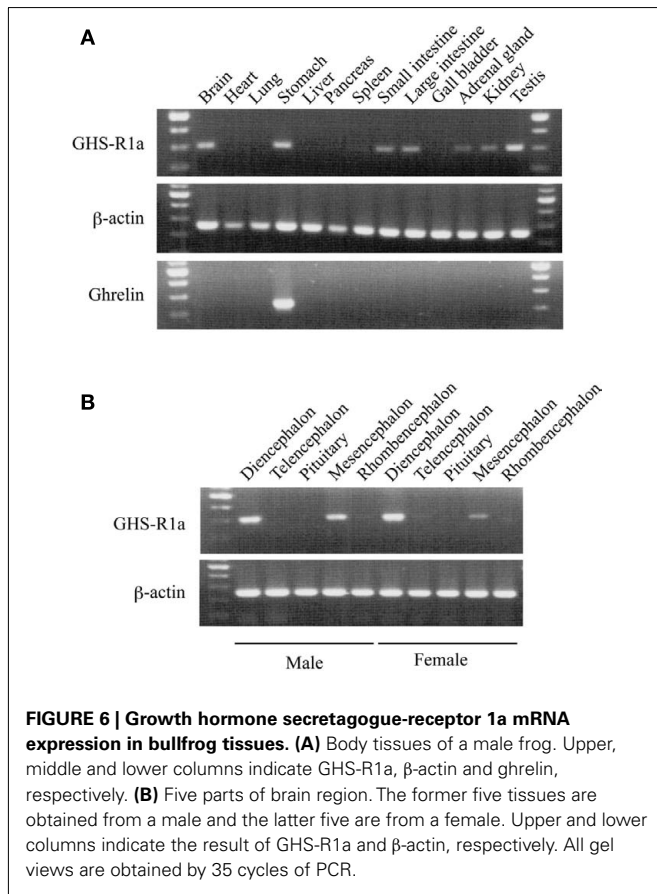
agonists, and induced intracellular Ca²⁺ signaling in transfected HEK293 cells. There are two types of acyl modification in bullfrog ghrelin: octanoylation (C8) and decanoylation (C10; Kaiya et al., 2001). The present result showed that the two modified peptides have equipotency toward bullfrog GHS-R1a, indicating that the two peptides can bind the receptor with the same affinity to the receptor without lipid selectivity. On the other hand, the two GHS-R1a agonists that were equipotent in stimulating calcium signalling, exhibited 1/10 less potency when compared to that of bullfrog ghrelin 28-C8. In goldfish (gf) ghrelin receptor (gfGHS-Ra), a similar interaction to ghrelin or GHSs has been reported between gfGHSR2a-1 and gfGHS-R1a-1: gfGHSR2a-1 shows high affinity to all ligands examined such as goldfish ghrelin, GHRP-6



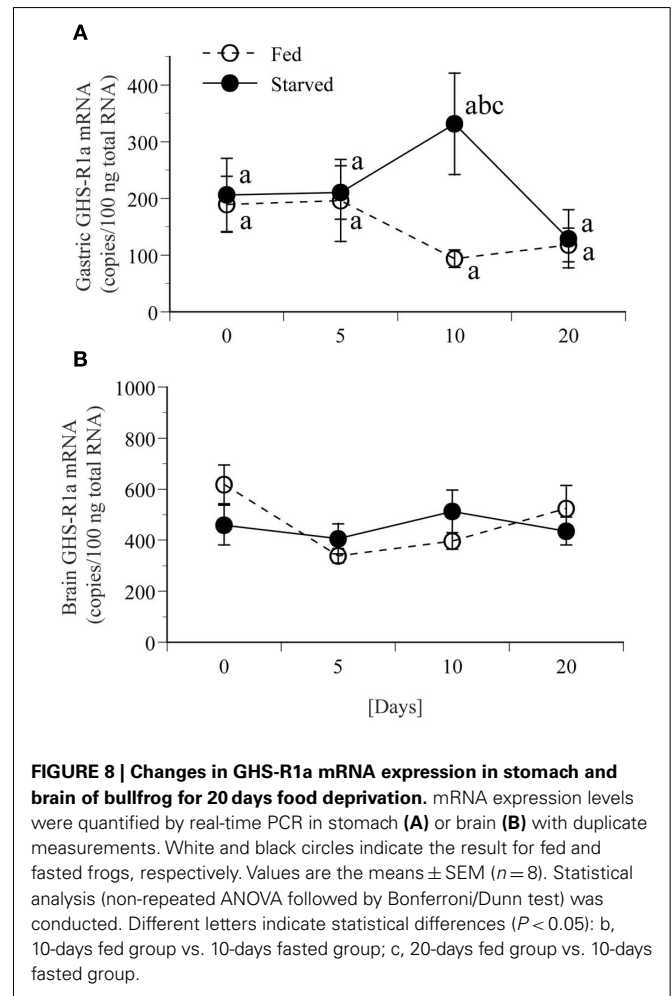
and hexarelin, but gfGHS-R1a-1 has low affinity to hexarelin. One reason would be due to the specific agonistic structure to human (mammalian) GHS-R1a in the evolutionary process. Therefore, low affinity to GHS-R1a agonists observed in bullfrog GHS-R1a could be due to the substitutions of key amino acid in positions that are important for determining the affinity to GHS-R1a agonists (Feighner et al., 1998). In frog receptors, Val¹³¹ for human GHS-R1a was substituted with Ile; which could be responsible for the low affinity. Recently, Ueda et al. (2010) reported several important amino acids in the human GHS-R1a structure for ghrelin or GHRP-6-inducing intracellular Ca^{2+} signaling and ghrelin binding, which include Cys¹¹⁶, Cys¹⁹⁸ and Glu¹⁸⁷, and they are conserved in both frog receptors.

Ligand selectivity between bullfrog and tree frog GHS-R1a was not observed as expected. We speculated that Thr³-ghrelin (bullfrog ghrelin) could show much higher affinity than Ser³-ghrelin (rat ghrelin) in bullfrog GHS-R1a, and inversely Ser³-ghrelin could activate tree frog GHS-R1a more potently than Thr³-ghrelin. The reason may be related to the fact that identified GHS-R1a mRNA

has not been detected (expressed) in the pituitary of both frogs. It was very strange because a previous *in vitro* experiment showed that bullfrog ghrelin has more potent GH- and PRL-releasing activities than rat ghrelin in bullfrog pituitary cells (Kaiya et al., 2001), indicating the direct effect of ghrelin to the pituitary, and it strongly suggests the presence of ghrelin receptor that selectively binds bullfrog ghrelin (Thr³-ghrelin) in the bullfrog pituitary. Ghrelin receptor mRNA has mainly been detected in the pituitary of almost all animals examined so far (Gnanapavan et al., 2002; Geelissen et al., 2003; Tanaka et al., 2003; Chen and Cheng, 2004; Kaiya et al., 2009a,b; Small et al., 2009). The present result suggests that our identified ghrelin receptor is the brain (hypothalamic) type ghrelin receptor, and it is possible that another (hypophyseal) type of ghrelin receptor is present, which preferentially binds Thr³-ghrelin in the pituitary of both frogs. However, despite several attempts, we were not able to identify the hypophyseal type until now. Further study would be required for the demonstration of the presence of a hypophyseal ghrelin receptor in frogs.

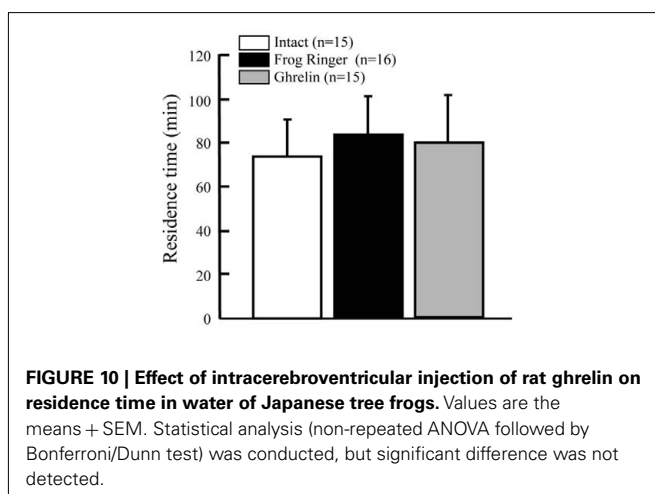
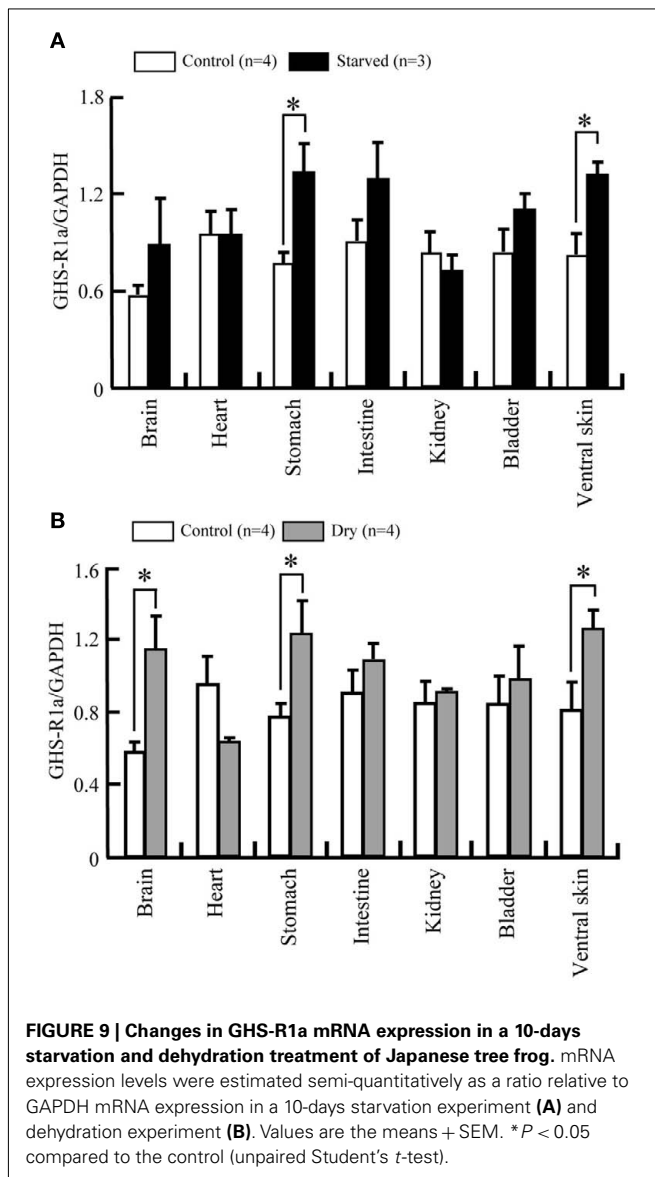


Growth hormone secretagogue-receptor 1a mRNA was detected in various tissues of each frog, and was mainly expressed in the brain, gastrointestinal tract, and kidney. Although its mRNA



distribution is highly similar to the pattern detected in other animals, its involvement in physiological events have to be further elucidated. In Japanese quail and chickens, GHS-R1a mRNA are expressed in the gastrointestinal tract, with the expression level differs in each region of the intestine (Kitazawa et al., 2009). In goldfish, tissue distribution of ghrelin receptor mRNA is different depending on the receptor type; GHS-R1a is mainly expressed in the brain and intestines, whereas GHS-R2a is not present in the intestine (Kaiya et al., 2010). GHS-R1a mRNA expression in frog tissues directly indicates that these are ghrelin's effectors, and it suggests the possible involvement of ghrelin in regulation of brain functions (Olszewski et al., 2008; Briggs and Andrews, 2011), gut motility (Peeters, 2005; Kitazawa et al., 2007, 2009) and renal function (Mori et al., 2000).

Specific GHS-R1a mRNA expression of frog was found in testis of bullfrog, ovary, heart and liver of tree frog. Recently, Izzo et al. (2011) reported testicular expression of ghrelin mRNA in a frog, *P. esculentus*. They showed localization of ghrelin mRNA in the cytoplasm of the interstitial Leydig cells and in the germinal compartment of Sertoli cells. In addition, the mRNA expression varies during reproductive cycle, and 17 β -estradiol affects testicular ghrelin mRNA expression. These results suggest an involvement



of the ghrelin system in frog reproduction. Rak et al. (2009) have suggested functional roles of ghrelin in the porcine ovarian follicles such as estradiol secretion, aromatase activity and cell proliferation. Sun et al. (2010) reported that ghrelin contributes to positive inotropic effect on cardiomyocytes. Recently, hepatoprotective effect of ghrelin has been suggested (Golestan Jahromi et al., 2010; Moreno et al., 2010). Further study is needed to clarify physiological actions of ghrelin in frogs. Recently, presence of ghrelin and GHS-R1a proteins have been demonstrated in the pancreas of *X. laevis* (Suzuki and Yamamoto, 2011). We could not confirm mRNA expression of GHS-R1a in the pancreas of bullfrog and tree frog in this study. Further detailed examination is necessary by taking the presence of another receptor type from identified GHS-R1a into consideration.

In bullfrog, GHS-R1a mRNA expression in the stomach increased 10 days after fasting. Similarly, in tree frog, a 10-days food deprivation led to increase in GHS-R1a mRNA expression in the stomach. Similar changes in plasma ghrelin levels, gastric ghrelin content and gastric ghrelin mRNA have been observed in our previous study using bullfrog (Kaiya et al., 2006). This may be because that stomach is the primary source for ghrelin production, the gastric GHS-R1a could first detect the ghrelin change. Taking together with the results from the current study, we hypothesized that this could be the metabolic regulation of the ghrelin system in bullfrog. A similar change observed in tree frog may suggest a physiological involvement of the ghrelin system during negative energy balance. In goldfish, GHS-R1a and 2a mRNA expressions do not change in the intestine for 7 days of food deprivation although intestine is the primary source for ghrelin production (Kaiya et al., 2010).

Growth hormone secretagogue-receptor 1a mRNA expression did not change in the brain of both frogs. In fact, little is known about expression changes in GHS-R1a mRNA in the brain of other animals. In rats, receptor density had not changed by nutritional state in the hypothalamus despite of relevant alterations in plasma ghrelin level observed (Harrold et al., 2008). Riley et al. (2008) reported no change in GHSR1a-LR mRNA expression in tilapia brain for 7 days fasting. On the other hand, in rats, GHS-R1a mRNA expression in arcuate nuclei of the hypothalamus increased 48 h after fasting (Nogueiras et al., 2004). Furthermore, Riley et al. (2008) have showed later that a high level of expression of GHSR1a-LR mRNA in whole brain has been observed in short-term 3-h before meal, although the increase is not evident 1–2 h before meal (Peddu et al., 2009). In goldfish, 7 days of fasting decreased *gfGHS-R1a-1* in the vagal lobe, whereas GHS-R1a-2 did not change. On the other hand, no changes were observed for both receptor mRNA expressions in the olfactory lobe (Kaiya et al., 2010). These results indicate that regulation of mRNA expression in goldfish brain during food deprivation is region-specific. In the present study, we used whole brain for gene expression analysis. More detail analyses including region-specific or time-dependent regulation may be required for detecting changes in receptor mRNA expression.

In tree frog, dehydration treatment led to increase in GHS-R1a mRNA expression in the brain, stomach and ventral skin. Little is known about the effect of the ghrelin system on dehydration. Recently, it becomes evident that ghrelin inhibits water intake in

rats, chickens and seawater-acclimated eels (Kozaka et al., 2003; Tachibana et al., 2006; Hashimoto et al., 2007; Hashimoto and Ueta, 2011; Nobata and Takei, 2011). In tree frog, ICV injection of AVT or angiotensin II (Ang II) extended the residence time in water dish, indicating increased water absorption from the ventral skin (Maejima et al., 2008, 2010). These results are supported by the presence of receptor for those hormones in the tree frog brain. Results from the present study show that GHS-R1a mRNA expression increased in the brain after dehydration, suggesting a possible involvement of ghrelin on water absorption in tree frog. However, ICV injection of ghrelin did not affect the residence time although the injected dose (25 pmol/g BW) was considered to be sufficient. As ghrelin is a peripheral, circulating gastric hormone rather than a neuroendocrine hormone, further study is required to examine the effect of peripherally injected ghrelin on water absorption.

In summary, we determined nucleotide sequence of ghrelin receptor (GHS-R1a) cDNA in the two species of anuran

amphibians. The two GHS-R1a are capable of binding ghrelin and inducing intracellular Ca^{2+} signaling, but expected ligand selectivity between Ser³-ghrelin and Thr³-ghrelin was not observed. Since the identified GHS-R1a mRNA was not detected in the pituitary, it is possible that another type of ghrelin receptor could be found in the pituitary. In frogs, GHS-R1a mRNA expression was up-regulated by fasting or dehydration, suggesting that ghrelin is involved in the regulation of appetite and osmoregulation. Further studies are required for understanding the physiological relevance of the ghrelin system in amphibian.

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