Molecular cloning and mRNA expression analysis of two GH secretagogue receptor transcripts in orange-spotted grouper (*Epinephelus coioides*)

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Abstract

GH secretagogue receptor (GHSR) is the receptor of ghrelin, a circulating GH-releasing and appetite-inducing hormone. In this paper, two *Ghsr* cDNAs, *gpGhsr1a* and *gpGhsr1b*, were identified and characterized in a teleost, the orange-spotted grouper (*Epinephelus coioides*). The *gpGHSR1a* is 1512 bp in length with an open reading frame (ORF) that encodes a protein of 383 amino acids with seven transmembrane (TM) domains, while the 1703 bp *gpGHSR1b* contains an ORF encoding for 303 amino acids with five TM domains. Comparison between cDNA and gene sequences showed that the two transcripts are two alternative splicing forms of a single *gpGhsr* gene. Tissue distribution and ontogeny of two *gpGhsr* mRNAs were examined by RT-PCR. The *gpGHSR1a* is mainly expressed in brain and pituitary gland, when compared with a more

widespread expression of *gpGHSR1b*. During embryonic and larval development, the *gpGhsr1b* mRNA appears before the *gpGhsr1a* mRNA. Furthermore, quantitative real-time PCR performed on brain showed that both transcripts have the highest expression level in the pituitary gland. The expression level of *gpGHSR1a* was generally higher than that of *gpGHSR1b*. GHSR expressing cells were also detected widely in grouper brain by *in situ* hybridization, with a broader distribution than previous reports in mammals. Finally, an *in vitro* study showed that expression of both *gpGHSR* transcripts in pituitary and hypothalamus is downregulated by GH and ghrelin but not by des-acetyl ghrelin, and this suggests that feedback regulation of GHSR also exists in teleostean fishes.

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Introduction

The secretion of growth hormone (GH) from the pituitary gland is mediated by a complex interplay of neuroendocrine factors (Peng & Peter 1997, Muller *et al.* 1999, Wong *et al.* 2006). In addition, a group of artificial molecules known as GH secretagogues (GHSs) stimulates GH release (Bowers *et al.* 1980, 1984, Smith *et al.* 1997) via a 'novel' receptor, the GH secretagogue receptor (GHSR). The full-length GHSR, namely GHSR1a, is a G-protein-coupled receptor, which was first isolated from human and porcine pituitary cDNA libraries (Howard *et al.* 1996). An alternative splice variant of the GHSR, termed GHSR1b, has also been found. It is a truncated receptor that has five transmembrane (TM) domains and is devoid of functional activity (Howard *et al.* 1996, McKee *et al.* 1997). The endogenous ligand of GHSR was subsequently identified from the rat stomach and named 'ghrelin' (Kojima *et al.* 1999). In mammals, ghrelin is a peptide of 28 amino acids (aa) with an essential *n*-octanoylation on the Ser3 residue (Bednarek *et al.* 2000, Yang *et al.* 2008) and implicated in many functions besides GH release (reviewed by van der Lely *et al.* 2004, Kojima & Kangawa 2005).

The existence of ghrelin has now been demonstrated in nine teleostean species (reviewed by Unniappan & Peter 2005, Kaiya *et al.* 2008): goldfish, *Carassius auratus* (Unniappan *et al.* 2002); Japanese eel, *Anguilla japonica* (Kaiya *et al.* 2003a); rainbow trout, *Oncorhynchus mykiss* (Kaiya *et al.* 2003b); two tilapia species, *Oreochromis mossambicus* (Kaiya *et al.* 2003c) and *Oreochromis niloticus* (Parhar *et al.* 2003); channel catfish, *Ictalurus punctatus* (Kaiya *et al.* 2005); black sea bream, *Acanthopagrus schlegelii* (Yeung *et al.* 2006); sea bass, *Dicentrarchus labrax* (Terova *et al.* 2008); and Atlantic halibut, *Hippoglossus hippoglossus* L. (Manning *et al.* 2008). Physiological roles of ghrelin in teleosts have also been widely studied, including effects on GH secretion (Riley *et al.* 2002, Kaiya *et al.* 2003a,b,c, Ran *et al.* 2004, Shepherd *et al.* 2007), appetite stimulation (Unniappan *et al.* 2002, 2004, Matsuda *et al.* 2006a, Jönsson *et al.* 2007, Miura *et al.* 2007, Shepherd *et al.* 2007), water intake (Kozaka & Ando 2003), reproduction (Mustonen *et al.* 2002, Parhar *et al.* 2003), immunity (Yada *et al.* 2006), growth (Riley *et al.* 2005, Fox *et al.* 2007), intestinal motility (Olsson *et al.* 2008), and locomotor behavior (Matsuda *et al.* 2006a,b).

However, the GHSR has only been identified from three species of teleosts: pufferfish, *Spheroides nephelus* (Palyha *et al.* 2000), black seabream (Chan & Cheng 2004), and zebrafish, *Danio rerio* (Olsson *et al.* 2008), and a GHSR1b transcript has only been found in black seabream (Chan & Cheng 2004), not in pufferfish (Palyha *et al.* 2000) or zebrafish (Olsson *et al.* 2008). Teleostean GHSRs have high similarities with their mammalian counterparts, and the ligand activation domain of GHSR is evolutionarily conserved from pufferfish to man (Palyha *et al.* 2000). The mRNA distribution of GHSR in brain differs between black seabream (Chan & Cheng 2004) and mammals (McKee *et al.* 1997, Gnanapavan *et al.* 2002). Furthermore, the signal transduction pathway (Chan *et al.* 2004) and the promoter activity (Yeung *et al.* 2004) of GHSR have also been studied in black seabream.

Compared with ghrelin, little work has been performed on teleostean GHSRs. Hence, some important basic aspects of the ghrelin/GHSR system, which are predicted to be specific for teleostean species, may have gone undetected so far. In this study, two transcripts of GHSR were obtained from the orange-spotted grouper, *Epinephelus coioides*, a marine fish widely cultured in Southern and Eastern China. We examined the mRNA expression of two transcripts in various tissues and during early development, and addressed the localization of *Ghsr* mRNA expression in the brain of this fish. Moreover, the effects of seabream GH, rat ghrelin, and rat des-acyl ghrelin on *Ghsr* mRNA expression were investigated in pituitary cells and hypothalamic fragments.

Materials and Methods

Sampling of adult and juvenile grouper

All adult and juvenile fish were obtained from Guangdong Daya-Bay Fishery Development Center (22°36'2" N; 114°32'45" E). Two-year old female orange-spotted groupers, total length of 30–35 cm and body weight of 820–950 g, held at 22–28 °C seawater, were anesthetized in ice and killed by decapitation. Unfertilized and fertilized eggs were collected following natural spawning of brood stock kept in ferroconcrete tanks. Embryos were sampled from 1 to 20 h after fertilization including 16-cell stage, morula stage, blastula stage, gastrula stage, neurula stage, crystal stage, hatch prophase, and hatching. Larvae were reared in indoor ferroconcrete tanks and sampled from newly hatched to 5 days post-hatching (dph). Sampling of grouper embryos and larvae were described in detail previously (Li *et al.* 2005). The samples were snap-frozen in liquid nitrogen immediately until RNA extraction.

cDNA and gene cloning of *Ghsr* in grouper

Total RNA was extracted from adult grouper hypothalamus using Trizol reagent (Invitrogen) and quantified based on absorbance of the samples at 260 nm. The RNA integrity was checked by agarose gel electrophoresis, and the purity was

established by calculating the ratio of the absorbance readings at 260 and 280 nm. First-strand cDNA was synthesized using Superscript III System (Invitrogen) with 4 µg total RNA as template and oligo dT as primer. The experimental strategy of grouper *Ghsr* cDNA cloning is shown in Fig. 1, and all primer sequences used in this study are shown in Table. 1. Degenerate primers, RpS1, RpS2, and RpA2, designed based on the alignment of known GHSR 1a sequences, were used to isolate a fragment of grouper GHSR 1a. The first-round PCR was performed using 1 µl first-strand cDNA as template with RpS1 and RpA2. The semi-nested second-round PCR was performed using 1 µl 100-fold diluted first-round PCR product as template with RpS2 and RpA2. In each case, the amplification condition consisted of an initial denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 15 s, 52 °C for 15 s and 72 °C for 1 min, and then a final extension at 72 °C for 5 min.

The full-length cDNA was obtained using Rapid Amplification of cDNA Ends (RACE) methods. Five gene specific primers (GSP), R3-1, R3-2 R5-1, R5-2, and R5-3, were designed according to the obtained *Ghsr1a* cDNA fragment. For 3'-RACE, the first-strand cDNA was synthesized from 4 µg total RNA using Superscript III System (Invitrogen) and Anchor Primer (AP, Gibco-BRL). The first-round PCR was performed using 1 µl first-strand cDNA as template with R3-1 and Abridged Universal Amplification Primer (AUAP, Gibco-BRL) under the following condition: an initial denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 15 s, 56 °C for 15 s, and 72 °C for 1 min 30 s, and then a final extension at 72 °C for 5 min. The second-round PCR was performed using 1 µl 100-fold diluted first-round PCR product as template with R3-2 and AUAP under the same cycling condition as the first-round PCR. For 5'-RACE, the first-strand cDNA was synthesized using Superscript III System (Invitrogen) with 4 µg total RNA as template and R5-1 as primer. Subsequently, the synthesized cDNA was purified using E.Z.N.A Gel Extraction Kit (Omega, Norcross, GA, USA). After that, in a 50 µl tailing reaction mixture, a homopolymeric tail was added to the 3'-end of the purified cDNA using Terminal deoxynucleotidyl Transferase (TaKaRa, Shiga, Japan) and dCTP (Invitrogen). The first-round PCR was performed using 2 µl of the dC-tailed reaction mixture as template with R5-2 and Abridged AP (Gibco-BRL) under the following condition: 94 °C for 3 min, 35 cycles of 94 °C for 15 s, 58 °C for 15 s, and 72 °C for 1 min, and then a final extension at 72 °C for 5 min. The second-round PCR was performed using 1 µl 100-fold diluted first-round PCR product as template with R5-2 and AUAP under the same cycle profile

Figure 1 Experimental strategy used to obtain complete *gpGhsr1a* and *gpGhsr1b* cDNA.

Table 1 Oligo-deoxynucleotide primers used in the experiments

	Primer	Sequence
Fragment		
<i>gpGhsr1a</i> cDNA partial cloning	RpS1	5'-CCTSTGYATGCCSCTSGACCT-3'
	RpS2	5'-CCACVRTSCTCAVCATCACMGCG-3'
	RpA2	5'-TGASAGRATRGGGTGAT-3'
<i>gpGhsr1a</i> cDNA 3'-RACE	R3-1	5'-CCAAAAGGCGGGTGC GCGCCC-3'
	R3-2	5'-AAGCAACAGGCAGACCATC-3'
<i>gpGhsr1a</i> cDNA 5'-RACE	R5-1	5'-ATTCCGTCCAGCCGTTGAG-3'
	R5-2	5'-CTTGATGGTCTGCCTGTTGCT-3'
	R5-3	5'-CCACTGCGTAATGCGTCATC-3'
<i>gpGhsr1a</i> and <i>gpGhsr1b</i> cDNA ORF cloning	Rorf1F	5'-CCGGGGTGCCTTCAGCAGT-3'
	Rorf1R	5'-AATGGCTTTGGGTGAGTGTTA-3'
<i>gpGhsr1a</i> tissue distribution	R1aTF	5'-ATCAAGATGCTGGTGGTGGT-3'
	R1aTR	5'-TCCGTCCAGCCGTTTGAG-3'
<i>gpGhsr1b</i> tissue distribution	R1bTF	5'-CACCTCGCCAGGCTGAAT-3'
	R1bTR	5'-AACGGCAACCAGCATAGAACG-3'
Reference gene 18S	18SF	5'-CCTGAGAAACGGCTACCACATCC-3'
	18SR	5'-AGCAACTTAGTATACGCTATTGGAG-3'
Universal primer	AUAP	5'-GGCCACGCGTCGACTAGTAC-3'
	AP	5'-GGCCACGCGTCGACTAGTAC(T) ₁₆ -3'
	AAP	5'-GGCCACGCGTCGACTAGTACGGGIIIGGGIIIGG-3'

as the first-round PCR except that an annealing temperature of 56 °C was used instead.

To confirm that the composite sequence was indeed derived from a single mRNA transcript, a RT-PCR was performed using 1 µl first-strand cDNA for partial cloning as template, with two GSPs, Rorf1F and Rorf1R, derived from the 5' and 3' ends of the composite sequence respectively. The amplification conditions consisted of an initial denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 15 s, 56 °C for 15 s, and 72 °C for 2 min, and then a final extension at 72 °C for 5 min.

For gene structure determination, genomic DNA was extracted from grouper liver using the classic Genome DNA Isolation kit (Bio Basic Inc., Markham, Ontario, Canada). A portion of *Ghsr* gene was amplified from 20 ng genomic DNA by PCR with GSPs Rorf1F and Rorf1R. The PCR condition was: an initial denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 15 s, 56 °C for 15 s, and 72 °C for 2 min, and then a final extension at 72 °C for 5 min.

PCR products were purified using E.Z.N.A Gel Extraction Kit (Omega) and subcloned into pGEM-T Easy vectors (Promega). The recombinant plasmids were sequenced from three independent clones using the ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA). The homology was analyzed using MegAlign and the phylogenetic tree was constructed using PHYLIP and viewed using tree view. Reliability of the tree was evaluated by the bootstrap method with 1000 replications.

RT-PCR and qRT-PCR: tissue distribution and ontogeny of Ghsr mRNA

Tissue distribution and ontogeny of grouper *Ghsr* mRNA were examined by semi-quantitative RT-PCR, and the

expression levels of two *GHSR* transcripts in grouper brain were quantified by real-time PCR. Total RNA was extracted from 7 brain regions, 18 peripheral tissues, and 16 developmental stages of embryos and larvae. For larval samples, total RNA was extracted from the whole body. The total RNA was then quality verified and quantified as stated above for the cDNA cloning. Each RNA extract (2 µg) was reversely transcribed into first-strand cDNA using ThermoScript RT-PCR System (Invitrogen) after it was treated by DNaseI (Invitrogen). The RNA sample was also tested for genomic DNA contamination by no enzyme reverse transcription control.

Firstly, for semi-quantitative RT-PCR, the GSPs R1aTF and R1aTR were designed to specifically amplify a *GHSR1a* fragment of 308 bp, and R1bTF and R1bTR were designed to specifically amplify a *GHSR1b* fragment of 188 bp. For tissue distribution, the semi-quantitative RT-PCR was validated using the RT product of hypothalamus as template by performing PCR in different cycles to determine the cycle numbers that generated half maximal PCR product and, for ontogeny, the RT product of 5 dph was used as template instead. In the study of tissue distribution, PCRs were performed on 36 and 42 cycles for *GHSR1a* and *GHSR1b* respectively and, in the study of ontogeny, PCRs were performed on 42 cycles for both transcripts, under identical PCR conditions with a cycle profile of 94 °C for 15 s, 57 °C for 15 s, and 72 °C for 30 s. Ribosomal RNA 18S was used as the internal control and amplified under the same condition as *GHSR* transcripts except that a tenfold diluted first-strand cDNA was used as template and the amplification cycle number was 18. RT-PCR products of both *GHSR1a* and *GHSR1b* were confirmed by Southern blot analysis. The procedure of Southern blot was as described by Li *et al.*

(2005), and signal was captured using Genescope system (SynGene, Cambridge, UK).

Secondly, real-time PCR was additionally used to quantify the mRNA expression levels of two *GHSR* transcripts in grouper brain and pituitary. The brain was cut into seven dissected regions including olfactory bulbs, telencephalon, diencephalon, cerebellum, medulla, spinal cord, and hypothalamus. The real-time PCRs were performed using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) in ABI PRISM 7900 Sequence Detection System (Applied Biosystems). Data obtained from three individuals of two *GHSR* transcripts were compared with data obtained from amplification of the reference gene, 18S. Primer pairs R1aTF/R1aTR, R1bTF/R1bTR, and 18SF/18SR were used to amplify the *GHSR*1a product of 308 bp, the *GHSR*1b product of 188 bp, and the 18S product of 250 bp respectively. The reaction mix (20 µl) contained 4 µl RT product, 0.4 µl (10 µM) for each forward and reverse primers, 10 µl Platinum SYBR Green qPCR SuperMix-UDG, and 0.4 µl ROX reference Dye. All real-time PCRs were performed under the same conditions: 95 °C for 10 min, 50 cycles of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min. Fluorescent data for *GHSR*1a, *GHSR*1b, and 18S were collected at 87, 82, and 80 °C respectively. Melt curve analysis was performed over a range of 55–95 °C to verify single product generation at the end of the assay. The standard curve quantification method was used to analyze the data. A standard curve for each gene was made from a tenfold dilution series of plasmid ranging from 10^{-4} to 10^{-10} ng DNA. Data for all genes were converted to copy numbers, and data for two *GHSR* transcripts were then normalized with the geometric mean of the copy numbers determined for the reference gene, 18S.

In situ hybridization (ISH) of GHSR expression cells in grouper brain

Anti-sense and sense cRNA probes were synthesized from linearized *GHSR* plasmids using an *in vitro* transcription DIG-labeling Kit (Roche). One of the anti-sense probes was designed to detect all isoforms of *GHSR* and the other was designed to detect the *GHSR*1a isoform specifically.

Samples of brain were quickly removed and fixed overnight in 4% paraformaldehyde in phosphate buffer (PBS). Samples were dehydrated in an ethanol series, immersed in xylene, and embedded in paraffin. Sagittal and coronal sections (8 µm) were cut and mounted onto 3-aminopropyltriethoxy-silane-coated slides.

Then the sections were deparaffinized with xylene, rehydrated through an ethanol series, and washed twice in PBS for 1 min. The sections were then treated with 6 µg/ml proteinase K in proteinase K buffer (0.1 M Tris-HCl, and 50 mM EDTA (pH 8.0)) at 37 °C for 30 min, washed once in PBS for 5 min, and fixed in 4% paraformaldehyde in PBS for 15 min. The sections were rinsed twice in DEPC-treated water, incubated in 0.1 M triethanolamine-HCl (pH 8.0)

with 0.25% acetic anhydride for 10 min and then washed in PBS for 5 min. The sections were prehybridized in hybridization buffer (50% formamide, 5×SSC, 1×Denhardt's solution, 0.1 mg/ml sonicated salmon sperm DNA, and 10% dextran sulfate) at 65 °C for 1 h. Subsequently, the probes diluted to 200 ng/ml were added into the hybridization buffer, and the sections were incubated at 48 °C for 16 h in a humid chamber. During hybridization, the sections were covered with PARAFILM (Alcan Packaging, Neenah, WI, USA), and sense probes were used as negative controls. After hybridization, the covers were removed by soaking the slides in 5×SSC. The sections were then washed twice in 2×SSC at 67 °C for 15 min, once in 0.5×SSC at 65 °C for 15 min, and then twice in 0.2×SSC at 65 °C for 30 min. The sections were incubated in buffer-1 (100 mM maleic acid (pH 7.5), 150 mM NaCl) for 5 min, immersed in 1.0% blocking reagent (Roche) prepared in buffer-1 at 37 °C for 1 h, and finally incubated with alkaline phosphatase-conjugated anti-DIG antibody (Roche) 1:2000 diluted in buffer-1 at 37 °C for 30 min. After that, the sections were washed twice in buffer-1 for 15 min and incubated in buffer-3 (100 mM Tris-HCl (pH 9.5) and 100 mM NaCl) for 3 min. The chromagen solution (337 µg/ml 4-nitroblue tetrazolium chloride and 175 µg/ml 5-Bromo-4-chloro-3-indolyl phosphate dissolved in buffer-3) was added, and the sections were incubated until a visible signal was detected. The reaction was stopped by addition of a reaction stop solution (10 mM Tris-HCl (pH 7.6) and 1 mM EDTA (pH 8.0)). The sections were dehydrated with an ethanol series and vitrified with xylene, then mounted with neutral balsam. All sections were viewed and photographed with an Axiovert 40 CFL microscope (Zeiss, Thornwood, NY, USA).

Regulation of Ghsr mRNA expression in pituitary and hypothalamus

Grouper pituitary cells were prepared by a modified enzyme digestion method (Wong *et al.* 1998). The pituitary glands were washed with M199 medium containing antibiotics (100 U/ml penicillin and 100 U/ml streptomycin) and digested with 1 mg/ml trypsin in Ca^{2+} -free HBSS at 28 °C for 40 min. The protease digestion was terminated by incubation in M199 medium containing 10% FCS at 28 °C for 5 min, following by further digestions in 10 ng/ml DNaseI, 2 mM EDTA, and 1 mM EDTA at 28 °C for 5 min respectively. Then, the loosened tissue was washed with 10 ng/ml DNaseI at 28 °C for 5 min and filtered through a 40 µm nylon mesh. The dispersed pituitary cells were harvested by mild centrifugation (200 g for 10 min) and finally re-suspended in M199 medium. The viability of the cells was assessed by Trypan blue exclusion, and only preparations showing more than 95% viability were used in subsequent experiments.

The grouper hypothalamus was dissected out and washed with M199 medium containing antibiotics. Then, it was

Figure 2 Comparisons of nucleotide and amino acid sequences between gpGHSR1a and gpGHSR1b. TM domains are boxed.

Figure 3 Phylogenetic tree of the gpGHSR1a amino acid sequence with other vertebrate species. The other amino acid sequences of GHSR1a were obtained from GenBank and phylogenetic tree was constructed using unrooted analysis with PHYLIP & Tree View. Reliability of the tree was evaluated by the bootstrap method with 1000 replications. Black seabream, *Acanthopagrus schlegelii* (AY151040), Cattle, *Bos taurus* (XM_592014), Chicken, *Gallus gallus* (NM_204394), Chimpanzee, *Pan troglodytes* (XM_516878), House mouse, *Mus musculus* (NM_177330), Human, *Homo sapiens* (NM_198407), Pig, *Sus scrofa* (Q95254), Rhesus monkey, *Macaca mulatta* (XP_001084886), Norway rat, *Rattus norvegicus* (NM_032075), Pufferfish, *Sphoeroides nephelus* (AAC33472), and Sheep, *Ovis aries* (NM_001009760).

diced into fragments ($<1 \text{ mm}^3$) with razor and washed thrice with M199 medium.

Dispersed pituitary cells (2.5×10^5 cells/well) and hypothalamic fragments were cultured in 24-well plates in M199 medium (pH 7.2, with 100 U/ml penicillin, 100 U/ml streptomycin, and 10% FCS) at 28°C with 5% CO_2 and saturated humidity. Samples were routinely incubated overnight to allow for recovery of membrane receptors after trypsin digestion. On the following day, the samples were washed with serum-free medium. The medium was removed again and replaced by medium containing stimulus, including seabream GH (Groupe, Thebarton, SA, Australia), rat ghrelin (Bachem AG, Switzerland), and rat des-*n*-octanoyl ghrelin (Bachem AG) in various concentrations at 28°C for 45 min.

After drug treatment, the medium was removed and the pituitary cells and hypothalamic fragments were collected and dissolved in Trizol reagent (Invitrogen). Total RNA extraction, first-strand cDNA synthesis, and expression levels analysis for two gpGHSR transcripts by real-time PCR were performed using the same protocols stated above. Data were normalized as percentage of each control groups and expressed as mean $\pm$ S.D. ($n=3$). Significant differences between each control and treatment groups were analyzed by ANOVA followed by

Figure 4 Diagram illustrating the intron/exon organization of the grouper *Ghsr* gene. Two exons are separated by a small intron. The deduced 383 aa gpGHSR1a protein is encoded by two exons, and the deduced 303 aa gpGHSR1b protein is encoded by exon I and part of the intron.

Fisher's least significance difference test (statistical analysis was done using SPSS 13.0, SPSS Inc., Chicago, IL, USA).

Results

Cloning of grouper Ghsr1a and Ghsr1b cDNA

In a partial cDNA cloning procedure, a 630 bp fragment was amplified from grouper hypothalamus using a semi-nested RT-PCR approach. Subsequently, two fragments of 617 and 744 bp were obtained in 3'- and 5'-RACE respectively. During the further cloning of open reading frame (ORF), however, two bands of about 1200 and 1500 bp were amplified, both of which were identified as transcripts of grouper GHSR (gpGHSR1a and gpGHSR1b). The *gpGhsr1a* cDNA was comprised of 1512 bp, including 87 bp in the 5'-untranslated region (UTR), 276 bp in the 3'-UTR, and 1149 bp in the ORF that encoded for a putative protein of 383aa with seven TM domains as indicated by hydrophobicity analysis. The *gpGhsr1b* cDNA was 1703 bp in length, with a 5'-UTR of 87 bp, a 3'-UTR of 707 bp, and an ORF of 909 bp, encoding a putative protein of 303aa that lacked TM-6 and TM-7 found in *gpGhsr1a* (Fig. 2). The *gpGhsr1b* cDNA had an extra 192 bp, which contained a stop codon between the two fragments, the same as *gpGhsr1a* does. As a result, though *gpGhsr1b* has a larger transcript size than *gpGhsr1a*, it actually encodes for a shorter truncated form of *gpGhsr1a*.

The aa sequence deduced from *gpGhsr1a* cDNA shows high homology with other teleostean GHSR1a (black

seabream: 93·0%; pufferfish: 85·6%) and lower homology with those from avian and mammalian species (chicken: 64·0%, pig: 57·1%, sheep: 54·4%, cattle: 55·7%, house mouse: 55·5%, Norway rat: 54·7%, rhesus monkey: 56·0%, chimpanzee: 55·7%, and human: 55·7%). Phylogenetic tree analysis of the GHSR1a aa sequences in various vertebrate species shows that gpGHSR1a clusters with high bootstrap in the lineage of other teleosts (black seabream and pufferfish), sharing the highest homology with black seabream, whereas the GHSR1a of avians (chicken) and mammals (pig, cattle, sheep, mouse, rat, monkey, chimpanzee, human) were grouped into two other distinct clusters (Fig. 3).

Structure of grouper Ghsr gene

In genomic PCR, a single product of 1340 bp was amplified. The obtained *Ghsr* gene fragment had two exons (exon I of 924 bp and exon II of 588 bp) separated by a small intron of 191 bp (Fig. 4). Comparison of the cDNA sequences and the gene sequence revealed that *gpGhsr1a* cDNA was composed of two exons: exon I contained the 5'-UTR up until Leu279 and exon II contained the left part of the G-protein-coupled receptor and the 3'-UTR. On the other hand, *gpGhsr1b* cDNA had completed the same sequence with the *gpGhsr* gene. For gpGHSR1b transcript, the small intron was retained, thus giving rise to the longer transcript. However, the presence of a stop codon in the intron resulted in the truncated receptor with only five TM domains (Fig. 2).

Tissue distribution of Ghsr1a and Ghsr1b mRNA in grouper

Assessment using RT-PCR followed by more sensitive Southern blot analysis in brain and peripheral tissues showed that *gpGhsr1a* and *gpGhsr1b* mRNA had different expression patterns. The expression of *gpGhsr1a* mRNA was predominantly observed in the seven brain regions and pituitary gland, and at much lower levels in heart, thymus, anterior intestine, middle intestine, posterior intestine, and liver, but there was no detectable expression in other tissues analyzed (Fig. 5). On the contrary, *gpGhsr1b* mRNA was present in the all brain regions and peripheral tissues studied (Fig. 5).

Figure 5 Tissue distribution of *Ghsr1a* and *Ghsr1b* mRNA in 2-years-old grouper. 1, olfactory bulbs; 2, telencephalon; 3, diencephalon; 4, cerebellum; 5, medulla; 6, spinal cord; 7, hypothalamus; 8, pituitary; 9, gill filament; 10, heart; 11, spleen; 12, liver; 13, stomach; 14, foreintestine; 15, midintestine; 16, posteriorintestine; 17, heart kidney; 18, kidney; 19, thymus; 20, gonad; 21, red muscle; 22, white muscle; 23, skin; 24, fat; M, 100 bp marker.

Figure 6 Expression of *Ghsr1a* and *Ghsr1b* mRNA in various embryonic and larval developmental stages in grouper. 1, unfertilized eggs; 2, fertilized eggs; 3, 16-cell stage; 4, morula stage; 5, blastula stage; 6, gastrula stage; 7, neurula stage; 8, crystal stage; 9, hatch prophase; 10, hatching; 11, 1-dph; 12, 2-dph; 13, 3-dph; 14, 4-dph; 15, 5-dph; 16, negative control; M, 100 bp marker.

Ontogeny of *Ghsr1a* and *Ghsr1b* mRNA in grouper

Expression of *gpGhsr1a* and *gpGhsr1b* mRNA was also examined during embryonic and larval development, including unfertilized eggs, just fertilized eggs, 16-cell stage, morula stage, blastula stage, gastrula stage, neurula stage, crystal stage, hatch prophase, hatching, and larva at 1, 2, 3, 4, and 5 dph. The *gpGhsr1a* mRNA was first detected from the neurula stage on with little change of expression even by 2 dph, then increased gradually from 3 dph and reached a higher expression at 5 dph (Fig. 6). In contrast, *gpGhsr1b* mRNA was found in fertilized but not in unfertilized eggs and, from blastula stage to 5 dph, it was expressed at a steady

level (Fig. 6). Southern blot analysis confirmed the authenticity of the RT-PCR result (Fig. 6).

Quantification of *Ghsr1a* and *Ghsr1b* mRNA expression in grouper brain

Since ghrelin exerts many of its known physiological functions on brain and in the pituitary gland, and since two transcripts of gpGHSR were highly expressed in these tissues, we used real-time PCR to quantify expression levels more accurately in these parts. Real-time PCR showed that both *gpGhsr1a* and *gpGhsr1b* mRNAs were expressed in pituitary gland and all brain regions (Fig. 7), and confirmed the expression sites detected by RT-PCR. Both *gpGhsr1a* and *gpGhsr1b* mRNA were most abundant in pituitary gland, for gpGHSR1a followed by hypothalamus, cerebellum, telencephalon, medulla, diencephalon, spinal cord, and olfactory bulbs, and for gpGHSR1b followed by cerebellum, hypothalamus, spinal cord, telencephalon, medulla, diencephalon, and olfactory bulbs. The expression levels of gpGHSR1a were higher than those of gpGHSR1b in all parts analyzed (Fig. 7).

Localization of *Ghsr* mRNA expression cells in grouper brain

ISH of GHSR expressing cells on sagittal (Fig. 8A) and coronal (Fig. 8B) sections revealed a widespread distribution in several brain regions and the pituitary gland in grouper. To assess the relative contribution of the two transcripts of GHSR in grouper brain, adjacent brain sections were examined in parallel for hybridization with the probe against common region and the probe specifically against the gpGHSR1a transcript respectively. However, the expression patterns as detected by the two sets of probes were virtually identical both locally and quantitatively.

GHSR expressing cells in grouper brain showed diverse shapes and volumes in different regions. In olfactory bulbs and telencephalon, the GHSR expressing cells were small, round, and distributed orderly with a low density in the internal cell layer and a high density in the external cell layer (Fig. 8A2, B1 and B2). GHSR expressing cells were found in several regions of the diencephalon. They are of fusiform shape with obvious fibers in/connecting to the preoptic area and anterior

Figure 7 Real-time PCR quantification of GHSR1a and GHSR1b expression in brain and pituitary gland of 2-years-old grouper. Results are represented as means $\pm$ s.d. ($n=3$ from three individual groupers). The gpGHSR copy numbers data were normalized by the geometric mean of copy data ($\times 10^{12}$) from reference gene 18S rRNA. Ob, olfactory bulbs; Te, telencephalon; Dp, diencephalon; Ce, cerebellum; Me, medulla; Sc, spinal cord; Hp, hypothalamus; Pit, pituitary. ■, gpGHSR1a; □, gpGHSR1b.

hypothalamus (Fig. 8A3 and B4). Small and round cells were found in posterior hypothalamus similar to those in the telencephalon (Fig. 8A4, B4 and B5). In mesencephalon and cerebellum, GHSR expressing cells were small, fusiform in shape with bipolar structure and distributed annularly (Fig. 8A5 and A6). Besides, one sort of GHSR expressing cell of huge shape appeared in the medulla (Fig. 8A7 and B6). Otherwise, the positive signal was intense in pituitary tissue; however, the shape of GHSR expressing cells was not clear in this region (Fig. 8A8).

Regulation of *gpGhr* mRNA in pituitary and hypothalamus

For *gpGHSR*1a, 10^{-7} M seabream GH reduced its mRNA expression only in primary cultured pituitary cells, and 10^{-5} M rat ghrelin decreased its mRNA expression both in primary cultured pituitary cells and hypothalamic fragments, but other tests did not effectively alter mRNA levels (Fig. 9A). For *gpGHR*-R1b, 10^{-7} M seabream GH down-regulated mRNA expression in both primary cultured

pituitary cells and hypothalamic fragments; 10^{-5} M rat ghrelin decreased its expression only in cultured hypothalamic fragments (Fig. 9B). However, rat ghrelin at a concentration of 10^{-6} M and 10^{-7} M and rat des-acyl ghrelin in all concentrations tested did not significantly change the mRNA expression of both *gpGHSR* transcripts (Fig. 9A and B).

Discussion

Two full-length cDNAs, *gpGHSR*1a and *gpGHSR*1b, for GHSR were cloned from grouper hypothalamus using RT-PCR and RACE methods. When compared with the full-length G-protein-coupled receptor *gpGHSR*1a, which has seven TM domains, *gpGHSR*1b is a truncated receptor that lacks TM-6 and TM-7. The meaning of structural multiformity of GHSR for physiology in different species is still unclear. GHSR1a has been identified in all the species studied before, including humans, pig (Howard *et al.* 1996), rat (McKee *et al.* 1997), pufferfish (Palyha *et al.* 2000), chicken (Geelissen *et al.*

Figure 8 (continued)

Figure 8 Sagittal and coronal sections schematic drawings and photos of the distribution of *Ghsr* mRNA expression cells in the brain of grouper. (A) sagittal sections. 1, schematic drawing; 2, telencephalon; 3, anterior hypothalamus; 4, posterior hypothalamus; 5, diencephalons; 6, cerebellum; 7, medulla; 8, pituitary. Scale bars of 5×photos are 20 μm. Scale bars of 40×photos are 10 μm. No positive signal was found with the sense probe (inset). (B) coronal sections. 1, olfactory bulbs–telencephalon; 2, telencephalon; 3, telencephalon–diencephalon–pituitary; 4, diencephalons–pituitary; 5, cerebellum–diencephalons–hypothalamus; 6, cerebellum–medulla. Scale bars of 5×photos are 100 μm. Abbreviations: OB, olfactory bulbs; dTA, dorsal telencephalic area; vTA, ventral telencephalic area; PoA, preoptic area; Eth, epithalamus; Vth, ventral thalamus; Dth, dorsal thalamus; PT, posterior tuberculum; HP, hypothalamus; PA, periventricular area; OT, optic tectum; Prt, pretectum; TM, tegmentum; ST, semicircular torus; LT, longitudinal torus; CT, cerebelli tectum; Va, valvula cerebelli; HB, hindbrain; Pit, pituitary.

Figure 9 *In vitro* regulation of gpGHSR1a and gpGHSR1b in hypothalamus and pituitary tissue of grouper. The hormone administration is described in the figure. Results are represented as means $\pm$ s.d. ($n=3$ from three wells). Statistically significant differences among groups, obtained by ANOVA, were assessed by Fisher's LSD test and are indicated as follows: * $P<0.05$ and ** $P<0.01$.

2003), mouse (Sun *et al.* 2004), black seabream (Chan & Cheng 2004), olive baboon (Luque *et al.* 2006), turkey (Richards *et al.* 2006), cattle (GenBank Acc XM_592014), rhesus monkey (GenBank Acc XP_001084886), and chimpanzee (GenBank Acc XM_516878). GHSR1b has only been reported so far in human, pig (Howard *et al.* 1996), and black seabream (Chan & Cheng 2004). In Mozambique tilapia and rainbow trout, two transcripts of GHSR may also exist (unpublished observation by Kaiya, reviewed by Kaiya *et al.* 2008). In zebrafish, a novel GHSR paralogous sequence that has 74% homology with the former reported GHSR1a has also been discovered in the genomic database and is named zebrafish GHSR2a (Kaiya *et al.* 2008). Moreover, another variant form of GHSR, designated GHSR1c or GHSR1aV, which lacks TM-6 when compared with GHSR1a due to a 48 bp deletion, has been detected in chicken and turkey (Geelissen *et al.* 2003, Tanaka *et al.* 2003, Richards *et al.* 2006). Our study provides new evidence for the existence of GHSR1b in teleostean fishes. However, GHSR1c has not been found in grouper and other reported teleosts either, inferring that GHSR1c may be a specific form of GHSR that only exists in birds.

Phylogenetic analysis showed that gpGHSR1a was evolutionary closer to other cloned GHSR1a sequences from teleostean species, but more distantly related to those from avian and mammalian species. Although the aa sequence of gpGHSR1a shares only about 55% identity with those of

mammals, the TM domains of GHSR1a are conserved in various species. Moreover, the extracellular region between TM-2 and TM-3 of GHSR1a is highly conserved, which shares 81.5% identity between grouper and human, and this region is reported to be the active domain for ligand binding (Feighner *et al.* 1998, Palyha *et al.* 2000).

The structure of the grouper *Ghsr* gene was very similar to that of black seabream (Chan & Cheng 2004), whose two exons are separated by a small intron; in human and rat genes (McKee *et al.* 1997, Peterseenn *et al.* 2001), the intron may comprise up to 2 kb. We also predicted the structures of *Ghsr* genes from other vertebrate species based on genomic databases using the Blast search method. In cow, chicken and some teleosts including zebrafish, fugu, *Takifugu rubripes*, and pufferfish, *Tetraodon nigroviridis*, *Ghsr* genes have a big hypothetical intron. However, in other teleosts such as medaka, *Oryzias latipes*, and stickleback, *Gasterosteus aculeatus*, the hypothetical intron of GHSR is less than only 200 bp. Therefore, we speculate that the small intron is characteristic of the *Ghsr* gene in teleostean fishes.

Both *Ghsr1a* and *Ghsr1b* mRNAs were highly expressed in the brain and pituitary gland of grouper. The appearance of gpGHSR mRNA in brain and pituitary is consistent with the main acting sites of ghrelin. Besides stimulating GH secretion (Kojima *et al.* 1999, Riley *et al.* 2002, Kaiya *et al.* 2003a,b, Ran *et al.* 2004, Shepherd *et al.* 2007), ghrelin has also been reported to be involved in other physiological functions controlled by central nervous system, including energy expenditure (Tschop *et al.* 2000), feeding behavior (Nakazato *et al.* 2001, Unniappan *et al.* 2002, 2004, Matsuda *et al.* 2006a, Jönsson *et al.* 2007, Miura *et al.* 2007, Shepherd *et al.* 2007), and sleep quality (Copinschi *et al.* 1997). The grouper active receptor form of ghrelin, gpGHSR1a, shows a similar expression pattern as that seen in black seabream (Chan & Cheng 2004). In teleosts, the expression levels of GHSR1a in brain regions can achieve 10–60% of that in pituitary gland. However, in human and rat, *Ghsr1a* mRNA is only predominantly expressed in pituitary gland and hypothalamus (McKee *et al.* 1997, Muccioli *et al.* 1998), but could not be detected in other brain regions by RNase protection assay most likely due to its low abundance and the low sensitivity of this procedure (McKee *et al.* 1997).

For ISH analysis, GHSR expressing cells in the brain and pituitary of grouper are widespread, in accordance with the result of real-time PCR. The GHSR positive cells were distributed widely and randomly in olfactory bulbs, telencephalon, diencephalon, cerebellum, medulla, spinal cord, hypothalamus, and pituitary gland. In some nuclei, the densities of GHSR-positive cells were higher than other regions. Previous research on mammalian species has shown that GHSR expressing cells can also be detected in several hypothalamic nuclei and other discrete regions (McKee *et al.* 1997, Zigman *et al.* 2006). Yet their distribution is confined in these sites and exhibits a clear pattern of zonal distribution (McKee *et al.* 1997), different from the situation of our study in teleosts. In the arcuate

nucleus of rat, GHSR expressing cells have been reported to co-localize with neuropeptide Y ($94\% \pm 1\%$), somatostatin ($30 \pm 6\%$), and GH-releasing hormone (20–25%; Willesen *et al.* 1999), and all these neuroendocrine factors are distributed more widely in teleostean brains than in their mammalian counterparts (Traverso *et al.* 2003, Chen *et al.* 2005, Ye *et al.* 2005, Lee *et al.* 2007). Hence, in the teleostean brain, these cells may play a more important endocrine role than in mammalian brains.

In peripheral tissues, the *gpGhsr1a* mRNA were detected in heart, thymus, along the intestine, and in liver, which corresponds to a ghrelin involvement in cardiovascular action (Tivesten *et al.* 2000), immune regulation (Koo *et al.* 2001, Dixit *et al.* 2004), and gastric acid secretion (Sibilia *et al.* 2002). The peripheral expression of GHSR1a in humans is limited to the thyroid, pancreas, spleen, myocardium, and adrenal besides brain and pituitary gland (Gnanapavan *et al.* 2002). However, in chicken, *Ghsr1a* mRNA has been found in almost all peripheral tissues (Geelissen *et al.* 2003, Tanaka *et al.* 2003, Richards *et al.* 2006), suggesting that the expression patterns of *Ghsr1a* mRNA are distinct among species.

On the contrary, the *gpGhsr1b* mRNA was observed in all peripheral tissues at similar expression levels. The tissue expression of gpGHSR1b is more widespread than previously reported for black seabream (Chan & Cheng 2004) but similar with the scenario in humans (Gnanapavan *et al.* 2002). GHSR1b is an inactive form of ghrelin receptor and reported to localize in the nucleus of the cell, while GHSR1a is on the plasma membrane (Smith *et al.* 2005). However, when GHSR1b is co-expressed with GHSR1a in a cell line, the signal transduction capacity of GHSR1a is attenuated (Chan & Cheng 2004) and this suggests a cross-talk function of the splice variant. Since the mRNA tissue distribution of *Ghsr1b* is much wider than *Ghsr1a* in grouper, we speculate that GHSR1b may mediate some unknown functions independent of ghrelin/GHSR1a system.

During embryonic and larval development, *gpGhsr1a* mRNA was expressed from the neurula stage on, coinciding with the development of the nervous system and prior to the appearance of its ligand, ghrelin, which is expressed from hatching in Atlantic halibut (Manning *et al.* 2008). The expression of *gpGhsr1b* mRNA can be detected as of the blastula stage, even earlier than *gpGhsr1a*. A role in embryonic development, independent of ghrelin/GHSR1a system, may be more relevant for gpGHSR1b. Besides, the expression of *Ghsr* mRNA has also been reported in chicken embryonic development, but the isoform of GHSR was not addressed in this report (Gahr *et al.* 2004).

In our *in vitro* study, seabream GH potently reduced the expression of two *gpGhsr* mRNA in pituitary and only *gpGhsr1b* mRNA in hypothalamus. The expression of *Ghsr* mRNA in pituitary gland and hypothalamus may decrease by GH *in vitro* (Geelissen *et al.* 2003) and *in vivo* (Bennett *et al.* 1997, Horikawa *et al.* 2000), and GH is reported to negatively feedback on many GH-releasing factors and their receptors in teleosts (Wong *et al.* 2006). Our study provides new evidence of GH feedback

regulation of GHSR in a teleost, and suggests that a more complicated regulation between ghrelin and GH may exist.

Rat ghrelin at 10^{-5} M downregulated the expression of two gpGHSR transcripts in pituitary gland and only the gpGHSR1a in hypothalamus. However, rat des-acyl ghrelin does not change the expression of both transcripts at all concentrations we used. Ghrelin is not a well-conserved peptide among vertebrate species (Unniappan & Peter 2005), but its N-terminal biological active core (GSSF with an acyl modification at the third residue) shows complete conservation among all species (Kaiya *et al.* 2008). The active core can functionally activate GHSR1a as efficiently as the full-length ghrelin and the entire sequence of ghrelin is not necessary for its activity (Bednarek *et al.* 2000). Otherwise, rat ghrelin has been reported to effectively activate seabream GHSR1a *in vitro* (Chan & Cheng 2004), and eel ghrelin can also activate rat GHSR1a *in vitro* and *in vivo* (Kaiya *et al.* 2003a). Therefore, rat ghrelin may influence the expression of gpGHSR via action on the gpGHSR1a. In chicken, both *Ghsr1a* and *Ghsr1c* mRNA can be downregulated by chicken ghrelin in pituitary cells (Geelissen *et al.* 2003), in agreement with the situation in our study. Our study provides evidence that ghrelin can also reduce the expression of both GHSR transcripts in hypothalamus. As des-acyl ghrelin did not change the expression of both GHSR transcripts in our study, we conclude that acylation is required for the ligand of the GHSR in this fish.

In summary, we have described and characterized two transcripts of *Ghsr* cDNA in grouper, providing new evidence for a splice variant in teleosts. The short intron may be one of the characteristics of *Ghsr* gene in teleostean fishes. Tissue distribution reflected that gpGHSR1a mediating some physiological functions of ghrelin; the function of the gpGHSR1b awaits further study but seems independent of the ghrelin/GHSR system. The expression of GHSR in grouper brain is both quantitatively and in distribution pattern different from that in mammals, as GHSR expressing cells are distributed more widely and randomly in fish brain than in mammalian brain. The *Ghsr* mRNA expression during fish embryonic and larval development was also examined, and it was shown that the appearance and expression profile of the two gpGHSR transcripts is different during development. In addition, *in vitro* experiments showed that the expression of both *gpGhsr1a* and *gpGhsr1b* mRNA are downregulated by rat ghrelin and seabream GH, strong evidence for a role of feedback regulation by these substances in teleosts.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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