Molecular identification of ghrelin receptor (GHS-R1a) and its functional role in the gastrointestinal tract of the guinea-pig

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Abstract

Ghrelin stimulates gastric motility *in vivo* in the guinea-pig through activation of growth hormone secretagogue receptor (GHS-R). In this study, we identified GHS-R1a in the guinea-pig, and examined its distribution and cellular function and compared them with those in the rat. Effects of ghrelin in different regions of gastrointestinal tract were also examined. GHS-R1a was identified in guinea-pig brain cDNA. Amino acid identities of guinea-pig GHS-R1a were 93% to horses and 85% to dogs. Expression levels of GHS-R1a mRNA were high in the pituitary and hypothalamus, moderate in the thalamus, cerebral cortex, pons, medulla oblongata and olfactory bulb, and low in the cerebellum and peripheral tissues including gastrointestinal tract. Comparison of GHS-R1a expression patterns showed that those in the brain were similar but the expression level in the gastrointestinal tract was higher in rats than in guinea-pigs. Guinea-pig GHS-R1a expressed in HEK293 cells responded to rat ghrelin and GHS-R agonists. Rat ghrelin was ineffective in inducing mechanical changes in the stomach and colon but caused a slight contraction in the small intestine. 1,1-Dimethyl-4-phenylpiperazinium and electrical field stimulation (EFS) caused cholinergic contraction in the intestine, and these contractions were not affected by ghrelin. Ghrelin did not change spontaneous and EFS-evoked [^{3}H]-efflux from [^{3}H]-choline-loaded ileal strips. In summary, guinea-pig GHS-R1a was identified and its functions in isolated gastrointestinal strips were characterized. The distribution of GHS-R1a in peripheral tissues was different from that in rats, suggesting that the functional role of ghrelin in the guinea-pig is different from that in other animal species.

Keywords: Growth hormone secretagogue receptor 1a, Guinea-pig, Rat, Tissue distribution, Gastrointestinal tract,

1. Introduction

Ghrelin is an endogenous ligand for growth hormone secretagogue-receptor 1a (GHS-R1a), which was first identified in pigs and humans [19], and is a 28-amino-acid peptide with *n*-octanoyl modification at the third serine residue (Ser^3) [26]. GHS-R is a G-protein-coupled receptor with seven transmembrane regions. Two GHS-R isoforms, a functional receptor, GHS-R1a (ghrelin receptor), and an alternative splice variant with undetermined function, GHS-R1b, have been identified [6]. Ghrelin is mainly produced in G-cells in oxyntic mucosa of the stomach and has potent activity for release of GH from the pituitary through activation of GHS-R1a. Accumulating evidence has also indicated that ghrelin is an important regulator of glucose metabolism, insulin release and cardiovascular functions, and it has been shown to be a peripheral circulating orexigenic hormone that increases body weight by stimulating food intake and by decreasing fat utilization [27].

Ghrelin and GHS-R1a have some structural similarities with motilin and the motilin receptor, respectively [1, 33]. Motilin is a gut hormone that is produced in the duodenum and induces phase III contractions in the stomach through activation of its own receptor (motilin receptor) [12, 20]. The similarity between the two gut peptides prompted examination of the physiological roles of ghrelin in regulation of gastrointestinal motility. In rodents, measurement of gastric motility in conscious and non-restrained animals indicated that ghrelin accelerated gastric emptying [23] and augmented spontaneous phase III-like contractions, and vagotomy or capsaicin abolished the ghrelin-induced contractions [13, 14]. Therefore, vagal afferent and efferent pathways are involved in the gastrointestinal-stimulating action of ghrelin in

rodents [13, 14]. Exogenous ghrelin also accelerates gastric emptying [28] and induces a premature gastric phase III of the migrating motor complex in humans [35]. On the other hand, exogenous ghrelin has no effect on gastrointestinal motility in conscious dogs [32]. Our recent study demonstrated that the guinea-pig is sensitive to ghrelin causing gastric contraction *in vivo* through activation of the capsaicin-sensitive vago-vagal reflex pathway similar to that in rats. Furthermore, ineffectiveness of des-acyl ghrelin and inhibition of ghrelin-induced action by a GHS-R1a antagonist indicated the involvement of GHS-R1a in ghrelin-induced gastric contraction [31]. These differences in ghrelin-induced gastrointestinal action are thought to be species-dependent, but it is possible that the differences reflect a relationship to feeding habit of the animals (rodents and humans, omnivorous; dogs, carnivorous; guinea-pigs, herbivorous). An immunohistochemical study using antibodies for rat ghrelin and rat GHS-R revealed the presence of ghrelin and GHS-R in intestinal enteric nerves of the guinea-pig [38]. In addition, ghrelin has been demonstrated to enhance endothelin-induced contraction in the guinea-pig renal artery [9]. Although these functional and immunohistochemical studies have shown the expression of GHS-R1a in guinea-pig tissues, the structure of guinea-pig GHS-R1a and its distribution and physiological function in the gastrointestinal tract have not been elucidated.

The aim of this study was to identify and characterize GHS-R1a in the guinea-pig. Tissue distribution of the receptor mRNA was determined by using quantitative real-time PCR and was compared with that in the rat. To determine the function of enteric GHS-R1a, the effects of ghrelin on gastrointestinal contractility and stimulation-induced neural responses were examined in isolated smooth muscle preparations.

2. Materials and methods

2.1. Animals and tissue preparations

Hartley guinea-pigs (*Cavia porcellus*) and Wistar rats of both sexes (weighing 200–250 g) were obtained from Sankyo Lab Service (Sapporo, Japan). All experimental procedures were approved by the Medical Ethics Committee of Rakuno Gakuen University. Guinea-pigs and rats were housed in stainless steel cages at a regulated temperature (22 ± 2°C) and 60%–65% relative humidity with a normal 12:12 hour light/dark cycle.

2.2. Guinea-pig GHS-R1a cDNA cloning

Total RNA was extracted with TRIzol reagent (Invitrogen, Grand Island, NY) from the cerebrum that had been stored in RNAlater (Ambion). Full-length cDNA was determined to amplify an approximately 700-bp fragment using degenerated primers that were designed on the basis of the portions that are highly conserved in other species of GHS-R1a. Then 3'- or 5'-RACE PCR was performed on the basis of the determined nucleotide sequence using a GeneRacer Kit (Invitrogen). Primers used in this study are shown in Table 1.

Cerebrum total RNA (1 μg) was transcribed with GeneRacer 3'-oligo using a QuantiTect RT Kit (QIAGEN, GmbH, Hilden, Germany) (final volume of 20 μl). PCR was performed with 2 μl of a template, a primer set (100 pmol/μl GHS-R-dSES1 and

GHS-R-dANT1) and ExTaq DNA polymerase (TaKaRa, Otsu, Japan). The reaction conditions were 94˚C for 2 min followed by 35 cycles of 94˚C for 0.5 min, 54˚C for 0.5 min and 72˚C for 1 min with final extension at 72˚C for 3 min. The amplified product was purified by the Wizard PCR preps DNA purification system (Promega, Madison, WI) and subjected to second-round nested PCR. Nested PCR was performed under the same conditions as those used for primary PCR with another primer set (100 pmol/μl GHS-R-dSES2 and GHS-R-dANT1), 2 μl PCR-prepsed template and ExTaq DNA polymerase. The obtained product was subcloned into the pCRII-TOPO vector (Invitrogen), and the nucleotide sequence was determined by automated sequencing (model 3130, Applied Biosystems, Foster City, CA) according to the protocol of the BigDye™ terminator cycle sequencing kit (Applied Biosystems). As a result, a 707-bp GHS-R-like fragment was identified.

For 3'-RACE PCR, primary PCR was performed with a gene-specific primer (GSP), gpGHSR-S1, and a 3'-primer using HotStar Taq Plus Mix (QIAGEN GmbH). The reaction conditions were 95˚C for 5 min followed by 35 cycles of 95˚C for 0.5 min, 57˚C for 0.5 min and 72˚C for 1 min with final extension at 72˚C for 3 min. After PCR preps of the amplified product, nested PCR was performed with another GSP, gpGHSR-S2, and a 3'-nested primer under the same conditions as those used for primary PCR. A 1154-bp GHS-R-like fragment was identified in this process.

To determine the 5'-side cDNA sequence, first-strand cDNAs were synthesized from 2.5 μg cerebrum total RNA with an anti-sense primer (gpGHSR-Q-AS) using a QuantiTect RT Kit. Primary PCR was conducted using a GSP, gpGHSR-AS1, a 5'-primer and HotStar Taq Plus Mix with amplification conditions of 95˚C for 5 min followed by 35 cycles of 95˚C for 0.5 min, 57˚C for 0.5 min and 72˚C for 1 min with

final extension at 72˚C for 3 min. After PCR preps of the product, nested PCR was performed using another GSP, gpGHSR-AS3, a 5'-nested primer and HotStar Plus Taq Mix under the same conditions as those used for primary PCR. A specific 450-bp product was identified.

To determine full-length cDNA, 3'RACE PCR was performed using cerebrum cDNA for 3'-RACE as a template. HotStar Plus Taq Mix containing 2.5% DMSO was used for amplification with the GSP gpGHSR-full-s and 3'primer. Then nested PCR was conducted with gpGHSR-full-s and 3'-nest primer. Reaction conditions were 95˚C for 5 min followed by 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.

2.3. Functional analysis of guinea-pig GHS-R1a

To examine functional activity of the identified receptor protein, we cloned an open reading frame (ORF) of the cDNA encoding the protein. RT-PCR was performed under the same conditions as those for full-length cDNA described above except for the use of another primer set, gpGHSR-code-s and gpGHSR-code-AS2. The isolated cDNA was subcloned into pcDNA3.1-V5-His-TOPO mammalian cell expression vector (Invitrogen). A vector having correct orientation of the insert for expression and correct GHS-R1a sequence was sub-cultured, and the plasmid vector was isolated using a HiSpeed Plasmid Midi kit (QIAGEN GmbH) and diluted to 1 μg/μl for a transfection experiment.

Intracellular Ca^{2+} concentrations were measured using FLIPR^{tetra} (Molecular Devices, Menlo Park, CA) as described previously [24]. As a positive control and for

comparison of the response with guinea-pig GHS-R1a, rat GHS-R1a was examined in the same way as that for guinea-pig GHS-R1a. As ligands, synthetic rat ghrelin, growth hormone-releasing peptide-6 (GHRP-6) and hexarelin were applied at final concentrations of 0.03 nM to 300 nM.

2.4. Quantitative real-time PCR (qPCR) for guinea-pig GHS-R1a and rat GHS-R1a

Total RNA was extracted separately by Trizol reagent from 30 tissues obtained from seven guinea-pigs and six rats that had been stored in RNAlater (Ambion). First-strand DNA was synthesized from 1 μg total RNA using a QuantiTect RT Kit (QIAGEN GmbH) with oligo-$dT_{12\text{-}18}$ primer. PCR was performed using the LightCycler 480 system (Roche Applied Science, Mannheim, Germany) with a QuantiFAST SYBR Green PCR Kit (QIAGEN GmbH) and a primer set for the guinea-pig (gpGHSR-Q-s and Q-AS, Table 1) and for the rat (rGHSR-Q-s and Q-AS, Table 1). Expected sizes of amplicons for the guinea-pig and rat GHS-R1a were 358 bp and 161 bp, respectively, and they were confirmed by 1.5% agarose gel electrophoresis. β-actin (Acc#AF508792, B-act-Q-s and B-act-Q-AS, Table 1) was used as an internal control for both animals. The amplification conditions were 95°C for 5 min followed by 40 cycles at 95°C for 10 sec and 60°C for 30 sec. The reaction mixture consisted of 1X master mix and 250 nM each of the primer and template (100 ng total RNA equivalent). For quantification of GHS-R1a cDNA copy number, linear regression analysis was performed using a serially diluted linearized pCRII vector cloned a guinea-pig GHS-R1a fragment, full-length rat GHSR-R1a or a β-actin fragment amplified by a specific primer set. These vectors were linearized by restriction with *Xba-I*. Data were calculated by Second Derivative Max

mode of the LightCycler software. The values were used if desired size of the amplicon was confirmed by 1.5% agarose gel electrophoresis containing ethidium bromide.

2.5. In vitro contraction study of gastrointestinal strips

Guinea-pigs were sacrificed by bleeding from the carotid artery under anesthesia with pentobarbital (40mg/kg, i.p.). The gastrointestinal tract of each guinea-pig was quickly isolated and placed in ice-cold Krebs solution. Longitudinal muscle strips freed from mucosa were prepared from the gastric fundus and antrum. Longitudinal muscle layers of the duodenum (20 mm distal from the pylorus), jejunum (middle of the small intestine), ileum (50 mm proximal from the ileocecal junction), proximal colon (50 mm distal from the cecum) and distal colon (50 mm proximal from the anus) were peeled off using forceps and a fine swab. Smooth muscle preparations (15 mm in length and 2 mm in width) were suspended vertically in an organ bath (5 ml) to measure the longitudinal muscle contraction. The organ bath contained warmed (37^{o}C) Krebs solution (mM): NaCl, 118; KCl, 4.75; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $NaHCO_2$, 25 and glucose, 11.5 equilibrated with 95%O_2 + 5%CO_2 (pH 7.4). Mechanical activity of the preparations was measured with an isometric force transducer (SB-11T, Nihon Kohden, Tokyo, Japan) and recorded on an ink-writing recorder and then analyzed using a computer-aided analysis system (Power Lab 2/25, Japan Bioresearch Center, Nagoya, Japan). The initial load was set at 0.5 g for each preparation. The preparations were rinsed with Krebs solution every 15 min and allowed to equilibrate for 1 h. Prior to the addition of ghrelin, each muscle strip was subjected to 3 or 4 stimulations with 50 mM KCl (K^+) until a reproducible contraction was obtained. In order to examine whether

ghrelin causes contraction of gastrointestinal smooth muscle preparations, rat ghrelin (10 nM-1 μM) was applied to an organ bath at 1-h intervals and evoked responses were observed. Increase in muscle tonus among preparations was normalized by a standard contraction of 50 mM K^+ and expressed as a relative contraction (%).

In the guinea-pig intestine, neural contractions were evoked by a ganglion stimulant (1,1-dimethyl-4-phenylpiperazinium, DMPP) and electrical field stimulation (EFS), and the effect of ghrelin on these neural responses was examined. EFS (2 Hz for 15 s, 0.5 ms in duration, and submaximum voltage of 15-20 V) was applied repetitively at 5-min intervals through two platinum electrodes placed on the left and right sides of the bath, sandwiching the preparations. After observing 4 reproducible EFS-induced contractions, ghrelin (1 μM) was applied at the middle of the stimulation interval and its effect on the contraction was observed for 20 min.

2.6. In vitro release study

The effects of ghrelin on release of acetylcholine were examined in longitudinal muscle preparations of the guinea-pig ileum loaded with [^{3}H]-choline as previously reported [31]. The isolated muscle preparations were incubated with 140 nM [^{3}H]-choline for 60 min in 1.3 ml warmed Krebs solution (37°C) equilibrated with 95% O_2 + 5% CO_2. After washing in fresh Krebs solution (37°C, bubbled with gas mixture) for 30 min, the preparations were immersed in 2 ml Krebs solution containing hemicholinium-3 (10 μM). The incubation medium (37°C, bubbled with gas mixture) was sequentially changed at 5-min intervals.

First, to investigate the effect of ghrelin on [^{3}H]-outflow of a non-stimulated

intestinal preparation, the preparation was incubated with 1 μM ghrelin for 5 min, and the [^{3}H]-effluxes before and after stimulation were compared. Ghrelin has been reported to stimulate NO release from nitrergic nerves in the rat stomach and guinea-pig stomach [31, 37]. Therefore, ghrelin-induced action was also examined in the presence of L-nitro arginine methylester (L-NAME, 100 μM). Next, the effect of ghrelin on EFS-evoked [^{3}H]-efflux was investigated to clarify ghrelin-induced modification of neurally evoked acetylcholine release. First, EFS (S1) was applied through two platinum ring electrodes fixed on the top and bottom of the preparations at 35 min later of the series of experiments, and a second stimulation (S2) was applied 60 min after S1 in the absence (control) or presence of 1 μM ghrelin. At the end of each experiment, the tissue was dissolved in Soluene (500 μl), and radioactivity in the tissue and incubation medium was measured in a scintillation counter. [^{3}H]-outflow was expressed as fractional rate, in which the amount of radioactivity in the incubation medium was divided by the total radioactivity present in the tissue, in the same collection period. [^{3}H] content of the tissue in each period was calculated by cumulatively adding the amount of [^{3}H] in each fraction to the [^{3}H] content of the tissue at the end of the experiments. An inhibitory or excitatory effect of ghrelin on [^{3}H]-efflux was evaluated by comparison of S2/S1 ratios in the absence (control) and presence of ghrelin (1 μM).

2.7.Chemicals

The following chemicals were used in the present experiments: atropine sulphate (Wako, Osaka, Japan), des-acyl rat ghrelin (Peptide Institute Inc. Osaka, Japan), rat ghrelin (Peptide Institute Inc.), growth hormone-releasing peptide-6 (GHRP-6,

Bachem, Bubendorf, Switzerland), hexarelin (Phoenix Pharmaceutical Inc., Belmont, CA, USA), 1,1-dimethyl-4-phenylpiperazinium iodine (DMPP, Wako), hemicholinium-3 (Sigma), hexamethonium chloride (Wako, Osaka, Japan), L-nitroarginine methylester (L-NAME, Sigma) and tetrodotoxin (Wako). All drugs were dissolved, diluted in distilled water, and applied to an organ bath at designated concentrations.

2.8. Statistical analysis

Pharmacological data are expressed as means ± S.E.M of more than four experiments. The significance of differences between the values was determined at $P < 0.05$ using Student's t-test (paired or unpaired) for single comparisons or ANOVA followed by Bonferroni Dunnett's test for multiple comparisons.

3. Results

3.1. Cloning of guinea-pig GHS-R1a

Nucleotide and deduced amino acid sequences of isolated cDNA are shown in Fig. 1. We identified a 1522-bp cDNA encoding a GHS-R-like protein, which is composed of a 46-bp 5'-untranslated region (UTR), an open reading frame of 1107 bp encoding a 368-amino-acid protein, and a 369-bp 3'-UTR (GenBank Acc# AB574182). A BLAST search indicated that this deduced protein is GHS-R1a. Figure 2 shows a comparison with other GHS-R1a sequences. Numerous consensus sequences for

GHS-R1a have been highly conserved in the deduced receptor protein. The protein showed the highest identity (93%) to horse GHS-R1a, moderate identity (85%) to dog GHS-R1a and the lowest identity (54%) to tilapia GHS-R1a-like receptor (GHSR1a-LR) (Fig. 3).

3.2. Functional analyses of guinea-pig GHS-R1a

Next, we examined whether the deduced protein is activated by ghrelin or GHS-R1a agonists. HEK 293 cells transiently expressing the identified protein were treated with 0.03 nM to 300 nM of rat ghrelin (Fig. 4A). Intracellular Ca^{2+} concentrations increased in guinea-pig GHS-R1a-like protein-expressing cells in a concentration-dependent manner. Rat GHS-R1a, which was used as a positive control for the same transfection procedure, responded to rat ghrelin as previously reported [26]. The half effective concentrations (EC_{50}) of rat ghrelin were 10 nM for guinea-pig GHS-R1a-like protein and 3 nM for rat GHS-R1a. We also examined responsiveness to GHS-R1a agonists, GHRP-6 and hexarelin, using HEK 293 cells stably expressing guinea-pig GHS-R1a-like protein (Fig. 4B). GHRP-6 and hexarelin increased intracellular Ca^{2+} concentration in a concentration-dependent manner (0.3 nM- 300 nM). Ranking order of potency was GHRP-6 = hexarelin > rat ghrelin. Since ghrelin-induced responses to the identified GHS-R1a-like protein were confirmed, this protein was designated guinea-pig GHS-R1a.

3.3. Tissue distribution of guinea-pig and rat GHS-R1a mRNAs

We quantified GHS-R1a mRNA in central and peripheral tissues from 3 male and 4 female guinea-pigs. There was no clear sex-related preferential expression of GHS-R1a mRNA among the tissues examined and the data from both sexes were mixed. GHS-R1a mRNA was mainly detected in the central nervous system including the cerebral cortex, thalamus, hypothalamus, pituitary, mesencephalon, pons, medulla oblongata, olfactory lobe and cerebellum (Fig. 5A). In these tissues, GHS-R1a mRNA was predominantly expressed in the pituitary, followed by the hypothalamus. Expression of mRNA was also found in the lung, liver, kidney, adrenal gland, stomach, duodenum, jejunum, ileum, cecum and colon, but it was only visualized by electrophoreses of 40-cycles PCR samples and was not able to be represented numerically (Fig. 5B). When the expression level and distribution pattern of GHS-R1a mRNA were compared to those in the rat, the distribution pattern in the brain was almost identical, but expression levels were higher in the cerebral cortex, thalamus, mesencephalon, pons and medulla oblongata of the rat (Fig. 5A). In peripheral tissues of the rat, GHS-R1a mRNA was detected in all tissues examined, and testicular expression level was extremely high (Fig. 5B).

3.4. Effect of ghrelin on isolated gastrointestinal strips

Ghrelin was applied for 5 min at 1-h intervals and evoked responses were observed in the respective gastrointestinal regions (Fig. 6). Ghrelin (1 μM) did not cause any mechanical changes in the gastric and colonic strips but caused a small contractile response in the small intestinal preparations. The relative amplitudes of contraction (50 mM K^+-induced contraction = 100%) were 0.6 ± 0.5% (n = 4) in the fundus, 0.54 ±

0.22% (n = 4) in the antrum, 3.5 ± 1.4% (n = 8) in the duodenum, 6.7 ± 2.4% (n = 6) in the jejunum, 6.5 ± 1.6% (n = 19) in the ileum, 1.1 ± 0.36% (n = 7) in the proximal colon and 0.9 ± 0.29% (n = 5) in the distal colon. In the case of the ileum, amplitudes of the responses to ghrelin varied from preparation to preparation. Eight of the 19 preparations were relatively sensitive to ghrelin and showed large contractions (over 5% of 50 mM K^+ contraction, 12.7 ± 2.5%, n = 8), but the other 11 preparations were relatively insensitive to ghrelin (2.1 ± 0.5%, n = 11) (Fig. 6). Concentration-response relationships for ghrelin were obtained by using ghrelin-sensitive ileal preparations (10 nM: 2.6 ± 0.9%, 100 nM: 8.7 ± 2.1%, 1 μM: 13.3 ± 1.2%, n = 5). Similarly, 3 of the 8 duodenal preparations and 3 of the 6 jejunum preparations were sensitive to ghrelin; the contractile responses were over 5% of 50 mM K^+-induced contraction. Des acyl-ghrelin (1 μM) was relatively ineffective in changing smooth muscle tonus both in the jejunum (1.5 ± 0.78%, n = 6) and ileum (1.6 ± 0.2%, n = 6).

3.5. Effect of ghrelin on DMPP and EFS-induced responses

Since ghrelin was effective in causing mechanical responses of the small intestine, modification of neuro-effector transmission by ghrelin was examined in intestinal preparations. Neural responses were evoked by DMPP, a ganglion stimulant. DMPP (1-100 μM) caused a transient concentration-dependent contractile response, which was decreased by hexamethonium (100 μM), atropine (1 μM) and tetrodotoxin (1 μM), in the small intestine and colon, suggesting that contraction is induced by activation of intrinsic cholinergic nerves. Ghrelin treatment (1 μM for 5 min) did not change the DMPP (10 μM, approximately EC_{50} value, control=100%)-induced contractions in the

duodenum (104 ± 4%, n = 4), jejunum (99.5 ± 1.7%, n = 4), ileum (100 ± 2.0%, n = 6), proximal colon (101 ± 3.8%, n = 4) and distal colon (96 ± 2.7%, n = 5) (Fig. 7A).

The effect of ghrelin (1 μM) on EFS-induced contraction was also examined in the jejunum and ileum. EFS (2 Hz)-induced contraction was markedly decreased by atropine (1 μM) and was abolished by tetrodotoxin (1 μM). After obtaining 4 reproducible EFS-induced contractions, the preparation was treated with ghrelin (1 μM) and its effect was determined. As shown in Fig. 7B, EFS-induced contractions in the jejunum and ileum were not significantly affected by treatment with ghrelin.

3.6. Effect of ghrelin on [^{3}H]-efflux from ileal strips

To investigate the effect of ghrelin on acetylcholine release from myenteric cholinergic neurons, [^{3}H]-efflux from [^{3}H]-choline-loaded ileal longitudinal muscle strips was measured both in the absence and presence of a NO synthase inhibitor, L-NAME (100 μM). Ghrelin (1 μM for 5 min) did not affect [^{3}H]-efflux from ileal strips in the control condition. Fractional rates before and after treatment with ghrelin were 0.81 ± 0.03% and 0.74 ± 0.06% (n = 4, P = 0.37), respectively. Ghrelin also did not affect the fractional rate in the L-NAME-treated preparations (control = 0.89 ± 0.07%, ghrelin = 0.97 ± 0.07%, n = 4, P = 0.45) (Fig. 8A). The effect of ghrelin on EFS-induced [^{3}H]-efflux was investigated to clarify modification of the evoked acetylcholine release. The fractional rates of [^{3}H]-efflux induced by S1 and S2 in control conditions were 1.78 ± 0.06% and 1.40 ± 0.08% (n = 4), respectively (S2/S1 = 0.79 ± 0.06, n = 4). Ghrelin (1 μM) treatment did not significantly change the S2/S1 ratio (0.88 ± 0.06, n = 4) compared with the control level (P = 0.34 vs. control). In

L-NAME-treated preparations, S2/S1 ratio was also not affected by ghrelin (1 μM) (Fig. 8B).

4. Discussion

In the present study, we isolated a cDNA encoding a 368-amino-acid GHS-R1a-like protein from the guinea-pig brain. Functional analysis of the receptor protein expressed in HEK 293 cells demonstrated that the identified cDNA encodes GHS-R1a and that GHS-R1a responds to ghrelin and GHSs by increasing intracellular Ca^{2+} concentration. Thus, we designated the identified protein as GHS-R1a for the guinea-pig. Expression of GHS-R1a mRNA in the central nervous system of the guinea-pig was comparable with that in the rat, but there were differences in expression level in the gastrointestinal tract. Molecular biological and physiological studies indicated that the functional role of ghrelin and peripheral GHS-R1a for the regulation of gastrointestinal motility is weak in the guinea-pig.

The amino acid composition of guinea-pig GHS-R1a (368 amino acids) is greater than that of GHS-R1a in other mammals reported so far (349 to 366 amino acids). The protein sequence of guinea-pig GHS-R1a was most similar to that of horse GHS-R1a with 93% identity but was quite different from that of dog GHS-R1a (85%). The different degrees of identity may reflect their herbivorous feeding habit. In addition, phylogenetic analysis revealed that guinea-pig GHS-R1a is classified differently from that in rodents such as rats and mice. *Rodentia* are divided into three groups: ctenohystrica, mouse-related and squirrel-related clades. *Hystricomorpha* including the guinea-pig is classified into ctenohystrica and is different from the mouse-related clade

[3]. This phylogenetical location of the guinea-pig has also been proposed by other groups [8, 17]. Our data on GHS-R1a structure classification also support this hypothesis.

Functional analysis of guinea-pig GHS-R1a-expressing cells showed that rat ghrelin caused an increase in intracellular Ca^{2+} concentration in a concentration-dependent manner. The EC_{50} value for rat ghrelin in guinea-pig GHS-R1a (10 nM) was slightly higher than that in rat GHS-R1a (3 nM). This is reasonable since the ligand used in this experiment was rat ghrelin. However, our previous study indicated no difference in the activity of ghrelin between GHS-R1a of different animal species, e.g., rat and chicken, to rat GHS-R1a [21]. The similar responsiveness is due to the fact that the N-terminal molecular structure of ghrelin including acyl modification, which is important for its biological activity, has been highly conserved; the N-terminal portion affects ghrelin affinity to the receptor [22, 30]. In addition to low responsiveness of rat ghrelin, ranking order of the effects of three GHS-R1a agonists on guinea-pig GHS-R1a (hexarelin = GHRP-6 > rat ghrelin) was different from that for rat GHS-R1a. Rat ghrelin shows almost the same affinity as that of GHRP-6 and hexarelin [11] or 10-times higher affinity than that of GHRP-6 and hexarelin to rat GHS-R1a [34]. Since homology between guinea-pig GHS-R1a and rat GHS-R1a is 92%, the discrepancy in affinity and ranking order suggests a specific three-dimensional structure of guinea-pig GHS-R1a for accepting guinea-pig ghrelin. Our preliminary search of the Ensembl database supported our prediction that guinea-pig ghrelin (# ENSCPOG00000020910) has a different amino acid sequence of the N-terminal portion from that of rat ghrelin. We are making progress in purifying the peptide and determining the molecular structure. It is likely that the different molecular structure affected ghrelin binding to the

guinea-pig GHS-R1a. It would be interesting to compare the biological activities of guinea-pig ghrelin between guinea-pig GHS-R1a and rat GHS-R1a.

Quantitative real-time PCR revealed that guinea-pig GHS-R1a mRNA is predominantly expressed in the pituitary, followed by the hypothalamus. In this study, we also examined mRNA expression of GHS-R1a in rats to compare the tissue distribution patterns in the two species. We found that mRNA distributions in the central nervous tissues were almost identical in the guinea-pig and rat. High expression levels of GHS-R1a in the pituitary and hypothalamus have already been demonstrated in mammals [16, 18] and the chicken [15]. This distribution pattern in the brain is responsible for stimulation of GH release and food intake by ghrelin. However, the fact that mRNA expression was observed only in the rat central nervous system (cerebral cortex, thalamus, mesencephalon and medulla oblongata) suggests rat-specific actions of ghrelin in those parts of the brain. On the other hand, GHS-R1a mRNA was expressed at detectable levels in all gastrointestinal regions of the rat but was not detected in the guinea-pig gastrointestinal tract. Expression of appropriate levels of GHS-R1a mRNA in all regions of the gastrointestinal tract has been demonstrated in humans [36], chickens and Japanese quails [25], and we previously demonstrated heterogeneous gastrointestinal region-dependent expression in avian species [25]. The homogeneous expression pattern of GHS-R1a mRNA in the rat gastrointestinal tract is comparable to that in humans but not in chickens [25, 36]. It is interesting that GHS-R1a mRNA expression was not detected in the gastrointestinal tract of the guinea-pig, but this is consistent with the results of our present physiological studies discussed later. Very low expression level of GHS-R1a in the gastrointestinal tract might affect feeding habit of the animal as a grass-eating animal. Further studies on GHS-R1a

expression in the stomach and small intestine of other species should be carried out to clarify the comparative physiological roles of ghrelin and GHS-R1a in gastrointestinal tract function.

In our recent *in vivo* study, we demonstrated that ghrelin caused gastric contraction through activation of the capsaicin-sensitive vago-vagal reflex pathway but that the contribution of peripheral gastric GHS-R to regulation of gastric contractility is small in the guinea-pig [31]. In isolated gastrointestinal tracts of the rat and chicken, exogenous ghrelin caused contraction through activation of both neural and myogenic GHS-R1a [10, 24, 25]. In addition, enteric neurotransmission to smooth muscle was modified by ghrelin in the gastrointestinal tract of rats [2, 7, 14] and chickens [24]. These results are consistent with results of a molecular biological study showing the expression of GHS-R1a in the gastrointestinal tract of chickens [25] and rats (present study). Therefore, low expression level of GHS-R1a in the guinea-pig gastrointestinal tract prompted us to examine the effect of ghrelin on smooth muscle contractility and neurotransmission. In gastric and colonic muscle strips, rat ghrelin was ineffective in causing contraction and changing spontaneous contraction as previously reported [31]. In contrast, rat ghrelin caused contraction of small intestinal strips (duodenum, jejunum and ileum), but the mean amplitude of the contractile response was only 6% of high K^+-induced contraction. However, about half of the small intestinal preparations showed definite contractile responses to ghrelin, and the responses increased depending on its concentration. Since des-acyl ghrelin was almost ineffective in all small intestinal preparations, it is thought that GHS-R1a mediates contractile responses in the small intestine. However, the fact that the expression level of GHS-R1a mRNA is low and

differs from preparation to preparation might be responsible for the irregular responses to ghrelin.

The effect of ghrelin on neurotransmission was examined using DMPP and EFS. Ghrelin did not change the cholinergic neural contraction elicited by DMPP and EFS in the small intestine and colon of the guinea-pig. The effect of ghrelin on acetylcholine release was also examined to confirm the results of the contraction study. In the ileum, ghrelin did not change spontaneous and EFS-evoked acetylcholine release, although the presence of ghrelin receptors on cholinergic nerves has been demonstrated immunohistochemically [38]. Dass et al. (2003) also observed the same discrepancy between expression of GHS-R protein and functional responses to ghrelin in the human colon [4]. Although the discrepancy is difficult to interpret, low expression levels of GHS-R1a protein and localization of GHS-R1a on non-motor neurons such as vagal afferents [5] could explain the discrepancy between expression of GHS-R1a and its physiological function in intestinal motility. In gastric preparations, ghrelin inhibited acetylcholine release through stimulation of NO release [31]. However, in the present study, since ghrelin showed no effect on acetylcholine release in the absence or presence of L-NAME, an inhibitory response to endogenous NO was not observed in the ileum. Taken together, the results suggest that the weak responsiveness of intestinal muscle from the guinea-pig to ghrelin, which is different from the responsiveness of rat and chicken intestinal muscle preparations, is partly due to low expression levels of GHS-R1a mRNA and protein. However, the possibility that weak responsiveness to ghrelin is due to low binding affinity of rat ghrelin to the guinea-pig GHS-R1a cannot be ruled out. After determining the guinea-pig ghrelin structure, functional studies using homologous ghrelin are needed in future.

In conclusion, guinea-pig ghrelin receptor (GHS-R1a) was identified and its functions were characterized in expressed cells and in isolated gastrointestinal strips. The distribution of GHS-R1a in the guinea-pig brain is almost the same as that in other animals, but the distribution in the gastrointestinal tract is different from that in humans, rats and chickens. This difference may reflect their grass-eating habit. Further studies should be carried out to clarify the relationships between GHS-R1a characteristics and eating habit.

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Figure legends

Fig. 1. Nucleotide and deduced amino acid sequences of guinea-pig GHS-R1a. An asterisk after the last amino acid indicates termination by a stop codon (TGA). The nucleotide sequence was deposited in the DDBJ/EMBL/GenBank™ databases with the accession number AB574182.

Fig. 2. Multiple amino acid sequence comparison of GHS-R1a and GHS-R1a-like receptors. *The boxed letters* indicate identical amino acids in more than half of the examined species. Amino acid sequences are available from the DDBJ/EMBL/GenBank databases: human (NM_198407), rat (NM_032075), mouse (NM177330), pig (NM_214180), cattle (NM_001143736), horse (XM_001494000), dog (NM_001099945), ferret (EF526307), chicken (AB095995), Western clawed frog (XM_002931572), goldfish-1a-1 (AB504275), goldfish-2a-1 (AB504277), catfish-1a (FJ707319), rainbow trout (AB362479), tilapia (AB361053).

Fig. 3. Phylogenetic analysis of guinea-pig GHS-R1a and related receptors. 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 on out-group. The numbers at branch points represent bootstrap values (1000 repetitions). The value in parenthesis indicates amino acid sequence identity to guinea-pig GHS-R1a. Amino acid sequences except those indicated in Fig. 2 are available in the DDBJ/EMBL/GenBank databases: opossum (NX_001363145), quail-1aS (AB469019),

catfish-2a (NM_001200322), human MTLR (NM_001507), human NMUR1 (NM_006056), human NTSR1 (NM_002531).

Fig. 4. Changes in intracellular Ca^{2+} concentration in rat or guinea-pig GHS-R1a-expressing mammalian cells. (A) Rat or guinea-pig GHS-R1a was transiently expressed in HEK 293 cells. These cells were treated with rat ghrelin (0.3 nM -300 nM) and increase in Ca^{2+} concentration was observed. (B) Guinea-pig GHS-R1a was stably expressed in HEK 293 cells by the selection of G418. Rat ghrelin or GHS-R1a agonists GHRP-6 and hexarelin were applied at concentrations from 0.3 nM to 30 nM. Intracellular Ca^{2+} changes were measured by the FLIPR system. Values are means ± SEM (n = 3).

Fig. 5. Tissue distributions of guinea-pig GHS-R1a and rat GHS-R1a in central (A) and peripheral tissues (B). mRNA expression levels were quantified by real-time PCR with duplicate measurements. Black and white columns indicate results for the guinea-pig and rat, respectively. Values are means ±SEM (testis; n=3, other tissues; n = 6-7 for guinea-pigs and n = 5-6 for rats). ND and NE mean “not detected” and “not examined”, respectively.

Fig. 6. Each trace shows a representative effect of rat ghrelin (1 μM, ▲) in smooth muscle strips isolated from the gastric fundus, gastric antrum, duodenum, jejunum, ileum, proximal colon and distal colon of the guinea-pig. There was a preparation-related difference in the mechanical responses to ghrelin in the ileum, i.e., Ileum-1 was insensitive but Ileum-2 was sensitive to ghrelin in causing contraction (See

text).

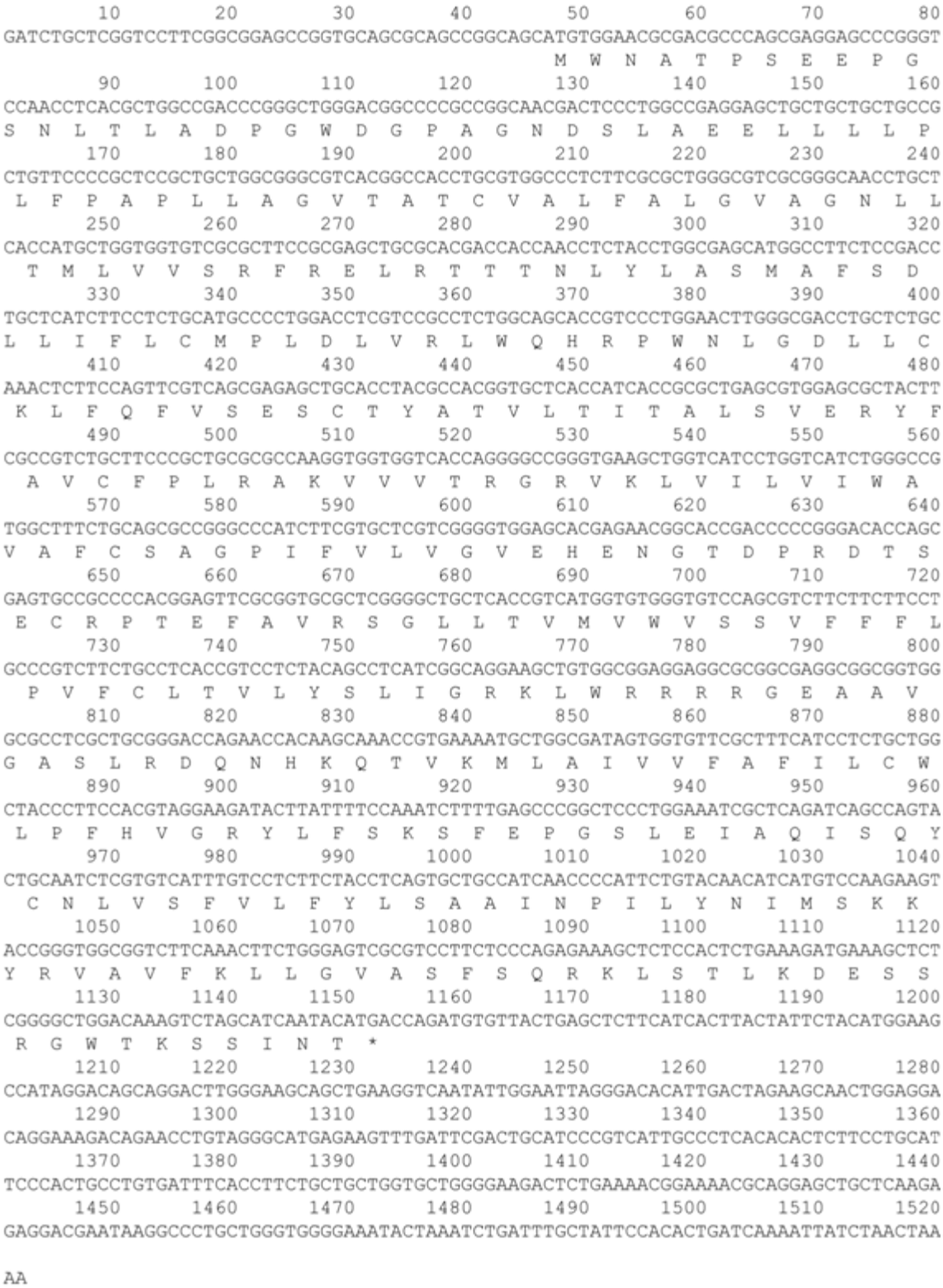
Fig. 7. Effects of rat ghrelin on neural contraction induced by DMPP and EFS. (A) After observing reproducible contractile responses to DMPP (10 μM), each intestinal preparation (duodenum, jejunum, ileum, proximal colon and distal colon) was treated with rat ghrelin (1 μM, for 5 min) and then DMPP was applied to examine the modification by ghrelin. Ordinate: Relative amplitude of contraction (control = 100%). (B) EFS (2 Hz for 15 s) was applied every 5 min and ghrelin (1 μM) was applied to the organ bath at 17.5 min, and modification of EFS-induced contraction was examined. Each symbol indicates contraction in the jejunum (●) and ileum (○). Ordinate: EFS-induced contraction was normalized using the response at 10 min. Symbols and vertical bars are means ± SEM of 4 experiments.

Fig. 8. Effects of rat ghrelin on [^{3}H]-efflux from ileal strips of the guinea-pig. (A) Effects of treatment with ghrelin (1 μM for 5 min, black bar) on spontaneous [^{3}H]-efflux were examined in the absence of L-NAME (normal, ■) and presence of L-NAME (100 μM, ●). Ordinate: [^{3}H]-efflux expressed as fractional rate. Abscissa: fraction number (5 min). (B) S2/S1 ratios were compared in the absence (EFS) and presence of ghrelin (1 μM, EFS + ghrelin) to examine modification of neurotransmission by ghrelin. The same experiments were also carried out in the absence of L-NAME (open column) and in the presence of L-NAME (100 μM, filled column). Ordinate: [^{3}H]-efflux expressed as fractional rate. Values are means ± SEM of 4 experiments.

Table 1　Primers used in this study

Name	Sequence (5' - 3')
GHS-R-dSES1	AAY YTY TAY CTS TSY AGY ATG GC
GHS-R-dSES2	TTR ATS GCN HCR CTS AGR TAR AA
GHS-R-dANT1	GAY CTS CTS ATY TTY CTS TGY ATG CC
gpGHSR-s1	TTC CAG TTC GTC AGC GAG AGC TGC
gpGHSR-s2	AGC TGC ACC TAC GCC ACG GTG CTC
gpGHSR-AS1	CAC GGT TTG CTT GTG GTT CTG
gpGHSR-AS3	GCT GAC GAA CTG GAA GAG TTT GCA
gpGHSR-full-s	GAT CTG CTC GGT CCT TCG GCG GAG
gpGHSR-code-s	ATG TGG AAC GCG ACG CCC AGC GAG
gpGHSR-code-AS2	TCA TGT ATT GAT GCT AGA CTT TGT
gpGHSR-Q-s	GCT GCG CGC CAA GGT GGT GGT CAC
gpGHSR-Q-AS	TAT CGC CAG CAT TTT CAC GGT TTG
rGHSR-Q-s	CTT TCT ACC GGT CTT CTG CCT
rGHSR-Q-AS	AGC AGA GGA TGA AAG CAA ACA
gpB-act-Q-s	CCA TCA TGA AGT GTG ACG TTG
gpB-act-Q-AS	AGA GTG AGG CCA GGA TAG AGC

Fig.1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M W N A T P S E E P G
90 100 110 120 130 140 150 160
CCAACCTCACGCTGGCCGACCCGGGCTGGGACGGCCCCGCCGGCAACGACTCCCTGGCCGAGGAGCTGCTGCTGCTGCCG
S N L T L A D P G W D G P A G N D S L A E E L L L L P
170 180 190 200 210 220 230 240
CTGTTCCCCGCTCCGCTGCTGGCGGGCGTCACGGCCACCTGCGTGGCCCTCTTCGCGCTGGGCGTCGCGGGCAACCTGCT
L F P A P L L A G V T A T C V A L F A L G V A G N L L
250 260 270 280 290 300 310 320
CACCATGCTGGTGGTGTCGCGCTTCCGCGAGCTGCGCACGACCACCAACCTCTACCTGGCGAGCATGGCCTTCTCCGACC
T M L V V S R F R E L R T T T N L Y L A S M A F S D
330 340 350 360 370 380 390 400
TGCTCATCTTCCTCTGCATGCCCCTGGACCTCGTCCGCCTCTGGCAGCACCGTCCCTGGAACTTGGGCGACCTGCTCTGC
L L I F L C M P L D L V R L W Q H R P W N L G D L L C
410 420 430 440 450 460 470 480
AAACTCTTCCAGTTCGTCAGCGAGAGCTGCACCTACGCCACGGTGCTCACCATCACCGCGCTGAGCGTGGAGCGCTACTT
K L F Q F V S E S C T Y A T V L T I T A L S V E R Y F
490 500 510 520 530 540 550 560
CGCCGTCTGCTTCCCGCTGCGCGCCAAGGTGGTGGTCACCAGGGGCCGGGTGAAGCTGGTCATCCTGGTCATCTGGGCCG
A V C F P L R A K V V V T R G R V K L V I L V I W A
570 580 590 600 610 620 630 640
TGGCTTTCTGCAGCGCCGGGCCCATCTTCGTGCTCGTCGGGGTGGAGCACGAGAACGGCACCGACCCCCGGGACACCAGC
V A F C S A G P I F V L V G V E H E N G T D P R D T S
650 660 670 680 690 700 710 720
GAGTGCCGCCCCACGGAGTTCGCGGTGCGCTCGGGGCTGCTCACCGTCATGGTGTGGGTGTCCAGCGTCTTCTTCTTCCT
E C R P T E F A V R S G L L T V M V W V S S V F F F L
730 740 750 760 770 780 790 800
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P V F C L T V L Y S L I G R K L W R R R R G E A A V
810 820 830 840 850 860 870 880
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G A S L R D Q N H K Q T V K M L A I V V F A F I L C W
890 900 910 920 930 940 950 960
CTACCCTTCCACGTAGGAAGATACTTATTTTCCAAATCTTTTGAGCCCGGCTCCCTGGAAATCGCTCAGATCAGCCAGTA
L P F H V G R Y L F S K S F E P G S L E I A Q I S Q Y
970 980 990 1000 1010 1020 1030 1040
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C N L V S F V L F Y L S A A I N P I L Y N I M S K K
1050 1060 1070 1080 1090 1100 1110 1120
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Y R V A V F K L L G V A S F S Q R K L S T L K D E S S
1130 1140 1150 1160 1170 1180 1190 1200
CGGGGCTGGACAAAGTCTAGCATCAATACATGACCAGATGTGTTACTGAGCTCTTCATCACTTACTATTCTACATGGAAG
R G W T K S S I N T *
1210 1220 1230 1240 1250 1260 1270 1280
CCATAGGACAGCAGGACTTGGGAAGCAGCTGAAGGTCAATATTGGAATTAGGGACACATTGACTAGAAGCAACTGGAGGA
1290 1300 1310 1320 1330 1340 1350 1360
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1370 1380 1390 1400 1410 1420 1430 1440
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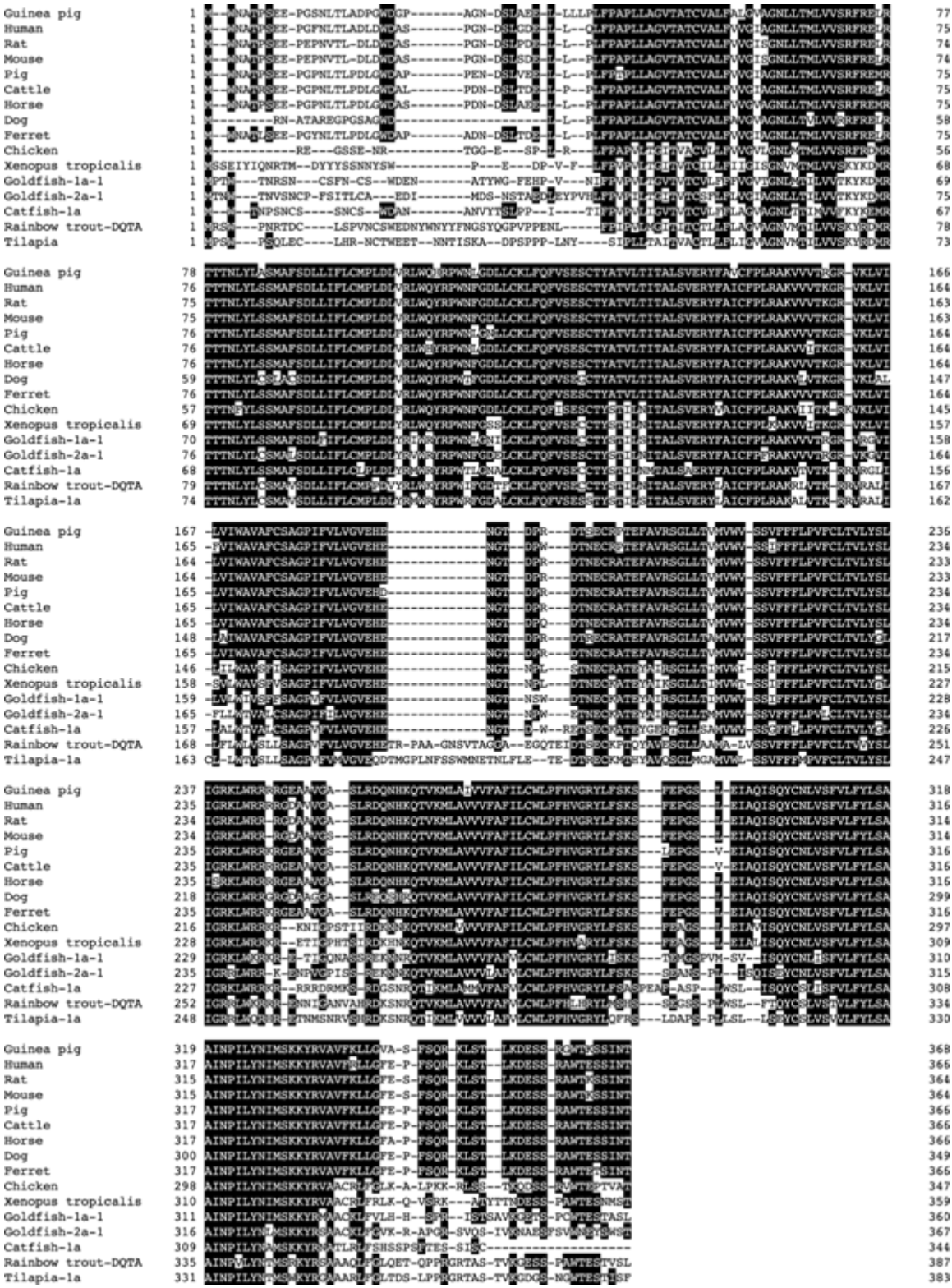
AA

Fig.2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Human
Rat
Mouse
Pig
Cattle
Horse
Dog
Ferret
Chicken
Xenopus tropicalis
Goldfish-1a-1
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Catfish-1a
Rainbow trout-DQTA
Tilapia-1a

Fig.3

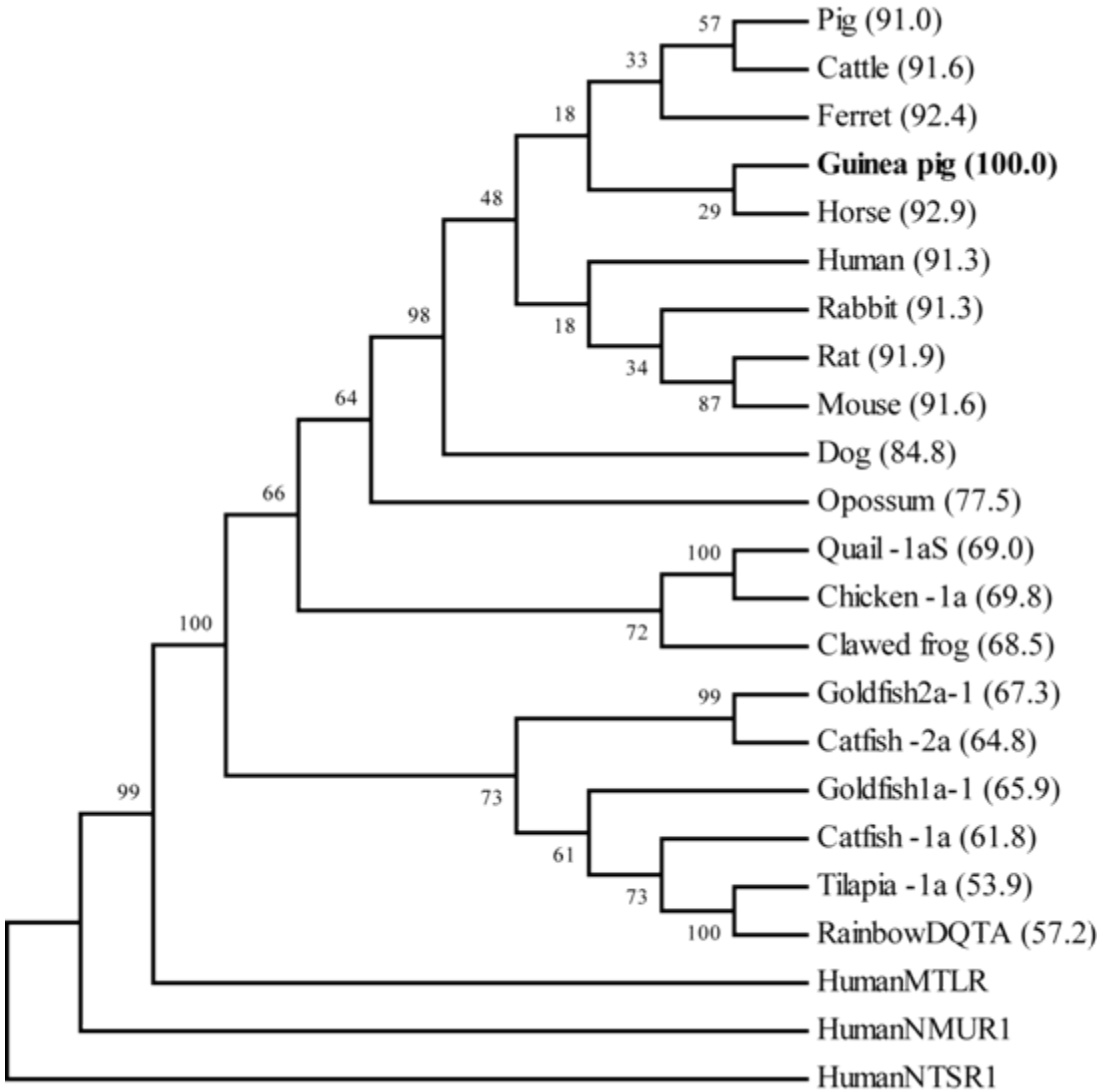

Pig (91.0)
Cattle (91.6)
Ferret (92.4)
Guinea pig (100.0)
Horse (92.9)
Human (91.3)
Rabbit (91.3)
Rat (91.9)
Mouse (91.6)
Dog (84.8)
Opossum (77.5)
Quail -1aS (69.0)
Chicken -1a (69.8)
Clawed frog (68.5)
Goldfish2a-1 (67.3)
Catfish -2a (64.8)
Goldfish1a-1 (65.9)
Catfish -1a (61.8)
Tilapia -1a (53.9)
RainbowDQTA (57.2)
HumanMTLR
HumanNMUR1
HumanNTSR1
57
33
18
48
29
18
98
34
64
87
66
100
100
72
99
73
61
73
99
100

Fig.4

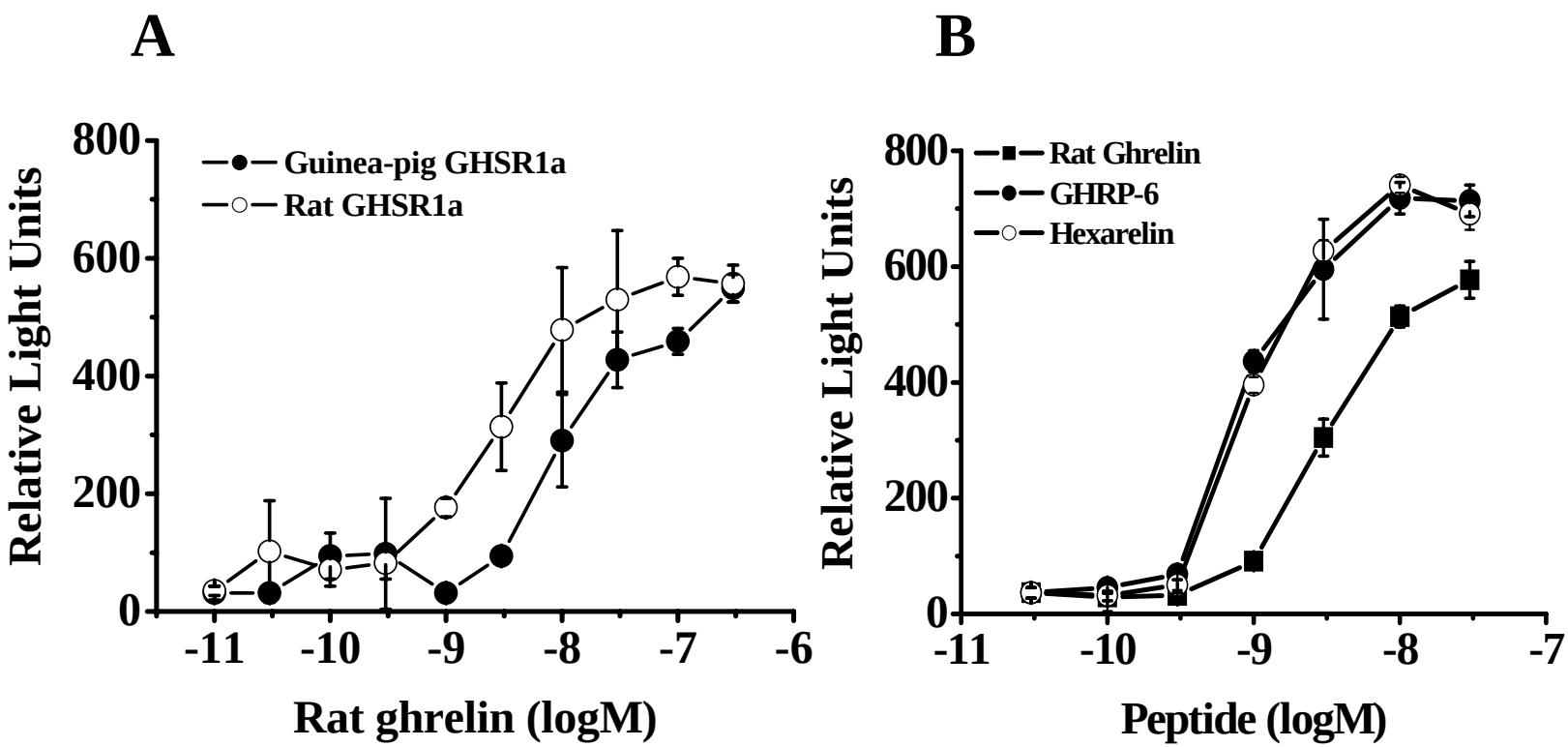
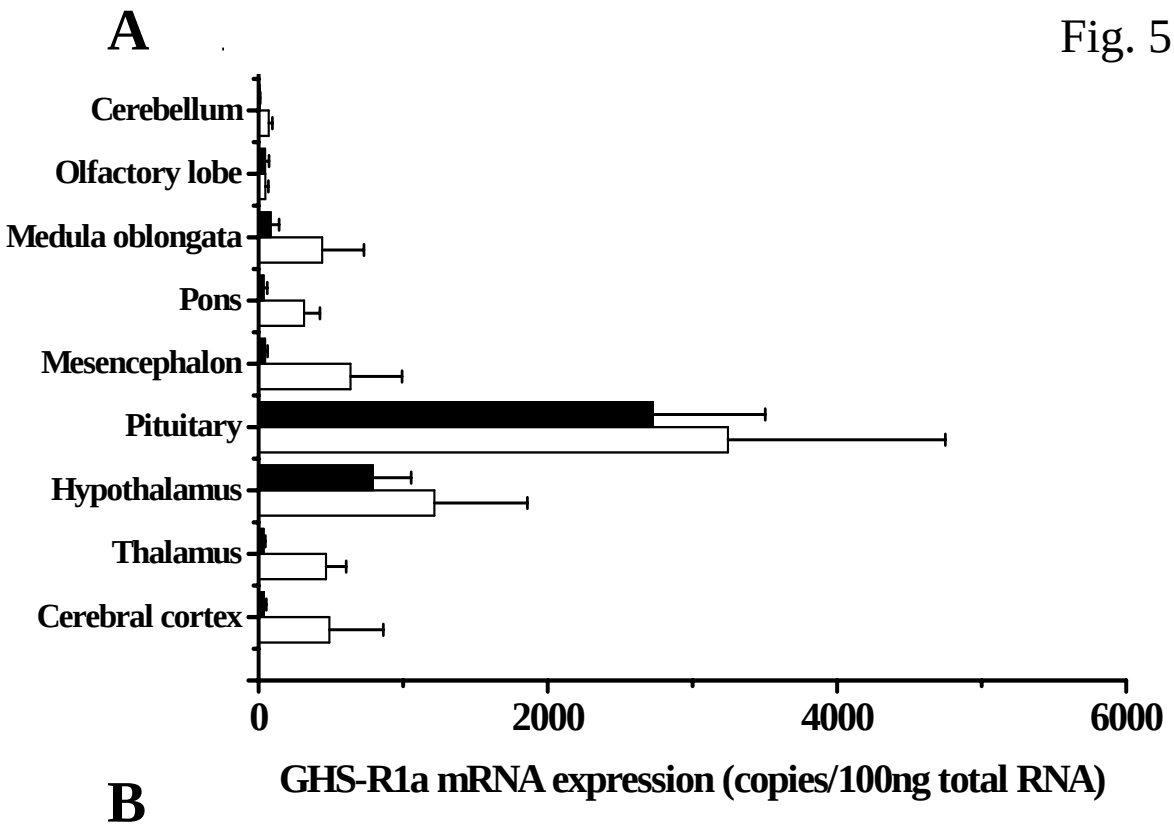

A
B
Relative Light Units
800
600
400
200
0
Guinea-pig GHSR1a
Rat GHSR1a
-11
-10
-9
-8
-7
-6
Rat ghrelin (logM)
Rat Ghrelin
GHRP-6
Hexarelin
Peptide (logM)

Fig. 5


A
Cerebellum
Olfactory lobe
Medula oblongata
Pons
Mesencephalon
Pituitary
Hypothalamus
Thalamus
Cerebral cortex
0
2000
4000
6000
GHS-R1a mRNA expression (copies/100ng total RNA)

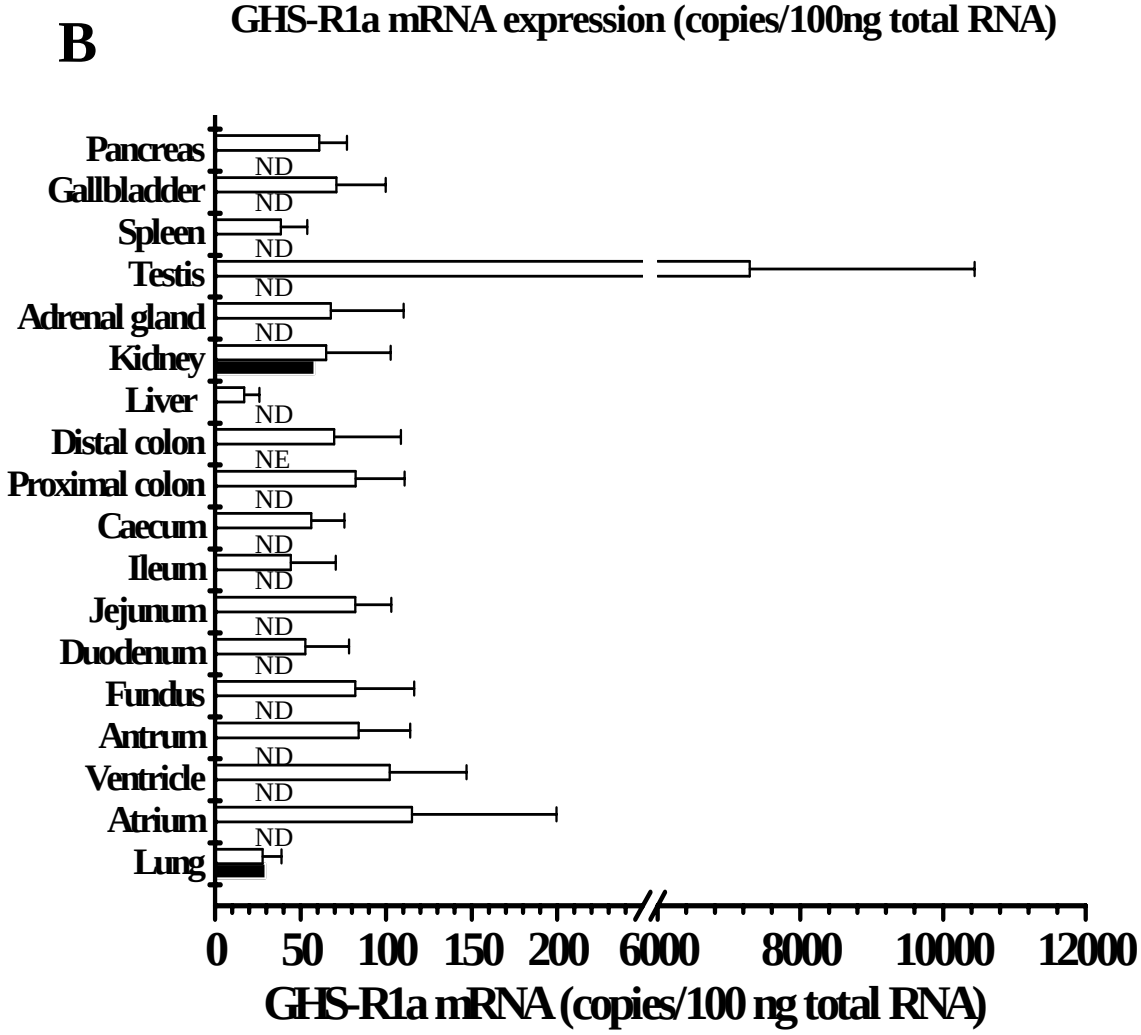

B
Pancreas
Gallbladder
Spleen
Testis
Adrenal gland
Kidney
Liver
Distal colon
Proximal colon
Caecum
Ileum
Jejunum
Duodenum
Fundus
Antrum
Ventricle
Atrium
Lung
ND
NE
0
50
100
150
200
6000
8000
10000
12000
GHS-R1a mRNA (copies/100 ng total RNA)

Fig.6

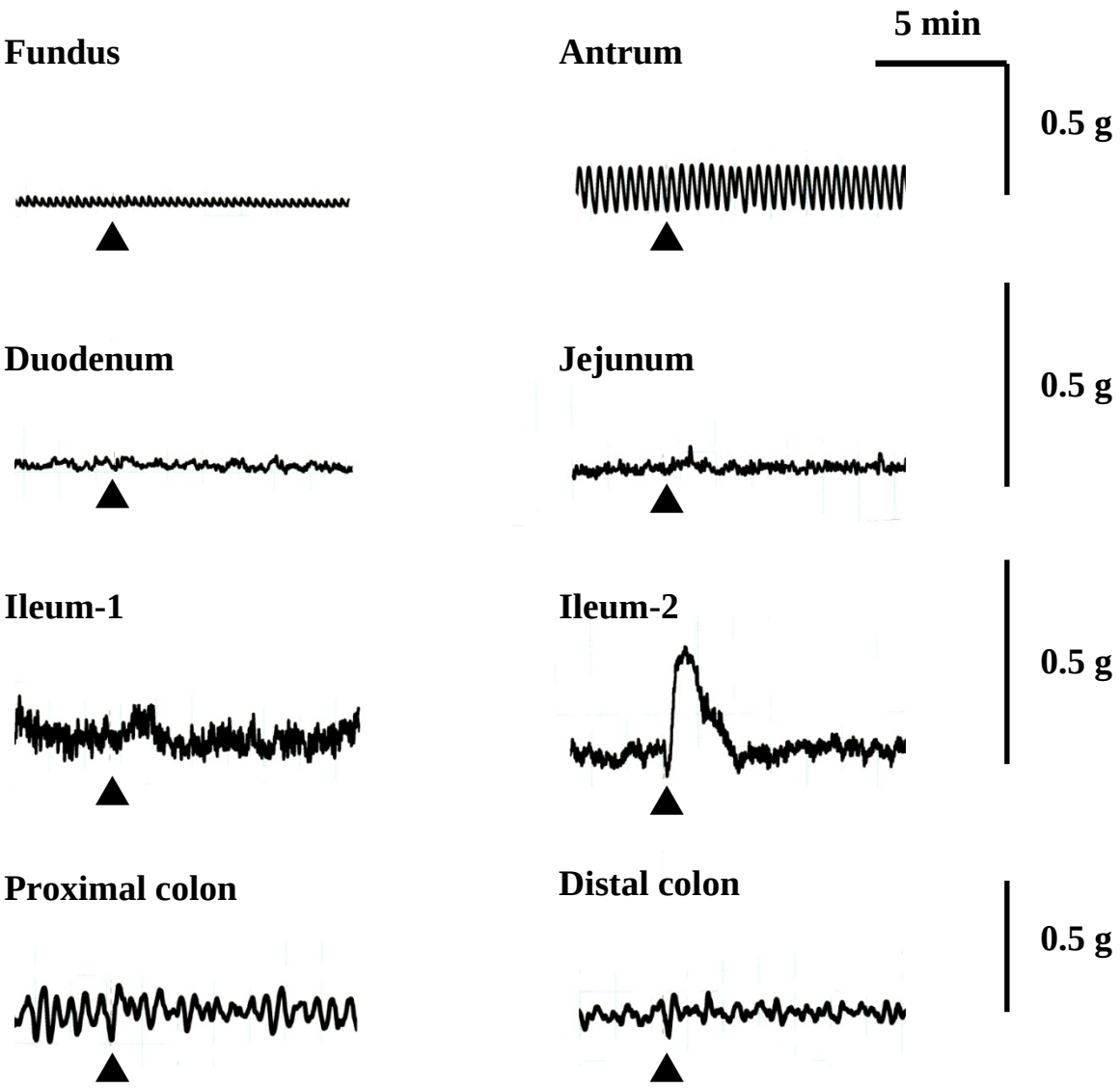

5 min
Fundus
Antrum
0.5 g
Duodenum
Jejunum
0.5 g
Ileum-1
Ileum-2
0.5 g
Proximal colon
Distal colon
0.5 g

Fig. 7

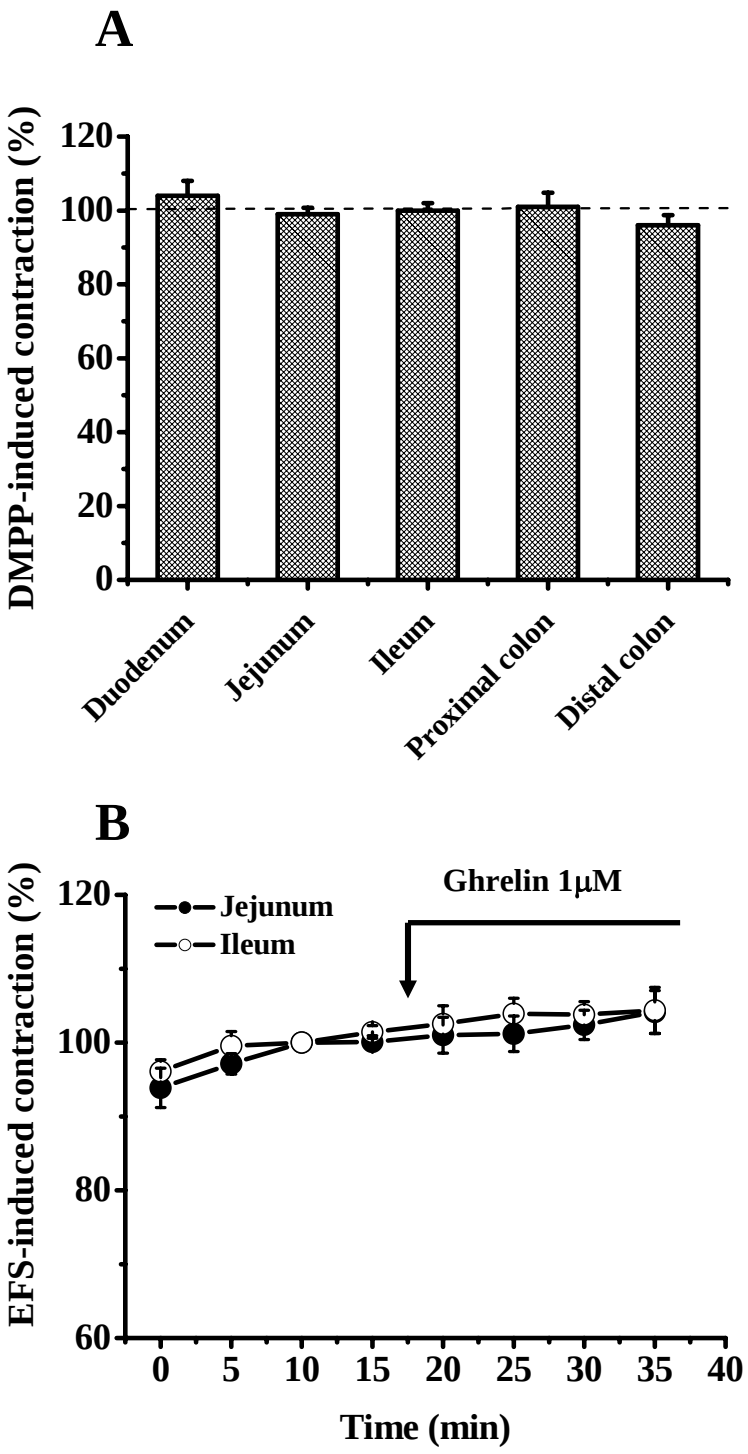
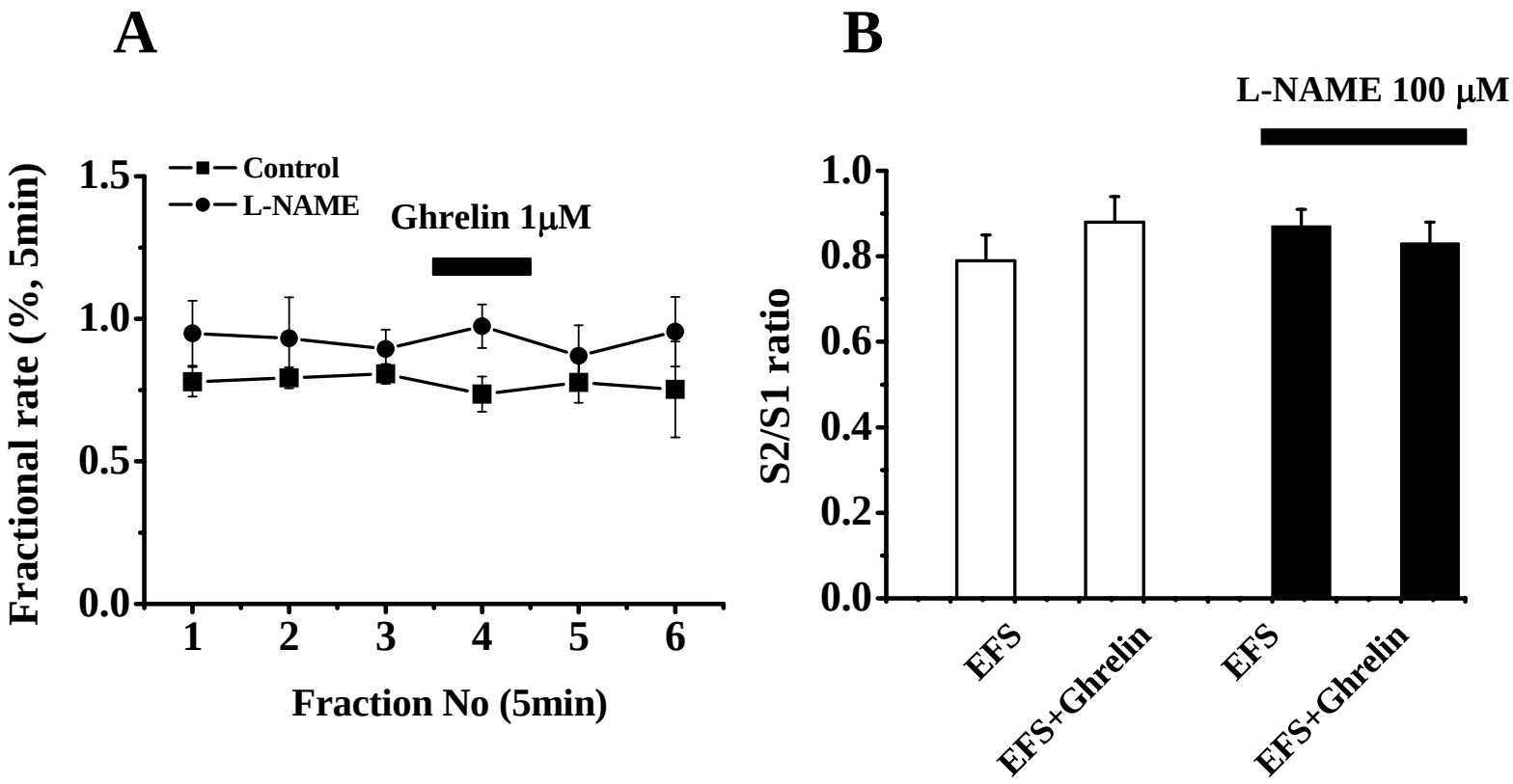

A
DMPP-induced contraction (%)
120
100
80
60
40
20
0
Duodenum
Jejunum
Ileum
Proximal colon
Distal colon
B
EFS-induced contraction (%)
Ghrelin 1μM
Jejunum
Ileum
120
100
80
60
0
5
10
15
20
25
30
35
40
Time (min)

Fig. 8


A
Control
L-NAME
Ghrelin 1μM
Fractional rate (%, 5min)
1.5
1.0
0.5
0.0
1
2
3
4
5
6
Fraction No (5min)
B
L-NAME 100 μM
S2/S1 ratio
1.0
0.8
0.6
0.4
0.2
0.0
EFS
EFS+Ghrelin
EFS
EFS+Ghrelin