Structural determination, distribution, and physiological actions of ghrelin in the guinea pig

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Abstract

We identified guinea pig ghrelin (gp-ghrelin), and examined its distribution and physiological actions in the guinea-pig. Gp-ghrelin is a 28-amino acid peptide (GASFR SPEHH SAQQR KESRK LPAKI QPR); seven amino acids are different from that of rat ghrelin at positions 2, 5, 10, 11, 19, 21, and 25, which include the conserved region known in mammals. The third serine residue is mainly modified by *n*-decanoyl acid. Both gp-ghrelin and rat ghrelin increased intracellular Ca^{2+} concentration of HEK293 cells expressing guinea pig growth hormone secretagogue receptor 1a (GHS-R1a), and the affinity of gp-ghrelin was slightly higher than that of rat ghrelin. In addition, gp-ghrelin was also effective in CHO cells expressing rat GHS-R1a with similar affinity to that of rat ghrelin. *Gp-ghrelin* mRNA was predominantly expressed in the stomach, whereas the expression levels in other organs was low. High levels of *GHS-R1a* mRNA expression were observed in the pituitary, medulla oblongata, and kidney, while medium levels were noted in the thalamus, pons, olfactory bulb, and heart. Immunohistochemistry identified gp-ghrelin-immunopositive cells in the gastric mucosa and pancreas. Intraperitoneal injection of gp-ghrelin increased food intake in the guinea pig. Gp-ghrelin did not cause any mechanical responses in isolated gastrointestinal smooth muscles *in vitro*, similar to rat ghrelin. In conclusion, the N-terminal structures that are conserved in mammals were different in gp-ghrelin. Moreover, the functional characteristics of gp-ghrelin, other than its distribution, were dissimilar from those in other

Rodentia.

Keywords: Guinea pig, Ghrelin, Growth hormone secretagogue receptor 1a (GHS-R1a), cDNA cloning, Functional characterization, Tissue distribution

1. Introduction

Ghrelin is a 28-amino-acid peptide with an *n*-octanoyl modification at the third serine residue (Ser-3), and is mainly produced in X/A-like cells in the oxyntic mucosa of the stomach. It is an endogenous ligand for growth hormone secretagogue receptor type-1a (GHS-R1a) [22, 23]. The modification at Ser-3 is accomplished by ghrelin-*O*-acyltransferase (GOAT)[54], and is essential for the binding of ghrelin to GHS-R1a and for eliciting its biological activity. The isoform of ghrelin that lacks Ser-3 modification is called des-acyl ghrelin or unacylated ghrelin, and is dominant in the stomach and plasma; however, its physiological role has not been fully understood yet. Ghrelin was initially known for its growth hormone-releasing activity, but accumulating evidence has indicated that it is an important regulator of glucose metabolism, endocrine/exocrine, gastrointestinal (GI) and cardiovascular functions. In addition, it is a peripheral orexigenic hormone that increases body weight by stimulating food intake and decreasing fat utilization [12, 22, 23, 32, 43].

Ghrelin has been identified in several mammalian and non-mammalian vertebrates, including fishes and birds [17]. In mammals, the N-terminal 10 amino acid sequence (GSSFLSPEHQ) is identical, along with the conserved amino acids at positions 16 (K), 20 (K), 21(P), 25 (L) and 28 (R) [18]. Since the N-terminal portion (GSSF including acyl modification) is known to be the active core of ghrelin [29], it is thought that the structure of ghrelin has been conserved throughout the evolutionary process of the molecule [18].

66 The guinea pig is a species of rodents widely used as an experimental model for studying
67 GI motor function because of the dense network of enteric neurons, easy separation of
68 longitudinal and circular muscles, and high sensitivity to several bioactive substances.
69 *Motilin* (a ghrelin-related peptide) mRNA has been identified in this species [52], along with
70 the expression of the motilin receptor proteins in the enteric nervous systems [52, 53].
71 However, this is different from that seen in rats and mice [11], where the motilin system
72 (motilin and motilin receptor) is absent, and the ghrelin system is thought to act as a
73 substitute for the motilin system, especially in the regulation of GI motility [7,42]. Our
74 previous study demonstrates that the guinea pig is sensitive to rat ghrelin but not to
75 unacylated rat ghrelin during gastric contraction *in vivo* through the activation of the
76 capsaicin-sensitive vago-vagal reflex pathway [33]. In addition, we have also identified the
77 GHS-R1a structure in the guinea pig (gpGHS-R1a), which was activated by rat ghrelin [21],
78 suggesting the presence of the ghrelin system in guinea pig. However, the sequence of guinea
79 pig ghrelin has not been identified.
80 Several studies on the distribution of ghrelin have been carried out so far, mainly on rats
81 and mice. Interestingly, the stomach of these animal species, which is the major site of
82 ghrelin production, is morphologically markedly different from those of other mammals. It
83 consists of a fore stomach (squamous mucosa) and a glandular stomach; the fore stomach
84 forms approximately 60% of the total stomach volume [3]. On the other hand, the guinea pig

is a monogastric herbivorous animal with a completely glandular stomach [9] and is unable to synthesize vitamin C [36], similar to humans. These differences in gastric morphology and nutritional profiles between the guinea pig and other rodents prompted us to investigate the distribution of ghrelin and its receptor, and to evaluate the functions of ghrelin in guinea pigs.

The aim of the present study was to identify the structure of gp-ghrelin and to examine the distribution of *ghrelin* (as the ligand) and *GHS-R1a* mRNAs (as the target), and the expression of the ghrelin peptide using molecular biological and immunohistochemical (IHC) approaches. Furthermore, the identified gp-ghrelin peptide was synthetized and its biological activities such as activation of GHS-R1a, regulation of food intake and *in vitro* GI motility were examined using the homologous system.

2. Materials and Methods

All experiments were performed in accordance with the institutional guidelines for Care and Use of Animals approved by the animal care and use committees of the Rakuno Gakuen University, the National Cardiovascular Center, and the Kissei Pharmaceutical Co., Ltd.

2.1. Animals and tissue preparations

Three–five week old guinea pigs (*Cavia porcellus*; Slc: Hartley) of either sex obtained from Japan SLC, Inc. (Shizuoka, Japan) were used for the molecular biological studies of

104 *ghrelin* and *GHS-R1a* mRNAs, and to examine the GI contraction *in vitro*. Throughout the
105 acclimation and experimental periods, the animals were housed in cages in an air-conditioned
106 animal room (room temperature, 24 ± 1 °C; relative humidity, 60% ± 5%; 12:12-h light/dark
107 cycle). After accommodation of over 1 week, they were used for the experiments.
108 For the IHC and food intake studies, male guinea pigs aged 5 weeks were obtained from
109 the same animal breeder (Japan SLC), and housed in cages in an air-conditioned animal room
110 for 1 week (room temperature, 24 ± 1°C; relative humidity, 55% ± 8%; 12:12-h light/dark
111 cycle.). Subsequently, the animals were divided into two groups: feeding experiment group
112 and blood sampling group (n = 6 in each). Each animal was kept in an individual cage. A
113 standard diet (LRC-4; Oriental Yeast, Tokyo, Japan) and tap water were available ad libitum
114 throughout the acclimation and experimental periods.
115
116 **2.2 Identification of gp-ghrelin**
117 **2.2.1. cDNA cloning**
118 The full-length of gp-ghrelin cDNA was determined by 3'- or 5'-rapid amplification of
119 the cDNA ends (RACE) by polymerase chain reaction (PCR) using a GeneRacer Kit (Life
120 Technologies, CA, USA). Total RNA was extracted from the glandular stomach with TRIzol
121 reagent (Life Technologies) and stored in RNAlater (Life Technologies).

First, we explored the information about the nucleotide sequence of gp-ghrelin (117 amino acids, 351 bps) in the Ensemble Genome Browser (http://www.ensembl.org/Cavia_porcellus/Info/Index) and found a contig, ENSCPOT00000022228.1. The nucleotide sequence of the coding region of gp-ghrelin was amplified using a primer set designated by the above information (gpGHRL-s1: 5'-ATG ACC TTG GCG GGG ACC ATC TGC-3'; and gpGHRL-AS1: 5'-CTT GTC TGC GGG GGC CTC TTC AGC-3'). Total RNA (1 μg) was reverse-transcribed with a GeneRacer 3'-oligo primer using QuantiTect RT Kit (QIAGEN GmbH, Hilden, Germany) at a final volume of 20 μl. Reverse transcription PCR (RT-PCR) was performed with a template (2 μl), a primer set (10 pmol/μl) and the HotStar Taq Plus Mix (QIAGEN). The reaction conditions were as follows: 95 °C for 5 min, subsequent 35 cycles at 95 °C for 0.5 min, 55 °C for 0.5 min, and 72 °C for 1.5 min, and a final extension at 72 °C for 3 min. The amplified product was sub-cloned into the pCRII-TOPO vector (Life Technologies) and the nucleotide sequence was determined by automated sequencing (model 3130; Applied Biosystems, CA, USA) according to the protocol of the BigDye™ terminator cycle sequencing kit (Applied Biosystems). Thus, the nucleotide sequences of the natural and the deposited were determined, following which the primers for the 3'- or 5'-RACE PCR were designed based on the defined nucleotide sequence.

For the 3’-RACE PCR, the template used in the afore-mentioned experiment was used. Primary PCR was performed with a gene-specific primer (GSP), gpGHRL-s1 (10 pmol/µl), and a 3’-primer supplied from the kit using HotStar Taq Plus Mix. The reaction conditions were as follows: 95 °C for 5 min, subsequent 35 cycles at 95 °C for 0.5 min, 58 °C for 0.5 min, and 72 °C for 1 min, and a final extension at 72 °C for 3 min. The amplified product was purified by the Wizard PCR preps DNA purification system (Promega, WI, USA), and subjected to a second-round nested PCR. The nested PCR was performed with another GSP, gpGHRL-s2 (5'-ACC ATC TGC AGC CTG TTG CTC CTC-3'), and a 3’-nested primer supplied by the kit under the same conditions described for the primary PCR. A nucleotide sequence of approximately 470 bp was determined.

For the 5’-RACE PCR, first-strand cDNAs were synthesized from total RNA (2.5 µg) obtained from the glandular stomach with oligo-dT_{12-18} primers or an anti-sense GSP (gpGHRL-AS1) used in the previous experiment using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany). The primary PCR was run using gpGHRL-AS1, 5’-primer supplied from the kit, and the HotStar Taq Plus Mix under the following amplification conditions: 95 °C for 5 min, subsequent 35 cycles at 95 °C for 0.5 min, 57 °C for 0.5 min, and 72 °C for 1 min, and a final extension at 72 °C for 3 min. After the purification of the PCR product, nested PCR was performed using another GSP, gpGHRL-AS2 (5'-CTC TTC AGC CTC TTC TCC AAG GAC-3'), a 5’-nested primer

159 supplied by the kit, and the HotStar Plus Taq Mix under the same conditions as in the primary
160 PCR. The amplicon was obtained from both oligo-dT- and gpGHRL-AS1-originated
161 templates. An approximately 390-bp product was determined. Finally, the nucleotide
162 sequence of the full-length cDNA was determined by assembling the results from the 5'- and
163 3'-RACE PCRs.
164
165 **2.2.2. Ghrelin purification**
166 Ghrelin activity during the purification process was monitored by measuring the changes
167 in intracellular Ca^{2+} concentration in the previously established cell line stably expressing rat
168 GHS-R1a (CHO-GHS-R62)[22].
169 Frozen guinea pig stomach (3.5 g) was boiled in Milli-Q-grade water for 10 min. After
170 cooling, the extracted solution was acidified with acetic acid (AcOH) to a concentration of 1
171 M and homogenized by a polytron homogenizer (Central Scientific Commerce, Inc., Tokyo,
172 Japan). After centrifugation at 13,200 × *g* for 30 min, the obtained supernatant was purified
173 using Sep-Pak Plus C18 cartridge (Waters, Milford, MA) and eluted in a gradient with 25%
174 to 60% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA).
175 The resulting fraction was lyophilized and purified by cation-exchange chromatography
176 (SP-Sephadex C-25, H^+-form; GE Healthcare Life Sciences). A successive elution was
177 performed with 1 M AcOH, 2 M pyridine, and 1 M pyridine-AcOH (pH 5.0), yielding SP-I,

178 SP-II, and SP-III fractions. The strong basic peptide-enriched SP-III fraction underwent
179 gel-filtration high-performance liquid chromatography (HPLC; TSKgel G2000SW; 21.5 ×
180 300 mm; Tosoh, Tokyo, Japan) with elution using 35% ACN/0.1% TFA. Active fractions
181 assessed by CHO-GHS-R62 were applied to carboxymethyl (CM)-ion-exchange HPLC
182 (TSKgel CM-2SW; 4.6 × 250 mm; Tosoh, Tokyo, Japan). The fractions were separated by
183 reverse-phase (RP)-HPLC using a Symmetry C18 column (3.9 x 150 mm; Waters) at a flow
184 rate of 1 ml/min under a linear gradient from 10% to 60% ACN/0.1% TFA for 40 min. They
185 were then further purified by RP-HPLC using a diphenyl column (2.1 × 150 mm;
186 219TP5125; Vydac, Hesperia, CA) at a flow rate of 0.2 ml/min under a linear gradient from
187 10% to 60% ACN/0.1% TFA for 40 min. Fractions corresponding to each absorbance peak
188 were collected. The purified peptide was applied to protein sequencing (model 494HT;
189 Applied Biosystems) to analyze the peptide sequence, which was confirmed by referring the
190 deduced amino acid sequence of the cDNA.

191 The molecular weight of the purified peptide was determined using matrix-assisted laser
192 desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (4800 MALDI
193 TOF/TOF^{TM} instrument; AB SCIEX, MA, USA). Briefly, a part of the purified fractions was
194 spotted on the measure plate together with 1 μl of saturated α-cyano-4-hydroxycinnamic acid
195 dissolved in 60% ACN/0.1% TFA. Acylation patterns were determined by measuring the
196 difference between the detected molecular mass and the theoretical molecular mass

197 calculated from the amino acid sequence of the purified peptide. The main gp-ghrelin
198 determined was a custom order to Peptide Institute Inc. (Osaka, Japan).
199
200 **2.3. Binding activity of gp-ghrelin to gpGHS-R1a and rat GHS-R1a**
201 We examined whether the synthesized gp-ghrelin binds to and activates gpGHS-R1a and
202 rat GHS-R1a. GpGHS-R1a was transiently expressed in human embryonic kidney (HEK) 293
203 cells as described previously [21], whereas rat GHS-R1a was stably expressed in Chinese
204 hamster ovary (CHO) cells (CHO-GHS-R62) [22]. HEK293 and CHO cells were cultured in
205 Dulbecco's Modified Eagle's Medium (DMEM; Gibco™, Life Technologies, MD, USA, cat.
206 no. 11995-065) and MEM Alpha (Gibco™, Life Technologies, cat. no. 12571-063),
207 respectively, containing 10% fetal calf serum (FCS) supplemented with antibiotics (10 U/ml
208 penicillin and 100 µg/ml streptomycin).
209 Cultured HEK293 and CHO cells were plated onto a collagen-coated 96-well black plate
210 and a normal 96-well black plate (Corning Inc., NY, USA) at densities of 3×10^4 cells and 5
211 $\times 10^4$ per well, respectively. At least 20 h after culturing the cells, cultured medium was
212 aspirated and then 100 µl fluorescent dye solution containing 4.4 µM Fluo-4AM (Invitrogen),
213 1% FCS and 1% Powerload Concentrate (Thermo Fisher Scientific) in a working buffer
214 (1xHanks' BSS; Gibco™, Life Technologies), and 20 mM HEPES buffer containing 250 µM
215 probenecid (Sigma-Aldrich Chemical, MO, USA) were loaded onto each well. The plate was

incubated for 1 h at 37 °C. Samples (120 µl) containing designated concentrations of ghrelin were prepared with working buffer containing 0.001% Triton X-100. After washing the wells three times with working buffer, changes in the intracellular Ca^{2+} concentrations in the GHS-R1a-expressing cells were measured using FLIPR^{tetra} (Molecular Devices, CA, USA) after adding 100 µl of designated concentrations of gp-ghrelin or rat ghrelin (Peptide Institute Inc.).

2.4. *In vitro* study for measurement of gastrointestinal contractility

2.4.1. Drugs used

The administration volume of each drug in the organ bath study was 1 % of the bath volume (5 ml). Acetylcholine chloride (ACh; Wako Pure Chemical Industries, Ltd, Osaka, Japan) was dissolved in distilled water and synthetic gp-ghrelin, whereas rat ghrelin (Peptide Institute Inc. Osaka, Japan) was dissolved in distilled water for the *in vitro* study.

2.4.2. *In vitro* study

Guinea pigs were anaesthetized using pentobarbital sodium (50 mg/kg, i.p., Kyoritsu Seiyaku, Tokyo, Japan), and sacrificed by bleeding from the carotid vein. After midline incision, smooth muscle strips from different parts of the GI tract (stomach, duodenum, jejunum, ileum, and colon) were prepared. The GI strips were suspended vertically in an organ bath (5 ml) to measure the mechanical activity of the longitudinal muscles. We used

two kinds of GI preparations: muscle strips with intact mucosa (whole tubular preparation of the intestine), and muscle strips without the mucosa because of the possible action of ghrelin on the neural pathway located in the mucosa [31]. The organ bath contained warm (37 °C) Krebs solution: NaCl, 118 mM; KCl, 4.75 mM; $MgSO_4$, 1.2 mM; KH_2PO_4, 1.2 mM; $CaCl_2$, 2.5 mM; $NaHCO_3$, 25 mM; and glucose, 11.5 mM, equilibrated with 95% O_2 + 5% CO_2 (pH, 7.4). Mechanical activity of the muscle preparation was recorded with an isometric force transducer (SB-612T; Nihon Kohden, Tokyo, Japan) and the data obtained were analyzed with a computer-aided analysis system (Power Lab 2/25, Japan Bioresearch center, Nagoya, Japan). 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 to establish spontaneous contractions. Prior to the addition of gp-ghrelin or rat ghrelin, each strip was subjected to three or four stimulations with 100 μM ACh until reproducible contractions were observed.

To examine the mechanical responses to ghrelins, each ghrelin was applied cumulatively from 0.1 nM to 1 μM at 3-min intervals, and the evoked responses were measured by the increase in muscle tonus compared with that in the absence of ghrelin. The results indicated the percentage changes of muscle tonus in response to the 100 μM ACh stimulation.

2.5. Food intake and blood glucose level

Gp-ghrelin was dissolved in saline containing 0.1% BSA and the formulations were

prepared just before each treatment. The animals were fasted for 16 h before the treatment and injected intraperitoneally with 20 or 80 μg/kg body weight of gp-ghrelin or an equal volume of saline 1 h after light onset (n = 6 each). The doses of ghrelin were determined based on the previous reports in rats [50,51]. The amount of food intake was measured at 2, 4, 10, and 24 h after ghrelin injection.

Blood samples were collected repeatedly from each animal (n = 6) at 30 min before and 5, 30, 60, and 120 min after the ghrelin treatments. Blood glucose concentration was measured to confirm the fasting condition using the 7180 clinical analyzer (Hitachi High-Technologies, Tokyo, Japan).

2.6. Quantification of *ghrelin* and *GHS-R1a* mRNA by real-time PCR in guinea pig organs

Total RNA was extracted separately from 28 tissues or organs obtained from seven guinea pigs stored in RNAlater, using Trizol reagent. First-strand cDNA was synthesized from 1 μg total RNA using a QuantiTect RT Kit with oligo-dT_{12-18} primer. RT-PCR was performed using the LightCycler 480 system (Roche Applied Science, Mannheim, Germany) with QuantiFAST SYBR Green PCR Kit (QIAGEN). Quantification of gpGHS-R1a and β-actin (internal control) for guinea pig was conducted according to the method described in our previous study [21].

For ghrelin, a primer set (gpGHRL-s1 and gpGHRL-AS1) that was used for the cloning of the coding region in ghrelin was used for the quantification analysis. The expected size of the amplicon was 351 bp. The reaction mixture consisted of 1× master mix and 250 nM each of the primer and the template (100 ng total RNA equivalent). The amplification conditions were as follows: 95 °C for 5 min, and subsequent 40 cycles at 95 °C for 10 s and at 60 °C for 30 s. For quantification of ghrelin cDNA copy number, a linear regression analysis was performed using serially diluted linearized pCRII vector cloned from the coding region of the gp-ghrelin described in Section 2.2.1. The vectors were linearized by restriction with *Xba* I. Data were calculated using the 2nd Derivative Max mode in the LightCycler software. The values were used if the desired sizes of the amplicons were confirmed by 1.5% agarose gel electrophoreses containing ethidium bromide, and a single peak was obtained in the melting curve analysis after quantification analysis.

2.7. Immunohistochemical study of ghrelin and GOAT

The animals were euthanized by exsanguination via the abdominal aorta under deep anesthesia with 2% isoflurane (Pfizer Japan, Tokyo, Japan). The stomach, intestine, and pancreas were quickly collected and fixed in 10% phosphate-buffered formalin. The tissues were embedded in paraffin, cut into 3-μm-thick sections on a microtome and mounted on gelatin-coated (super-frost) glass slides. The sections were treated with 3% H_2O_2 solution to

block endogenous peroxidase, followed by washing in PBS. Specifically, the sections for GOAT immunostaining were pretreated with heat-induced epitope retrieval (microwave oven at 500 W in Target Retrieval Solution; Agilent Technologies, CA, USA) for 15 min. The sections were incubated overnight at 4 °C with the primary antibody as follows: anti-rat ghrelin antibody (rabbit polyclonal antibody; 1:3,000; Transgenic Inc., Fukuoka, Japan); anti-human GOAT antibody (goat polyclonal antibody; 1:100; Abcam plc, Cambridge, UK). The sections were incubated in Envision + Dual link System-HRP (Agilent Technologies) for 30 min at room temperature, washed with PBS, and stained with 3,3'-diaminobenzidine + Liquid (Agilent Technologies) for 1–3 min. Then, the sections were counter-stained with Mayer's hematoxylin. Control experiments for investigation of specificity of the antibodies were performed by substitution of the primary antibodies with a suitable dilution of normal rabbit or goat serum (Agilent Technologies). When the normal serum was used, there were no remarkable staining of ghrelin or GOAT in this study. Morphometric observation was done using a microscope (BX-53; OLYMPUS, Tokyo, Japan) with a digital camera (DP70; OLYMPUS, Japan).

For immunofluorescent double staining of ghrelin and GOAT, sections were prepared as described earlier and incubated for 1 h at room temperature together with the anti-ghrelin and anti-GOAT antibodies. The sections were then incubated in the appropriate secondary antibodies conjugated with Alexa Fluor 488 (Donkey anti-goat IgG; Abcam plc) and Alexa

311 Fluor 594 (Donkey anti-rabbit IgG; Abcam plc) for 1 h at room temperature. The sections
312 were cover-slipped using Fluoroshield Mounting Medium (Abcam plc). Stained sections
313 were visualized using a fluorescence microscope (IX-71; OLYMPUS) with a digital camera
314 (DP80; OLYMPUS).
315
316 **2.8. Immunoelectron microscopy for ghrelin-immunoreactive cells of the stomach**
317 The fundic region of the stomach was cut into small pieces, fixed in Karnovsky fixative
318 for 2 h at 4 °C, and then post-fixed in 1% osmium tetroxide in cacodylate buffer for 1.5 h.
319 After dehydration through a graded series of increasing concentrations of ethanol, the
320 samples were embedded in epoxy resin. Ultra-thin sections were cut, mounted on nickel grids
321 and treated with 5% sodium metaperiodate for 30 min, after which they were washed with
322 distilled water and PBS, and blocked for 60 min using a blocking solution (Protein Block
323 Serum-Free; Agilent Technologies); they were incubated overnight at 4 °C with rabbit
324 polyclonal anti-rat ghrelin antibody (1:6,000; Transgenic Inc.). Next, the sections were
325 incubated for 30 min with biotinylated goat anti-rabbit IgG (1:200; Agilent Technologies) and
326 for 60 min with gold (10 nm)-conjugated streptavidin (BBI Solutions, Cardiff, UK), followed
327 by a 5 min treatment with 1.25% glutaraldehyde/PBS. Immunostained sections were
328 counter-stained with uranyl acetate and lead citrate and examined under a JEOL 1200EX
329 electron microscope (JEOL, Tokyo, Japan). The sizes of 120 ghrelin-containing granules

were measured in six cells.

2.10. Statistical analysis

Experimental data are expressed as means ± standard error of mean (SEM) of more than three experiments. The significance of differences between the values was determined at $P < 0.05$ using the Student's *t*-test (unpaired) for single comparisons (two groups) and the Bartlett's test/Dunnett's test or Dunnett's rank test for multiple comparisons (over three groups) using StatLight (ver. 2.10, Yukms, Tokyo, Japan).

3. Results

3.1. Structure of gp-ghrelin

3.1.1. Cloning of gp-ghrelin cDNA

We succeeded in amplifying the coding region on gp-ghrelin cDNA using the information provided in the Ensemble Genome Browser; subsequent RACE PCR revealed the complete length of the gp-ghrelin cDNA (Fig. 1; accession #LC322126). The complete pre-proghrelin cDNA was 514 bp long (5'untranslated region, 52 bp; coding region, 354 bp; 3' untranslated region, 108 bp) and contained a putative polyadenylation site (AATAAA) followed by a poly-A tail. The nucleotide sequence of gp-ghrelin had 93% identity with rat, mouse, and dog, 89% with pig, 75% with cattle and sheep, and 71.5% with humans, respectively.

349 The deduced amino acid sequence of the coding region consisted of 117 amino acids and
350 the sequence of the putative mature ghrelin peptide consisting of 28 amino acids was
351 predicted as follows: GASFR SPEHH SAQQR KESRK LPAKI QPR. Alternative spliced
352 transcripts lacking CAG corresponding to the 14th glutamine, known as des-Gln14 ghrelin in
353 humans and mice [13, 14] were also identified (Fig. 1).
354
355 **3.1.2. Isolation and structural determination of gp-ghrelin in stomach**
356 The strong basic peptide-enriched SP-III fraction obtained by SP-Sephadex C-25
357 ion-exchange chromatography was isolated by gel-filtration HPLC, which separated the
358 substances based on their molecular weight (Fig. 2A). Intracellular Ca^{2+} mobilization activity
359 in CHO cells stably expressing rat GHS-R1a (CHO-GHS-R62) were distributed in fractions
360 from 13 to 17. The active fractions were separated by CM-ion-exchange HPLC (Fig. 2B).
361 Apparent ghrelin activities were observed in 20 consecutive fractions from 50 to 69, which
362 were divided into seven groups from “a” to “g” for further purification. Each group was
363 subjected to preparative RP-HPLC, followed by another characteristic RP-HPLC using a
364 diphenyl column. RP-HPLC profiles of the most yielded peptide from Group “e” were
365 illustrated in Figure 2C (preparative RP-HPLC) and Figure 2D (RP-HPLC using a diphenyl
366 column).
367 Peptide sequencing was also performed in this purified peptide from Group “e.” The

368 sequence was readable up to the 21st residue: GAXFRSPEHHXAQQXXEXXXL (X,
369 undetermined residue), and was consistent with the amino acid sequence deduced from the
370 cDNA. The third amino acid residue was not identified, indicating that its side chain might
371 have been modified by some fatty acids. Peaks corresponding to peptide absorbance were
372 manually collected in RP-HPLC using a diphenyl column, and subjected to MALDI-TOF
373 mass analysis.
374 The expected molecular forms were determined based upon the theoretical molecular
375 mass of the 28-amino acid gp-ghrelin (monoisotopic molecular weight = 3,225.73). The
376 difference in mass units between 3,225.73 and the analyzed value strongly suggests that the
377 hydroxyl group of the third serine is replaced by an acyl moiety. The mass unit of the
378 predominant molecular form in Group "e" was 3,380.8 ($M + H^+$ form), and the difference
379 was 155.07. This indicates that ghrelin (28 amino acids, 1-28) was attached to the saturated
380 decanoyl moiety (C10:0). Therefore, the structure of the major gp-ghrelin is deduced to be
381 GAS (C10:0) FR SPEHH SAQQR KESRK LPAKI QPR.
382 Molecular weights of other yielded peptides were also measured and the results are
383 shown in Table 1. Unfortunately, no ghrelin activity was seen in the finally isolated fractions
384 from Groups "d" and "g," which may be attributed to the low amounts or loss of ghrelin
385 peptide due to unknown biochemical properties during the process. In Groups "a," "b," and
386 "c," these molecules were deduced as ghrelin [1–27]; des-Arg28 with unsaturated decanoic

387 acid (C10:2) in Group “a,” and saturated decanoyl moiety (C10:0) in Groups “b” and “c,”
388 based on the elution position of ion-exchange HPLC. Ghrelin [1–27], as des-Gln14 ghrelin
389 with saturated decanoic acid (C10:0), was detected in Group “f” (Table 1).
390 Multiple comparisons of mature ghrelin sequences among vertebrate species are shown in
391 Figure 3. The N-terminal ten amino acid sequence of mammalian ghrelin (GSSFLSPEHQ) is
392 identical, and amino acids at positions 14 (Q), 15 (R), 16 (K), 17 (E), 20 (K), 21(P), 27 (P),
393 and 28(R) are conserved among the mammalian ghrelin identified so far [17,18]. Gp-ghrelin
394 consisted of 28 amino acids (GASFR SPEHHSAQQR KESRKLPAKI QPR), out of which
395 Ser-3 has been modified by *n*-decanoic acid, as shown earlier. Seven amino acids were
396 different from rat ghrelin at positions 2, 5, 10, 11, 19, 21, and 25 (Fig. 3). The amino acid at
397 position 21, one of the conserved amino acids among mammals, was changed from Pro to Ile.
398
399 **3.2. Effects of gp-ghrelin on cells expressing gpGHS-R1a or rat GHS-R1a**
400 Gp-ghrelin and rat ghrelin activated HEK293 cells that transiently expressed gpGHSR-R1a
401 with increased intracellular Ca^{2+} concentrations in a concentration-dependent manner (Fig.
402 4A). The maximum responses of both ghrelins were almost the same; nevertheless, the EC_{50}
403 of gp-ghrelin (1.65 nM) was slightly lower than that of rat ghrelin (4.3 nM). Similarly,
404 gp-ghrelin and rat ghrelin also activated the CHO cells stably expressing rat GHS-R1a in a
405 concentration-dependent manner (Fig. 4B). The maximal responses of both ghrelin were

406 almost the same, but the EC_{50} of gp-ghrelin (2.4 nM) was slightly lower than that of rat
407 ghrelin (9.2 nM).
408
409 **3.3. Distribution of *ghrelin* and *GHS-R1a* mRNAs**
410 As indicated in Figure 5A, *ghrelin* mRNA was highly expressed in the fundus and body
411 of the stomach. Low expression was observed in the olfactory bulb, heart (ventricle and
412 atrium), caecum, colon (proximal), and liver, while the expression levels in other organs were
413 extremely low.
414 The expression pattern of *gpGHS-R1a* mRNA was different from that of *ghrelin* mRNA
415 (Fig. 5B). High expression levels (relative expression level to β-action, above 0.1) were
416 observed in the pituitary, medulla oblongata, and kidney, whereas the expression levels were
417 low (relative expression level from 0.01 to 0.1) in the thalamus, pons, olfactory bulb, and
418 heart (ventricle and atrium); the remaining organs (below 0.01) showed negligible levels of
419 expression.
420
421 **3.4. Immunohistochemical detection of ghrelin and GOAT**
422 A comparison of amino acid sequences indicated that rat ghrelin had a high identity with
423 gp-ghrelin, as a result of which an IHC study was conducted using anti-rat ghrelin polyclonal
424 antibody. Ghrelin-immunopositive (ip) cells were observed in the mucosal layer in all regions

425 of the stomach (Fig. 6A); cardia (Fig. 6B), body-lesser curvature (Fig. 6C), body-greater
426 curvature (Fig. 6D), and pylorus (Fig. 6E). In the mucosal layer, ghrelin-ip cells were mainly
427 observed in the glandular bases of the body of the fundic and the pyloric glands. These
428 ghrelin-ip cells did not have a luminal connection, thereby exhibiting the morphology of a
429 closed-type cell. The gastric region with the highest number of ghrelin-ip cells was the
430 body-lesser curvature (64.4 ± 4.5 cells/mm^2, n = 9), followed by the body-greater curvature
431 (29.9 ± 2.5 cells/mm^2), the cardia (20.1 ± 1.4 cells/mm^2), and the pylorus (14.6 ± 2.4
432 cells/mm^2). Although ghrelin-ip cells were also observed in the duodenum, the numbers were
433 quite low (2.5 ± 1.2 cells/mm^2) when compared with those of the stomach. The ghrelin-ip
434 cells in the duodenum were also closed-type. No ghrelin-ip cells were found in the ileum,
435 cecum and colon.

436 On the other hand, in the pancreas, ghrelin-ip cells were observed in endocrine cells
437 within the Langerhans islets (Fig. 6F), but not in the exocrine gland cells. No ghrelin-ip cells
438 were found in the other organs (brain, heart, lung, liver, spleen, and kidney) of the guinea pig
439 (data not shown).

440 GOAT-ip cells were observed in the mucosal layer in all regions of the stomach, similar to
441 the ghrelin-ip cells (Fig. 7A). In addition, a certain number of GOAT-ip cells were also
442 observed in the duodenal mucosa (Fig. 7E). To determine whether GOAT were co-labeled
443 with ghrelin-ip cells, double staining with GOAT and ghrelin was performed. Almost all

444 GOAT-ip cells in the stomach were stained with ghrelin (Fig. 7D). However, a few number of
445 GOAT-ip cells in the duodenum did not show ghrelin immunoreactivity (Fig. 7H). GOAT-ip
446 cells were not observed in any region of the pancreas, including the Langerhans islets (data
447 not shown).
448
449 **3.5. Immunoelectron microscopy for ghrelin-immunoreactive cells in the stomach**
450 Immunoelectron microscopic observations of the fundic gland revealed the localization of
451 immunogold staining for ghrelin in round and compact electron-dense granules in the
452 endocrine cells (Fig. **8**). The endocrine cells were small, round to ovoid in shape with many
453 granules in the cytoplasm, and were positioned close to the capillaries. An accurate size
454 analysis of the ghrelin-containing granules showed a mean diameter of 174.1 ± 7.7 nm (n =
455 120 granules).
456
457 **3.6. Effects of gp-ghrelin on GI contractility in isolated muscle strips of guinea pig**
458 Gp-ghrelin did not cause any mechanical responses (0.1 nM–1 μM) in the mucosa-intact
459 or mucosa-removed gastric antral circular muscle strips (Fig. 9A). Rat ghrelin was also
460 ineffective in eliciting any mechanical responses in both types of preparations (Fig. 9A). The
461 effects of gp-ghrelin in the ileal longitudinal muscle strips and whole ileum are shown in
462 Figure 9B. Both muscle preparations were insensitive to any concentration of gp-ghrelin or

463 rat ghrelin (Fig. 9B). The whole duodenal preparation was also examined but no effect was
464 seen at any of the concentrations of gp-ghrelin; the relative changes in muscle tonus were
465 2.6% ± 2.2% (0.1 nM), 6.2% ± 3.7% (1 nM), 8.1% ± 5.2% (10 nM), 12.4% ± 8.5% (100 nM),
466 and 13% ± 12.3% (1 μM; n = 4). Muscle tonus in the presence of 1 μM gp-ghrelin was not
467 significantly different from that of the control. The effects of gp-ghrelin and rat ghrelin on the
468 longitudinal muscle strips from proximal and distal colon are shown in Figure 9C. Both
469 gp-ghrelin and rat ghrelin failed to cause any significant mechanical responses in the colonic
470 preparations.
471
472 **3.7. Effects of gp-ghrelin on food intake and blood glucose level**
473 The effect of intraperitoneal administration of gp-ghrelin on food intake was examined.
474 As shown in Figure 10A, the amount of food intake by ghrelin-treated guinea pigs was
475 significantly increased when compared to the saline-treated animals. The stimulatory effect
476 began within 2 h after administration and continued until over 24 h.
477 Blood glucose concentrations in serum were increased following gp-ghrelin treatments
478 when compared to saline treatment (Fig. 10B). The effects were noted 30 min after gp-ghrelin
479 injection (20 μg/kg). Obvious dose-dependent effects were seen 120 min after 20 or 80 μg/kg
480 gp-ghrelin injection.
481

4. Discussion

The ghrelin system (ghrelin and GHS-R1a) works as a physiological regulator of GI, cardiovascular, endocrine, and exocrine functions, as well as glucose metabolism in vertebrates [12, 22, 23、**32, 43]**. Many studies have focused on the ghrelin system in mice and rats, but not in guinea pigs, well-used laboratory rodents. Previously, we identified gpGHS-R1a and reported its characteristics in the GI tract of guinea pig [21]. A genomic project on guinea pigs is in progress, but the structure of endogenous ghrelin had not been clarified yet.

In the present study, we determined the structure of gp-ghrelin by peptide purification from stomach extracts, in combination with its cDNA cloning. It presented as a 28-amino acid peptide with a decanoyl modification at Ser-3. The N-terminal 10-amino acid sequence (GSSFL SPEHQ) of mammalian ghrelin is highly conserved and is identical among all mammals known so far [17,18]. However, three amino acids at positions 2, 5, and 10 in the gp-ghrelin region (G**A**SF**R**SPEH**H**) were changed from Ser to Ala, Leu to Arg, and Gln to His, respectively. Furthermore, an additional four amino acids at positions 11, 19, 21, and 25 were found to be different from those of rats and mice (Fig. 3). Eight amino acids in the guinea pig differed from those in dog, pig, and human, whereas fourteen amino acids were dissimilar to those in cattle and sheep. As guinea pigs belong to the order of Rodentia, their

structural homology is very high to that of rats and mice. From a food habit point of view, the guinea pig is an herbivorous animal; however, it is interesting to note that its homology is very different from that of cattle and sheep, suggesting a low correlation between ghrelin structure and food habits.

Functional analysis of gp-ghrelin with gpGHS-R1a or rat GHS-R1a revealed that it was capable of increasing intracellular Ca^{2+} concentrations in GHS-R1a-expressing cells, and the affinity was slightly higher than that of rat ghrelin. In the N-terminal conserved region, the sequence created by the first four amino acids (GSSF) with acyl modification (active core of ghrelin) is known to be the minimum unit required for biological activity [29]. It is interesting to note that gp-ghrelin also activated rat GHS-R1a with a similar concentration-response relationship as that of rat ghrelin; nevertheless, a considerable number of amino acids were found to be different within the N-terminal region. These findings suggest that substantial amounts of changes in the amino acid sequence, including that in the N-terminal structure of gp-ghrelin, do not conspicuously affect the binding affinity and biological activity of ghrelin to mammalian GHS-R1a.

Gp-ghrelin has been predominantly modified by *n*-decanoic acid in the present study. Although octnoyl modification is common in most of vertebrates possessing ghrelin [17, 18], a similar dominant decanoyl modification of ghrelin has been reported in the teleost, tilapia [16]. **We cannot completely exclude the possibility that octanoyl ghrelin was degraded**

during the purification process. However, our preliminary data showed that in contrast to decanoyl ghrelin, octanoyl ghrelin could not be detected in tissue extracts of the stomach by radioimmunoassay. This fact strongly suggests that decanoyl ghrelin is dominant molecular form in guinea pig stomach. The sources and mechanisms of ghrelin modification have gradually been clarified; ingested fatty acids are used in mammals, birds, and fish [15, 35, 56], while GOAT preferably modifies C6 rather than C8 to ghrelin [24]. However, the reason why ghrelin is predominantly modified by decanoic acid in the guinea pig remains unclear.

High expression levels of *ghrelin* mRNA were observed in the stomach of guinea pigs, similar to that in other mammals [4, 17, 23]. In the present study, immunohistochemistry revealed the presence of ghrelin-ip cells in the mucosal layer, especially in the lesser and greater curvatures of the gastric body, similar to that observed in humans [46], rats [4,25], mice, hamsters [55], dogs [39], horses, cows, and pigs [10]. In addition, as reported in the rat stomach [40] and chicken proventriculus [49], the ghrelin-ip cells in the stomach and duodenum were of the closed-type. Ghrelin-ip cells lacking contract with intestinal lumen is thought that the endocrine of ghrelin is regulated by neural and hormonal stimulations but not by stimulations from the lumen. Although ghrelin was not detected [25] or was infrequently observed in the pyloric regions in rats [4], a certain number of ghrelin-ip cells were seen in this region in the guinea pig. The presence of moderate numbers of ghrelin-ip cells have been

539 reported in the pyloric region of pigs [10]. Thus, the species-dependent distribution of
540 ghrelin-ip cells in the pyloric region may reflect the differences in gastric structure, food
541 habits (herbivore), and species-related ghrelin functions.

542 Electron microscopic observation showed that the size and the ultrastructural features of
543 the ghrelin-ip granules in guinea pig were similar to those of X/A-like cells in rats [4, 47],
544 mice, hamsters [55], dogs, and humans [39]. The results of the present study indicated that
545 although the distribution of ghrelin-ip cells in the stomach of the guinea pig was slightly
546 different from that in rats and mice, the main distribution pattern and ultrastructural
547 characteristics were almost the same.

548 GOAT is the enzyme that post-translationally modifies Ser-3 of unacylated ghrelin with
549 octanoic acid [54]. *GOAT* mRNA and protein were detected in identical mucosal oxyntic cells
550 containing ghrelin in the gastric mucosa of rats [41] and pigs [27]. In the guinea pig stomach,
551 GOAT immunoreactivity was found in the ghrelin-ip cells, implying the co-localization of
552 both molecules in the gastric mucosal cells; thus, there is a possibility that GOAT participates
553 in the acylation of ghrelin, as has been observed previously in rats [41] and pigs [27].
554 However, GOAT-ip cells without ghrelin immunoreactivity were found in the duodenum of
555 the guinea pig. Such cells have also been reported in mouse stomach and rat duodenum [41,
556 44]. These results suggest physiological roles of GOAT other than ghrelin acylation, as shown

557 in the study where phenotypes of GOAT knockout mice were different from those of ghrelin
558 knockout mice [19].
559 *GHS-R1a* mRNA is distributed heterogeneously among the organs of the guinea pig,
560 indicating the multi-functionality of ghrelin. High levels of *GHS-R1a* mRNA expression were
561 observed in the pituitary, medulla oblongata, heart, liver, and kidney. The high expression of
562 GHS-R1a in the pituitary was similar to that in rats, suncus, and chickens [8, 21, 22, 45], and
563 suggests the regulation of pituitary endocrine function, such as growth hormone release [23].
564 Expression levels of GHS-R1a in the medulla oblongata, center of autonomic nerves, heart,
565 and kidney suggest that ghrelin affects the regulation of cardiovascular functions such as
566 heart contractility, heart rate, blood pressure, and blood flow [23, 26]. In the guinea pig,
567 ghrelin has been reported to regulate the tonus of the renal blood vessels [6].
568 Exogenous gp-ghrelin induced a significant increase in food intake and blood glucose level
569 in the guinea pig, as reported in other species [2, 5, 34]. Exogenous ghrelin elevates blood
570 glucose levels in humans and rodents, and the hyperglycemic effect is responsible for the
571 reduction of plasma insulin levels through ghrelin-induced attenuation of Ca^{2+} signaling in
572 pancreatic β-cells [5]. In the guinea pig, ghrelin immunoreactivity was found in the
573 Langerhans islets of the pancreas, as reported in rats [38, 48]. Although GOAT-ip cells were
574 not found in the islets of the guinea pig in this study, it is reported that detection of the
575 proteins (ghrelin and GOAT) is difficult in the tissues except for stomach and intestine where

576 expression levels of the proteins are high [19]. However GOAT immunoreactivity has been
577 reported in the islet cells of other rodents [1, 30], therefore sensitive detection methods for
578 mRNA would be possible to detect the GOAT expression in the islet of guinea pig. The
579 ghrelin and GOAT mRNAs are demonstrated to be co-expressed in the porcine pancreas [27].
580 Considering these results, the closed localization of ghrelin and insulin secretory cells
581 (β-cells) in the guinea pig indicates the regulation of insulin release by endogenous ghrelin in
582 a paracrine manner. Since plasma insulin concentrations were not measured in this study, we
583 were unable to investigate the relationship between insulin and exogenous ghrelin.
584 Ghrelin forms a peptide family with motilin (a mediator of interdigestive motor
585 contraction) [2, 37]. Therefore, the GI-motor-stimulating actions of ghrelin have been the
586 focus of several studies, which have demonstrated that ghrelin stimulates GI motility both *in*
587 *vivo* and *in vitro* in mice and rats [7, 20, 28]. Previously, we reported that rat ghrelin did not
588 cause the contraction of isolated GI strips in guinea pig [21]. One plausible reason for this
589 insensitivity to rat ghrelin might be the structural differences in ghrelin between rats and
590 guinea pigs. However, in the present study, homologous gp-ghrelin also proved ineffective in
591 causing any contractions in the isolated guinea pig GI preparations, similar to the rat ghrelin.
592 This finding suggests that the ineffectiveness of rat ghrelin in the guinea pig GI tract is not
593 due to structural differences, but due to the low expression levels of GHS-R1a in the GI tract
594 of the guinea pig. In the suncus, it has been suggested that ghrelin acts on intrinsic primary

595 afferent neurons in the mucosal layer [31]; however, in the current study, gp-ghrelin was
596 found to be ineffective in the mucosa-intact GI strips.
597 In conclusion, to our best knowledge, this is the first comprehensive description of the
598 peptide structure, tissue distribution, and functions of ghrelin in a rodent, especially the
599 guinea pig. Even though gp-ghrelin has unique N-terminal structural features when compared
600 to other mammalian ghrelin, it has a functional relationship with gpGHS-R1a and rat
601 GHS-R1a, and fully activates the two receptors. In addition, its distribution, localization, and
602 morphological characterization in the producing cell was similar to that in other rodents.
603 However, in spite of its stimulatory effect on feeding, no marked effects were observed on the
604 GI tract *in vitro*. This is different from other rodents such as mice and rats, and may reflect
605 upon the evolution process of the ghrelin system and the feeding habit.
606
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Figure Legends

Figure 1. Nucleotide sequence and deduced amino acid sequence of the guinea pig ghrelin.

The guinea pig ghrelin cDNA (Acc # LC322126) consists of 514 bp, which encodes a 117-amino-acids pre-proghrelin. Mature guinea pig ghrelin consisting of 28 amino acids is *underlined*. The glutamine (Gln, Q) residue surrounded by a circle indicates the deleted amino acid in the case of des-Gln14 ghrelin. The polyadenylation signal (AATAAA) is *boxed*.

Figure 2. Purification profiles of guinea pig ghrelin

(A) Gel-filtration high-performance liquid chromatography (HPLC) of the strong basic peptide-enriched SP-III fraction. (B) Carboxymethyl (CM) cation-exchange HPLC (pH 4.8) of the active fraction (frs.13-17) from gel-filtration HPLC. (C) RP-HPLC for group "e" (frs. 61–63) from CM-HPLC. (D) Final RP-HPLC using a diphenyl column. A peak indicated by the arrowhead was isolated. *Black bars* in each graph indicate changes in intracellular Ca^{2+} concentrations of CHO-GHSR62 cells.

Fig. 3. Comparison of amino acid sequences of mature guinea pig ghrelin with mature ghrelin in vertebrates

Asterisks and *dots* indicate amino acids identical in all species or identical over the half

838 number among species. In parenthesis indicates the number of amino acid identical to ghrelin
839 in the animal compared to guinea pig ghrelin, and the percentage indicates the identity of
840 amino acid to gp-ghrelin when compared with ghrelin in each animal. Amino acid sequences
841 were obtained from the DDBJ/EMBL/GenBank™ databases (acc #: NM_012488 for mouse,
842 NM_021669 for rat, XM_002722463 for rabbit, AY903701 for cattle, AB060699 for sheep,
843 AY028942 for pig, NM_001032903 for Rhesus monkey, XM_002758630 for marmoset,
844 AB089201 for cat, AB060700 for dog, EU375448 for giant panda, XM_001375640 for
845 opossum, EU677468 for wallaby, AB029434 for human, EF613551 for duck, AY338465 for
846 goose, AB075215 for white leghorn chicken, AY338467 for emu, AB161457 for red-eared
847 slider turtle, AB058510 for bullfrog, AB062427 for Japanese eel, FJ560488 for large yellow
848 croaker, AB077764 for Mozambique tilapia, DQ665912 for European seabass, AB096919 for
849 rainbow trout, AF454389 for goldfish, NM_001083872 for zebrafish, AB254128 for
850 hammerhead shark, AB254129 for blacktip reef shark, AB4800033 for the red stingray).
851
852 **Figure 4. Changes in intracellular Ca^{2+} concentrations in rat or gpGHS-R1a-expressing**
853 **mammalian cells induced by rat ghrelin and gp-ghrelin**
854 (A) gpGHS-R1a was transiently expressed in HEK 293 cells. These cells were treated with
855 rat ghrelin (■) and gp-ghrelin (●) (0.01–100 nM), and increases in Ca^{2+} concentration were
856 measured. (B) Rat GHS-R1a was stably expressed in CHO cells and the effects of rat ghrelin

857 (■) and gp-ghrelin (●) (0.01–100 nM) were examined. Intracellular Ca^{2+} changes were

858 measured by the FLIPR system. Values are expressed as means ± standard error of mean (n =

859 3).

860

861 **Figure 5. Tissue-dependent differential expression of guinea pig *ghrelin* mRNA and**

862 ***GHS-R1a* mRNA in the central and peripheral tissues.**

863 *Ghrelin* (A) and *GHS-R1a* (B) mRNAs expression levels were quantified by real-time

864 polymerase chain reaction and indicated as relative values normalized against the expression

865 levels of *β-actin* mRNA. Values are expressed as mean ± standard error of mean from most

866 of the organs in 4–5 guinea pigs (*Ghrelin* mRNA: ovary, n = 3; *GHS-R1a* mRNA: liver,

867 kidney, and ovary, n = 2; uterus, n = 3).

868

869 **Figure 6. Distribution of ghrelin-immunoreactive cells in the stomach and pancreas of**

870 **guinea pig.** A: Schematic diagram of sampling site in stomach. ■, stratified squamous

871 epithelium; ▭, cardiac gland mucosa; ▨, fundic gland mucosa; and ▭, pyloric gland

872 mucosa. B: Cardia. C: Body-lesser curvature. D: Body-greater curvature, E: Pylorus. F:

873 Pancreatic islet. Ghrelin-immunoreactive cells (arrows). Scale bar: B–E = 100 μm; F = 50 μm

874

875 **Figure 7. Distribution of GOAT-immunoreactive cells in the stomach and duodenum of**

guinea pig.

Stomach (A–D) and duodenum (E–H). Immunofluorescence staining for GOAT (B and F, red), ghrelin (C and G), and merged (D and H, yellow). Immunoreactive cells (arrows). Scale bar: A and E = 100 μm

Figure 8. Immunoelectron micrographs of a ghrelin cell in the guinea pig gastric mucosa.

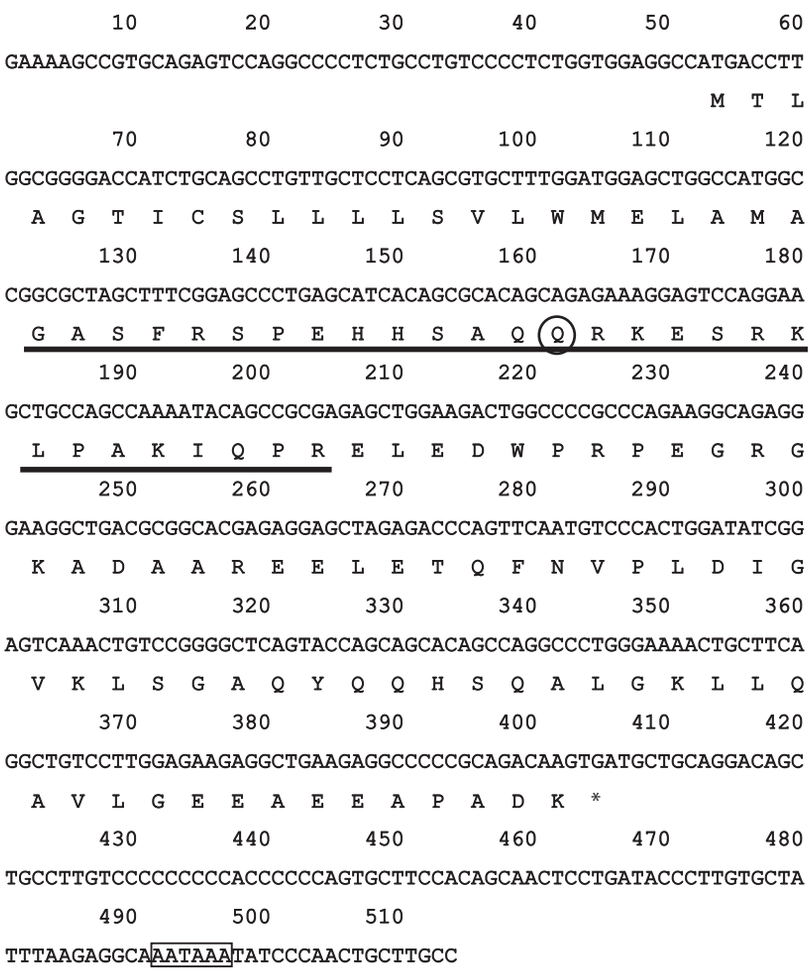
A: Cells with immunogold labeling for ghrelin. B: Ghrelin-immunoreactive granules (arrow). Scale bar: A, 1 μm; B, 200 nm.

Figure 9. The effects of rat ghrelin and guinea pig ghrelin on smooth muscle contractility in isolated guinea pig gastrointestinal tract.

(A) Rat ghrelin (□, ■) or gp-ghrelin (○, ●)-induced mechanical responses of gastric antrum circular muscle with intact mucosa (M+) or without mucosa (M-). (B) Rat ghrelin (□, ■) or gp-ghrelin (○, ●)-induced mechanical responses of whole ileum(whole, with mucosa) and longitudinal muscle (strips, without mucosa). (C) Rat ghrelin or gp-ghrelin-induced mechanical responses of longitudinal muscle strips (without mucosa) from proximal and distal colon. Values are expressed as mean ±standard error of mean from three to seven experiments.

Figure 10. Effect of intraperitoneal injection of ghrelin on cumulative food intake (A) and blood glucose concentration (B) in guinea pigs. Values are expressed as mean ± standard error of mean from six experiments for food intake, and three experiments for blood glucose concentration. *: $P < 0.05$, **: $P < 0.01$ compared with the values of the saline group.

Figure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A G T I C S L L L L S V L W M E L A M A
130 140 150 160 170 180
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G A S F R S P E H H S A Q Q R K E S R K
190 200 210 220 230 240
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L P A K I Q P R E L E D W P R P E G R G
250 260 270 280 290 300
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K A D A A R E E L E T Q F N V P L D I G
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V K L S G A Q Y Q Q H S Q A L G K L L Q
370 380 390 400 410 420
GGCTGTCCTTGGAGAAGAGGCTGAAGAGGCCCCCGCAGACAAGTGATGCTGCAGGACAGC
A V L G E E A E E A P A D K *
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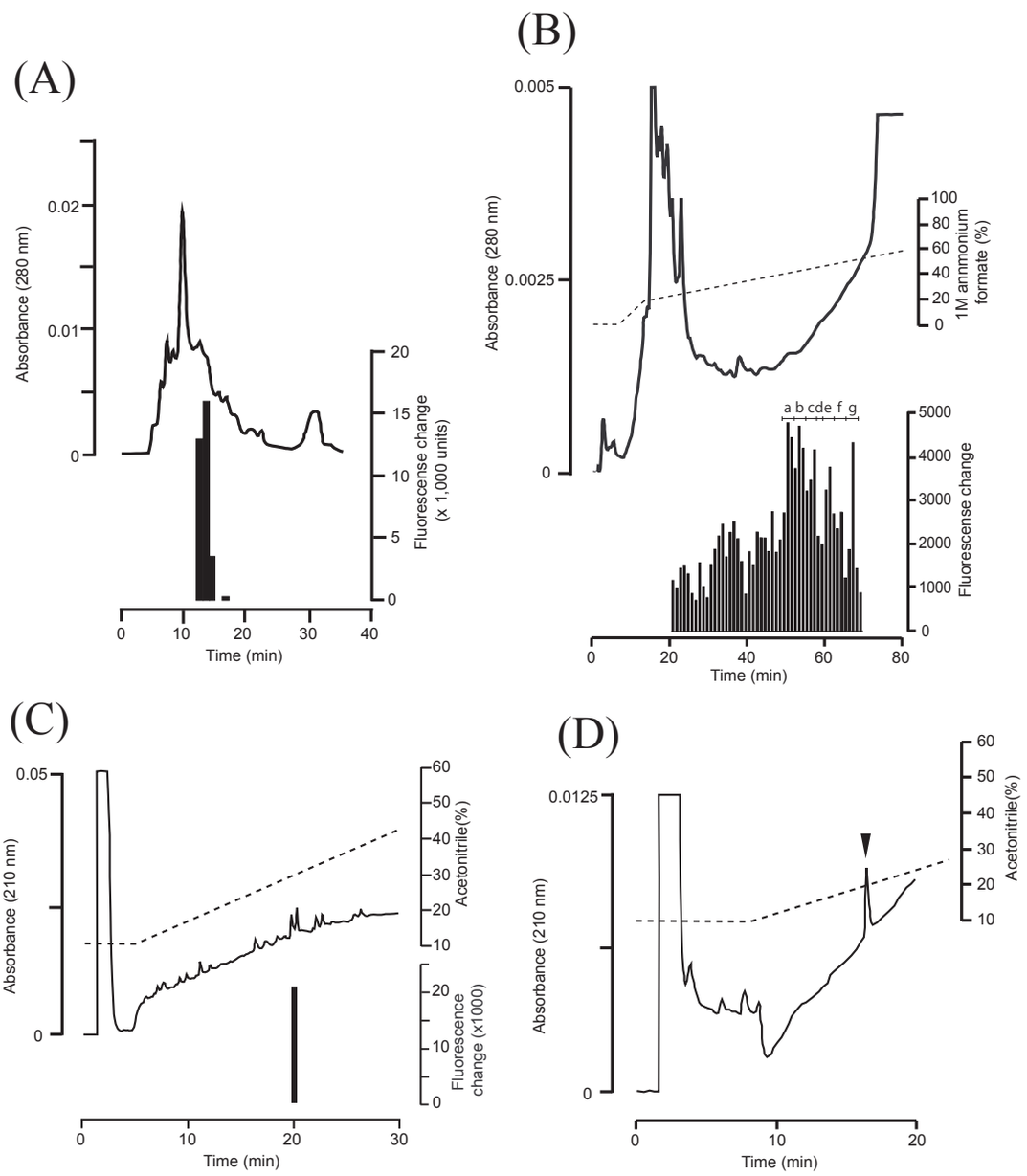
Figure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0.01
0
20
15
10
5
0
Fluorescense change
(x 1,000 units)
0
10
20
30
40
Time (min)
(B)
0.005
0.0025
0
Absorbance (280 nm)
100
80
60
40
20
0
1M annmonium
formate (%)
a b cde f g
5000
4000
3000
2000
1000
0
Fluorescense change
0
20
40
60
80
Time (min)
(C)
0.05
0
Absorbance (210 nm)
60
50
40
30
20
10
Acetonitrile(%)
20
10
0
Fluorescence
change (x1000)
0
10
20
30
Time (min)
(D)
0.0125
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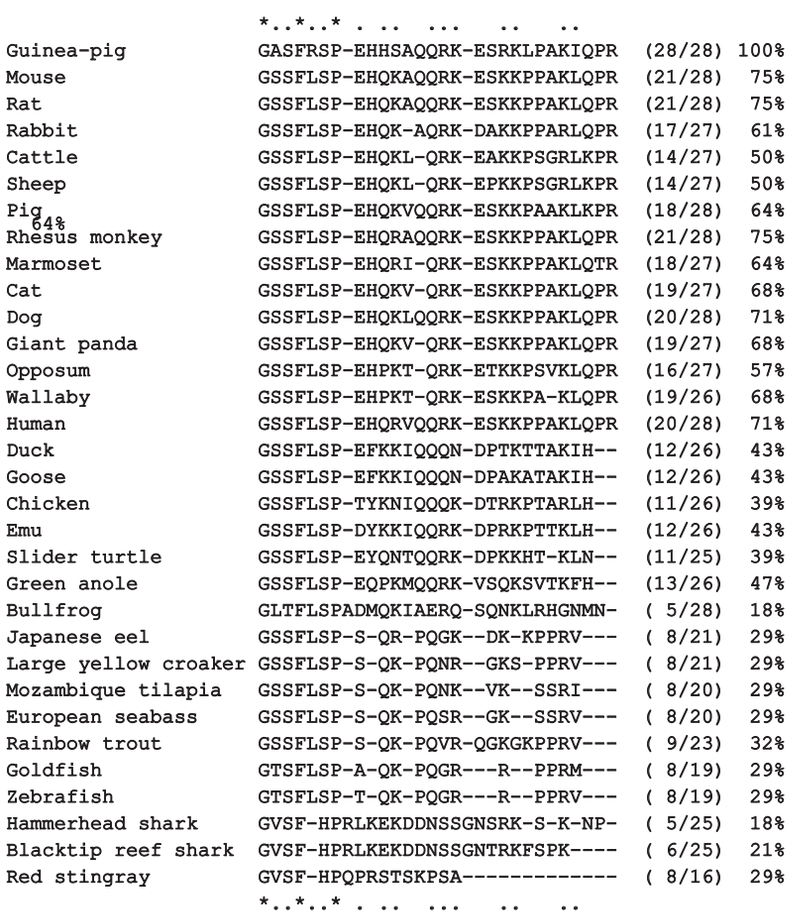
Absorbance (210 nm)
60
50
40
30
20
10
Acetonitrile(%)
0
10
20
Time (min)

Figure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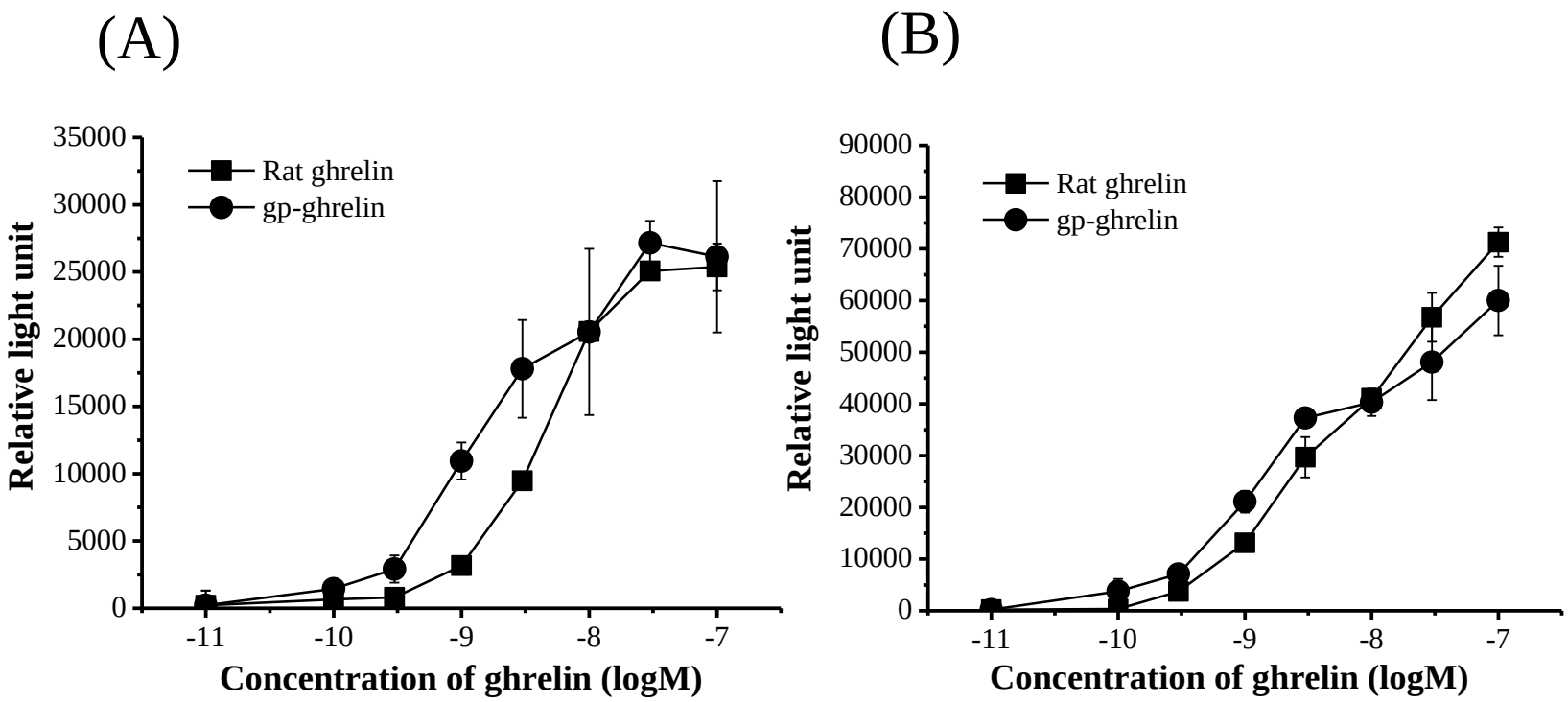
Rat GSSFLSP-EHQKAQQRK-ESKKPPAKLQPR (21/28) 75%
Rabbit GSSFLSP-EHQK-AQRK-DAKKPPARLQPR (17/27) 61%
Cattle GSSFLSP-EHQKL-QRK-EAKKPSGRLKPR (14/27) 50%
Sheep GSSFLSP-EHQKL-QRK-EPKKPSGRLKPR (14/27) 50%
Pig GSSFLSP-EHQKVQQRK-ESKKPAAKLKPR (18/28) 64%
64%
Rhesus monkey GSSFLSP-EHQRAQQRK-ESKKPPAKLQPR (21/28) 75%
Marmoset GSSFLSP-EHQRI-QRK-ESKKPPAKLQTR (18/27) 64%
Cat GSSFLSP-EHQKV-QRK-ESKKPPAKLQPR (19/27) 68%
Dog GSSFLSP-EHQKLQQRK-ESKKPPAKLQPR (20/28) 71%
Giant panda GSSFLSP-EHQKV-QRK-ESKKPPAKLQPR (19/27) 68%
Opposum GSSFLSP-EHPKT-QRK-ETKKPSVKLQPR (16/27) 57%
Wallaby GSSFLSP-EHPKT-QRK-ESKKPA-KLQPR (19/26) 68%
Human GSSFLSP-EHQRVQQRK-ESKKPPAKLQPR (20/28) 71%
Duck GSSFLSP-EFKKIQQQN-DPTKTTAKIH-- (12/26) 43%
Goose GSSFLSP-EFKKIQQQN-DPAKATAKIH-- (12/26) 43%
Chicken GSSFLSP-TYKNIQQQK-DTRKPTARLH-- (11/26) 39%
Emu GSSFLSP-DYKKIQQRK-DPRKPTTKLH-- (12/26) 43%
Slider turtle GSSFLSP-EYQNTQQRK-DPKKHT-KLN-- (11/25) 39%
Green anole GSSFLSP-EQPKMQQRK-VSQKSVTKFH-- (13/26) 47%
Bullfrog GLTFLSPADMQKIAERQ-SQNKLRHGNMN- (5/28) 18%
Japanese eel GSSFLSP-S-QR-PQGK--DK-KPPRV--- (8/21) 29%
Large yellow croaker GSSFLSP-S-QK-PQNR--GKS-PPRV--- (8/21) 29%
Mozambique tilapia GSSFLSP-S-QK-PQNK--VK--SSRI--- (8/20) 29%
European seabass GSSFLSP-S-QK-PQSR--GK--SSRV--- (8/20) 29%
Rainbow trout GSSFLSP-S-QK-PQVR-QGKGKPPRV--- (9/23) 32%
Goldfish GTSFLSP-A-QK-PQGR---R--PPRM--- (8/19) 29%
Zebrafish GTSFLSP-T-QK-PQGR---R--PPRV--- (8/19) 29%
Hammerhead shark GVSF-HPRLKEKDDNSSGNSRK-S-K-NP- (5/25) 18%
Blacktip reef shark GVSF-HPRLKEKDDNSSGNTRKFSPK---- (6/25) 21%
Red stingray GVSF-HPQPRSTSKPSA---------- (8/16) 29%
....* . .. .

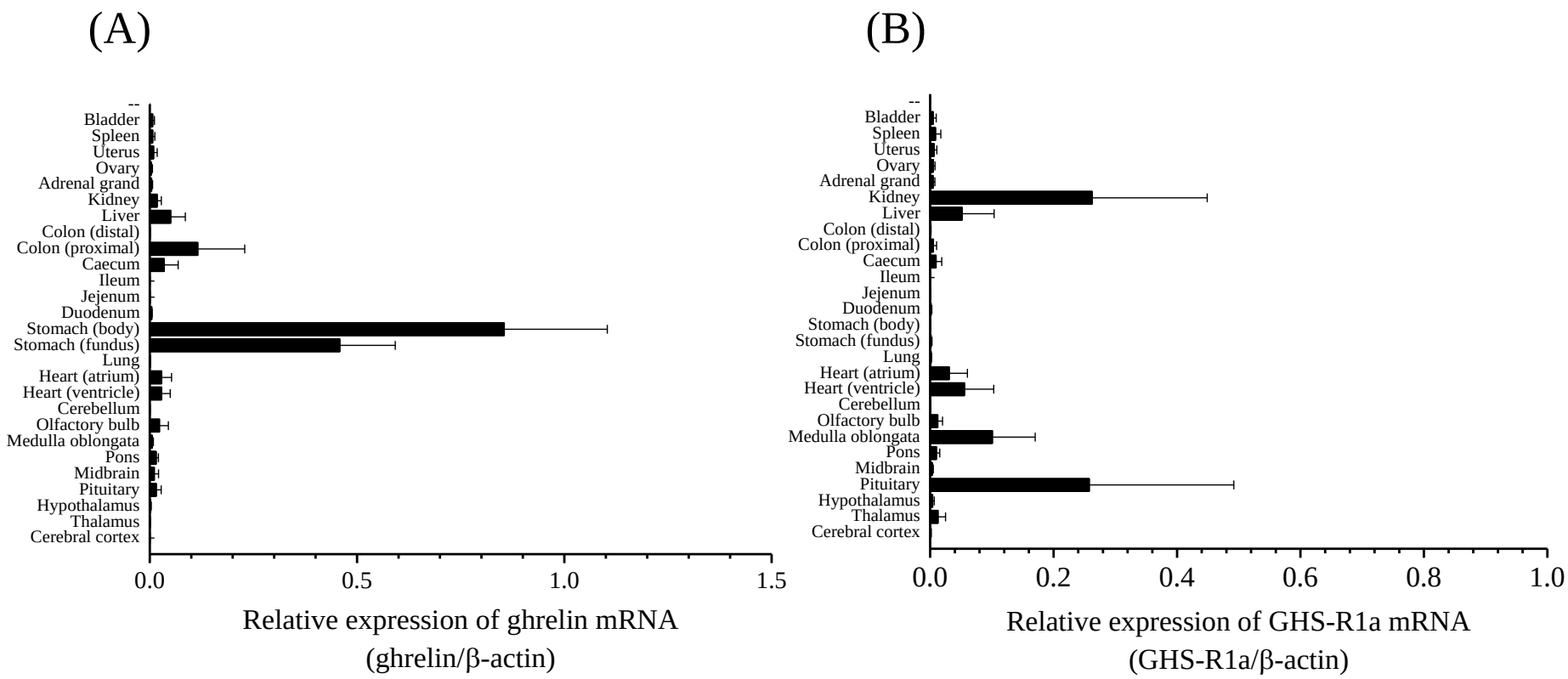
Figure

Fig.4


(A)
Rat ghrelin
gp-ghrelin
Relative light unit
35000
30000
25000
20000
15000
10000
5000
0
-11
-10
-9
-8
-7
Concentration of ghrelin (logM)
(B)
Rat ghrelin
gp-ghrelin
Relative light unit
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
-11
-10
-9
-8
-7
Concentration of ghrelin (logM)

Figure

Fig.5


(A)
--
Bladder
Spleen
Uterus
Ovary
Adrenal grand
Kidney
Liver
Colon (distal)
Colon (proximal)
Caecum
Ileum
Jejenum
Duodenum
Stomach (body)
Stomach (fundus)
Lung
Heart (atrium)
Heart (ventricle)
Cerebellum
Olfactory bulb
Medulla oblongata
Pons
Midbrain
Pituitary
Hypothalamus
Thalamus
Cerebral cortex
0.0
0.5
1.0
1.5
Relative expression of ghrelin mRNA
(ghrelin/β-actin)
(B)
--
Bladder
Spleen
Uterus
Ovary
Adrenal grand
Kidney
Liver
Colon (distal)
Colon (proximal)
Caecum
Ileum
Jejenum
Duodenum
Stomach (body)
Stomach (fundus)
Lung
Heart (atrium)
Heart (ventricle)
Cerebellum
Olfactory bulb
Medulla oblongata
Pons
Midbrain
Pituitary
Hypothalamus
Thalamus
Cerebral cortex
0.0
0.2
0.4
0.6
0.8
1.0
Relative expression of GHS-R1a mRNA
(GHS-R1a/β-actin)

Figure

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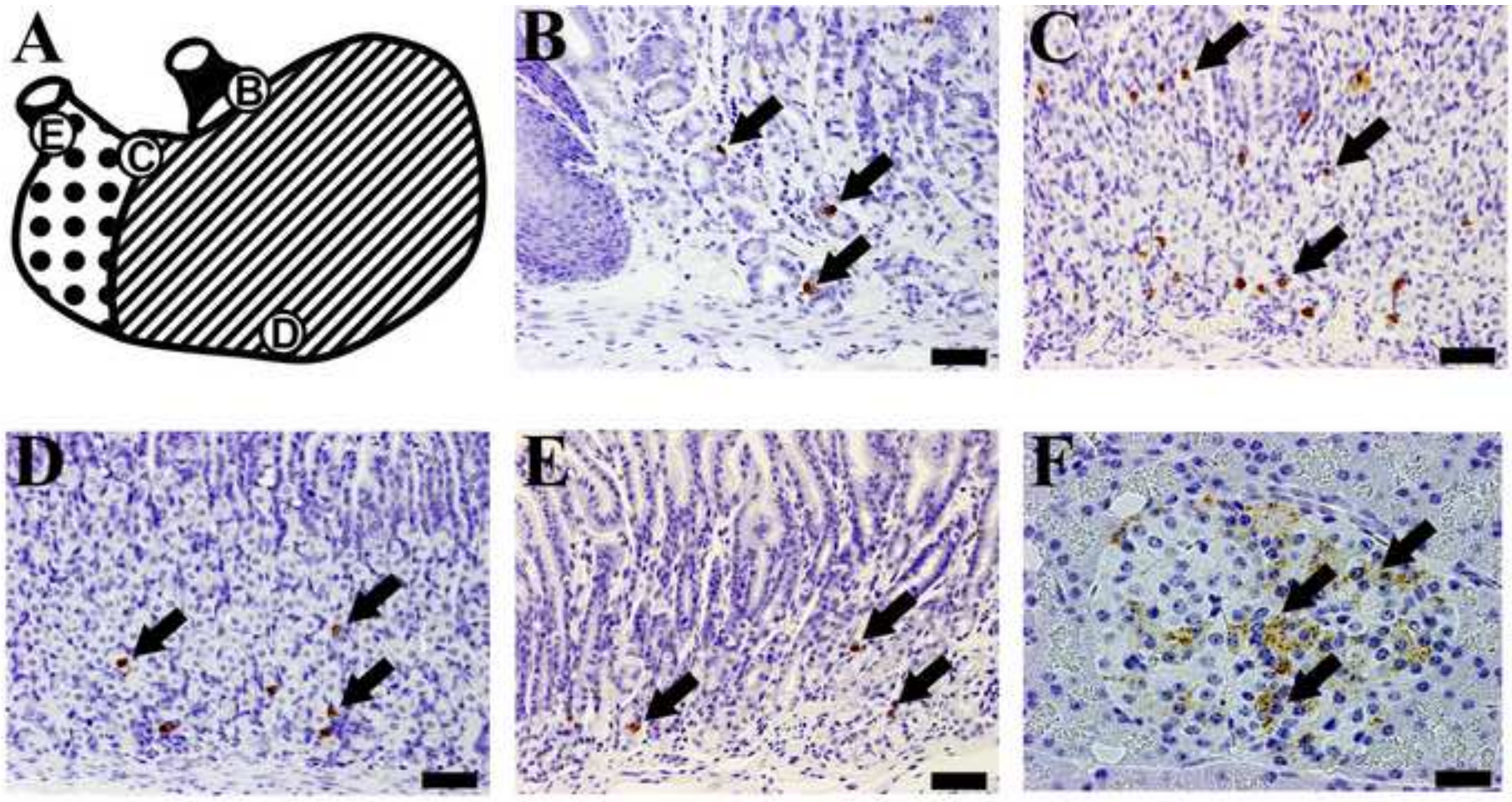

Figure

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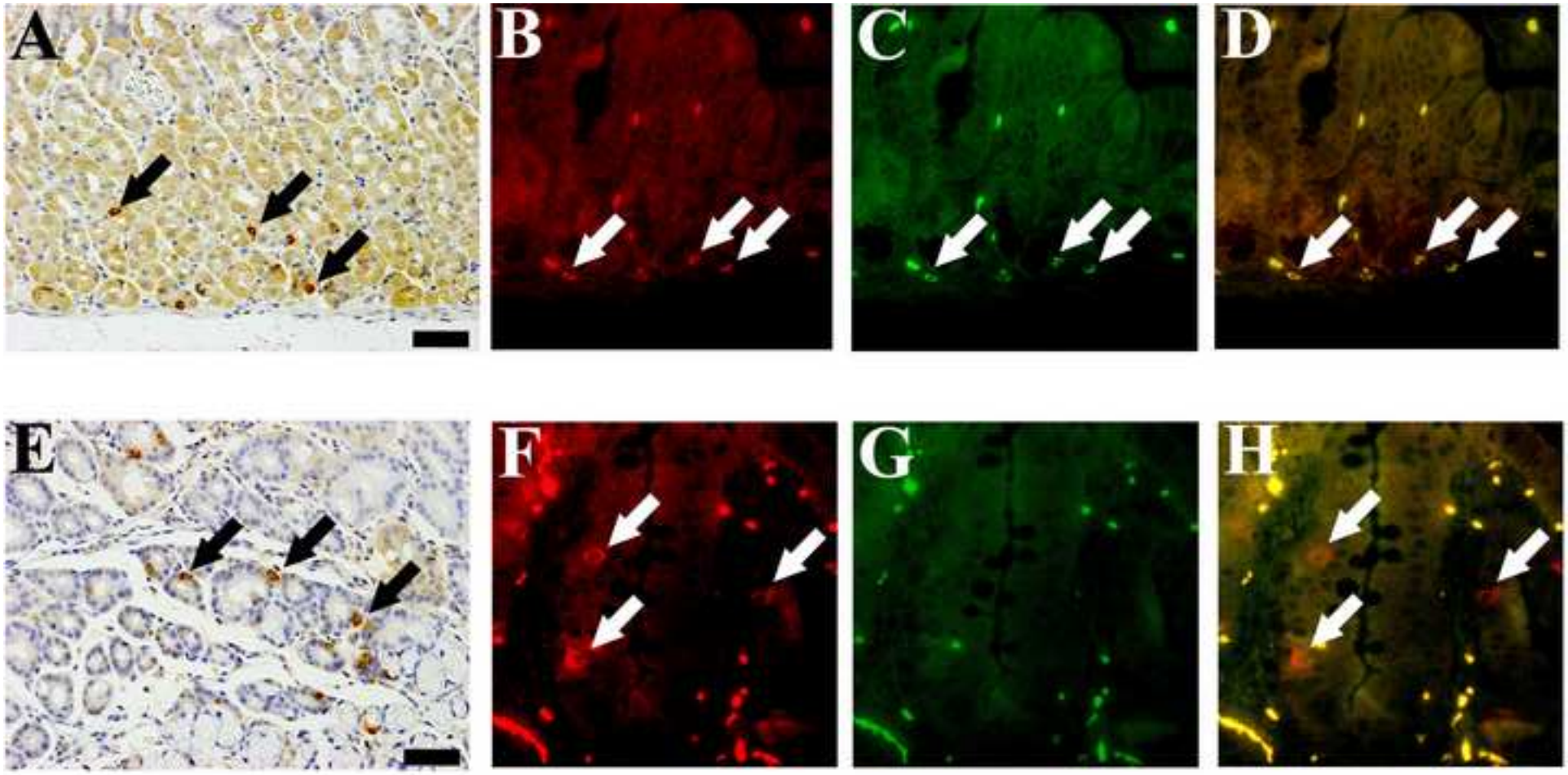

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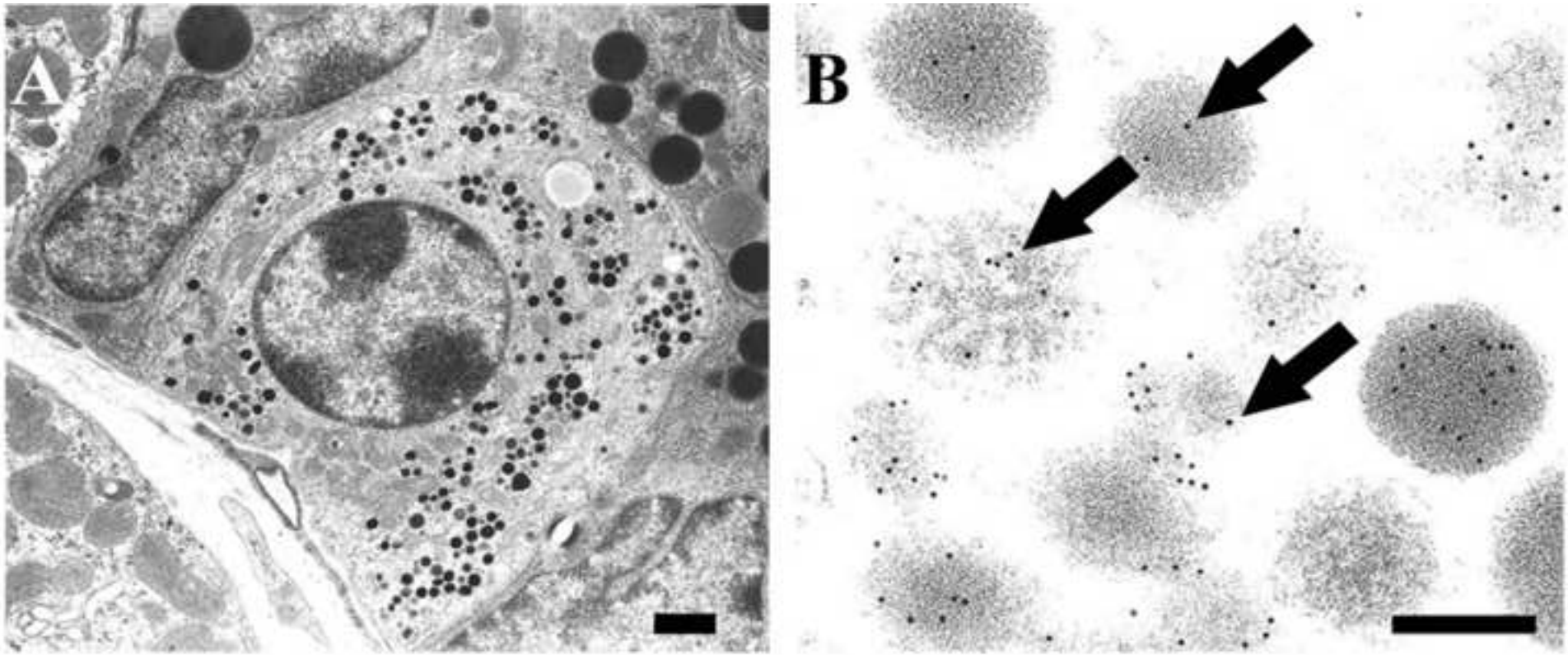
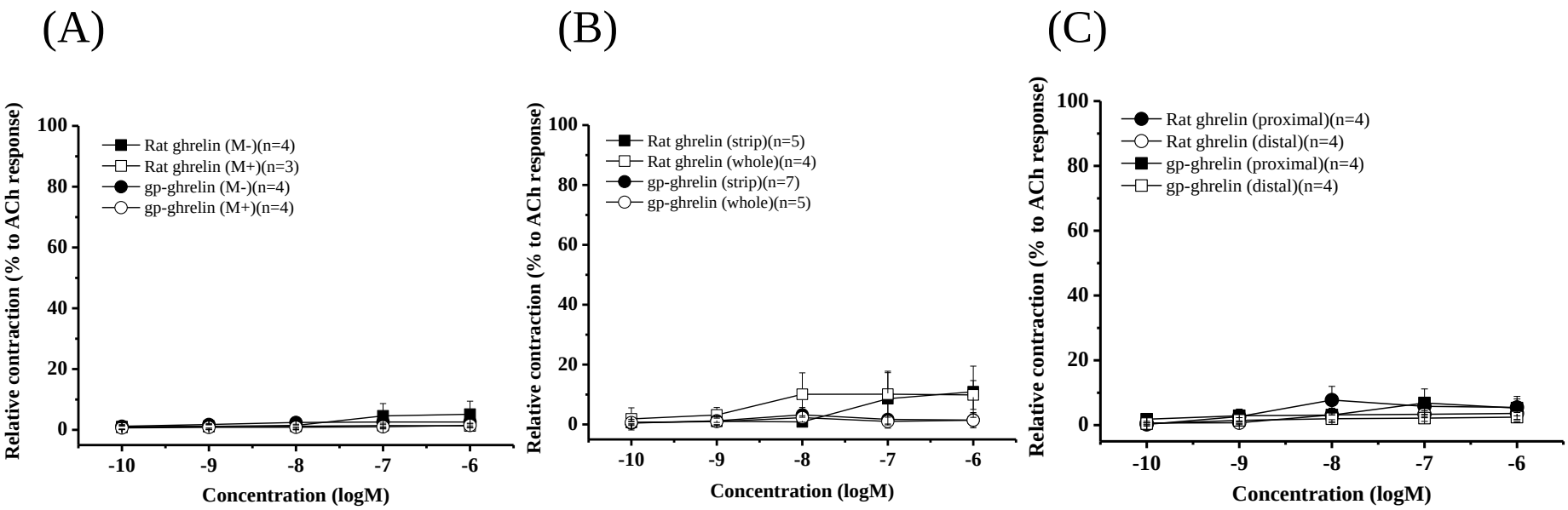


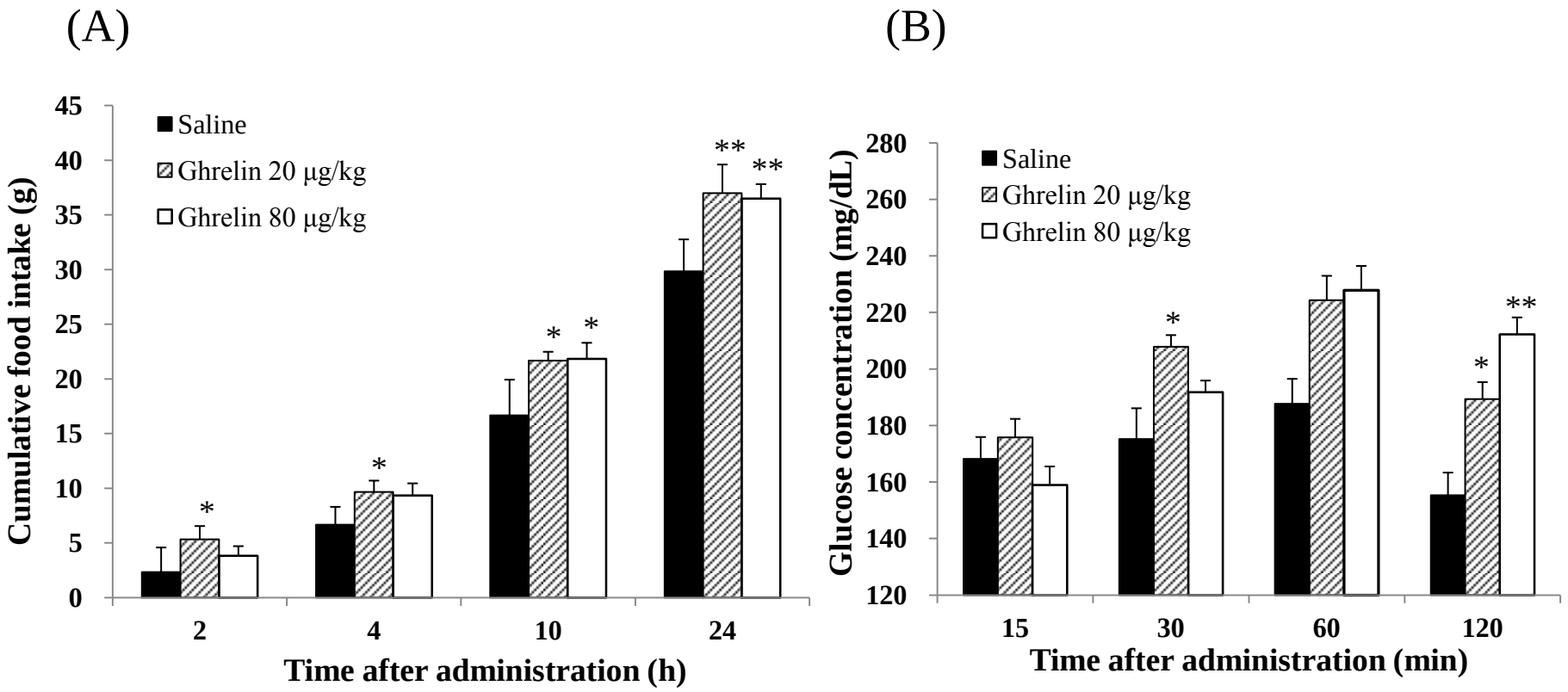
Figure

Fig.9


(A)
Relative contraction (% to ACh response)
Concentration (logM)
Rat ghrelin (M-)(n=4)
Rat ghrelin (M+)(n=3)
gp-ghrelin (M-)(n=4)
gp-ghrelin (M+)(n=4)
(B)
Relative contraction (% to ACh response)
Concentration (logM)
Rat ghrelin (strip)(n=5)
Rat ghrelin (whole)(n=4)
gp-ghrelin (strip)(n=7)
gp-ghrelin (whole)(n=5)
(C)
Relative contraction (% to ACh response)
Concentration (logM)
Rat ghrelin (proximal)(n=4)
Rat ghrelin (distal)(n=4)
gp-ghrelin (proximal)(n=4)
gp-ghrelin (distal)(n=4)

Figure

Fig.10



Table

Table 1 Ghrelin isoforms identified in purification process of the extract of the guinea pig stomach

Groups	Pufication proceses	Detected Mass [M+H]	Expected molecular form
A	CM fr.50-52, RP fr.21-22, Vy fr.1	3222.0	des-Arg28 ghrelin[1-27](C10:2)
B	CM fr.,53-55 RP fr.23, Vy fr.7	3224.4	des-Arg28ghrelin[1-27](C10:0)
C	CM fr.56-58, RP fr.21-23, Vy fr.13	3224.5	des-Arg28ghrelin[1-27](C10:0)
D	CM fr.59-60, RP fr.21-23, Vy fr.17-19	N.D.	
E	CM fr.61-63, RP fr.23, Vy fr.20	3380.8*	ghrelin[1-28](C10:0)
F	CM fr.64-66, RP fr.23, Vy fr.24	3252.5	des-Gln14 ghrelin[1-27](C10:0)
		3380.0	ghrelin[1-28](C10:0)
G	CM fr.67-69, RP fr.24-25, Vy fr.27-31	N.D.	

Note

CM: carboxylmethyl (CM)-HPLC; RP: preparative reverse-phase HPLC with Symmetry column; Vy: reverse-phase HPLC with Vydac column

N.D.: not detected; * indicate the most yielded, major form.