# Gastric motility in ghrelin receptor knockout mice

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**Abstract.** The aim of this study was to investigate the effects and possible mechanisms of ghrelin receptor (GHS-R) deficiency on gastric motility in GHS-R deficient ($Ghsr^{-/-}$) mice. $Ghsr^{-/-}$ and control ($Ghsr^{+/+}$) mice were genotyped by PCR. The percentage of gastric emptying (GE%) was calculated following the intraperitoneal administration of ghrelin. *In vitro*, the contractile response of smooth muscle strips to ghrelin and electrical field stimulation (EFS) and the intraluminal pressure change of isolated stomach to carbachol were observed in an organ bath. The staining of nerve cells in the gastric muscle layer was performed by immunofluorescence. Delayed gastric emptying was observed in the $Ghsr^{-/-}$ mice; ghrelin enhanced the GE% in the $Ghsr^{+/+}$ mice but had no effect on the GE% in the $Ghsr^{-/-}$ mice. *In vitro*, the response of the strips to ghrelin and EFS and the intraluminal pressure change to cabarchol was reduced in the $Ghsr^{-/-}$ mice. GHS-Rs were predominantly expressed on nerve cells in gastric muscle layers. The number of nerve cells was observed to be decreased in the $Ghsr^{-/-}$ mice. The delayed gastric emptying may relate to the loss of GHS-Rs and the reduction in the number of nerve cells in the gastric muscle layers of the GHS-R-deficient mice.

## Introduction

Growth hormone secretagogue receptor 1a (ghrelin receptor, GHS-R1a) is a specific G protein-coupled receptor (1). Ghrelin is an endogenous ligand for GHS-R1a (1) that has been identified in tissues of the central nervous system, including the hypothalamus and anterior pituitary gland (2,3), as well as in multiple peripheral organs and tissues (4,5), including the stomach and intestine (6), pancreas (7) and kidney (8).

Ghrelin was initially identified due to its stimulatory effect on the release of growth hormone (9). Following this discovery, a wide variety of biological functions of ghrelin were found. Ghrelin is known to stimulate appetite and acid secretion (10,11) and a positive energy balance (12), has cardiovascular actions (13) and controls digestive motility (14,15).

The effect of ghrelin on gastrointestinal tract motility has been of increasing interest. The central and peripheral administration of ghrelin increases the gastric emptying rate (16,17). However, the majority of studies on the effect of ghrelin on gastrointestinal tract motility have been performed in normal animals. Although some studies have been carried out on GHS-R gene-knockout mice (18,19), the changes in gastrointestinal tract motility in $Ghsr^{-/-}$ mice have not yet been reported. The aim of this study was to investigate the effects and possible mechanisms of GHS-R deficiency on gastric motility in $Ghsr^{-/-}$ mice.

## Materials and methods

*Chemicals*. Ghrelin and carbachol were obtained from Tocris Cookson (Bristol, UK). GHS-R1a (F-16; goat anti-mouse) and neurofilament heavy polypeptide (NF-H; H-5; mouse anti-mouse) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Phenol red solution (0.5%) and methylcellulose (400 cp, 2%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). FITC-conjugated secondary antibody (goat anti-mouse) and TRITC-conjugated secondary antibody (rabbit anti-goat) were obtained from Jackson ImmunoResearch, Inc. (Baltimore Pike, PA, USA).

*Animals*. Male and female $Ghsr^{+/-}$ mice (mixed 129S3/SvImJ and C57BL/6J background) were obtained from the Shanghai Research Center for Model Organisms (Shanghai, China). The offspring of heterozygous parents underwent genotype identification for subsequent experiments. GHS-R-knockout mice ($Ghsr^{-/-}$ mice) and their normal littermates ($Ghsr^{+/+}$ mice) were used (20). Animals (10 weeks old, 20-24 g) were kept under specific pathogen-free conditions with a normal 12/12 h light/dark cycle (21) for at least 7 days prior to the start of experimentation. Animal procedures were conducted according to the ethical guidelines of Shanghai Jiao Tong University. Measurements were performed in conscious animals following an 18-h fasting period.

*Generation and identification of $Ghsr^{-/-}$ mice*. A previously reported approach (19) was used to generate the $Ghsr^{-/-}$ mice. This procedure was performed in the Shanghai Research Center for Model Organisms.

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The genotypes of the $Ghsr^{+/+}$ and $Ghsr^{-/-}$ mice were identified by PCR. Genomic DNA was extracted from mice tails. The primers were: P1 (5'-GTGCGCACTGTCTCCTCTGATTTG-3'); P2 (5'-GTGCTTTGGGGTGCGTGTGATGGA-3') and P3 (5'-CACGCCCACCAGCACGAAGA-3'). The PCR process consisted of 35 amplification cycles (95˚C for 5 min, 94˚C for 50 sec, 61˚C for 50 sec and 72˚C for 3 min), with a final elongation period of 10 min at 72˚C. The expected PCR product sizes were 1.9 kbp for the $Ghsr^{-/-}$ mice and 1.2 kbp for the $Ghsr^{+/+}$ mice, while two PCR products were expected for $Ghsr^{+/-}$ mice. The PCR products were separated by electrophoresis on a 1.4% agarose gel, after which images were captured.

*Gastric emptying*. All the $Ghsr^{+/+}$ and $Ghsr^{-/-}$ mice received gavage feeding of 0.4 ml prewarmed (35˚C) phenol red meal (50 mg/100 ml in distilled $H_2O$ with 1.5% methylcellulose; viscosity 400 centipoise). The mice were sacrificed by cervical dislocation 20 min after gavage feeding. Four animals were sacrificed immediately after the gavage feeding to serve as internal controls. The entire stomach was carefully isolated, ligated just above the cardia and below the pylorus, and removed. Gastric emptying measurements were performed as described previously (22,23). The stomach and its contents (phenol red meal plus possible gastric secretions) were homogenized with 10 ml NaOH (0.1 N). The mixture was kept at room temperature for 1 h. The supernatant (5 ml) was added to 0.5 ml trichloroacetic acid solution (20%, w/v) to precipitate any proteins. After centrifuging (2500 x g, 20 min), 5 ml supernatant was added to 4 ml NaOH (0.5 N) to develop the maximum intensity of color. The solutions were read at a wavelength of 560 nm with a spectrophotometer (Shanghai Yixian Co., Shanghai, China). The percentage of gastric emptying (GE%) was determined as: GE% = (1-X/Y) x 100, where X and Y are the absorbances of phenol red collected from the stomachs of animals sacrificed 20 min after gavage and immediately after gavage feeding, respectively.

The gastric emptying rates were studied following the intraperitoneal administration of 0, 20, 40 or 80 $\mu$g/kg ghrelin. Ghrelin was dissolved in 0.9% NaCl. The intraperitoneal administration volume (ghrelin plus saline solution) was 0.2 ml.

*Organ bath*

*Contractility measurements of smooth muscle strips*. $Ghsr^{+/+}$ and $Ghsr^{-/-}$ mice were fasted for 18 h and sacrificed by cervical dislocation. Circular muscle strips, freed from mucosa (length 10 mm, width 1 mm) were cut from the gastric antrum and suspended vertically in an organ bath filled with Krebs solution (121.5 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM $KH_2PO_4$, 25.0 mM $NaHCO_3$ and 11.0 mM glucose). The organ bath chamber was gassed with 95% $O_2$/5% $CO_2$ and warmed to 37˚C. Figures obtained from the isometric force transducer (Harvard Apparatus, South Natick, MA, USA) were continuously recorded and stored on a computer and analyzed using the SMUP-E biological signal processing system (Chengdu Equipment Factory, Chengdu, China). The initial load was set at 0.5 g for each strip. The Krebs solution was changed every 15 min and the organ bath was allowed to equilibrate for 1 h. Electrical field stimulation (EFS) was applied to the preparation through a pair of platinum ring electrodes fixed on the top and bottom of the bath. A frequency spectrum (4 Hz) was obtained using pulse trains (duration 1 msec, train 10 sec, 2-min intervals) (24,25).

The contractile responses of the smooth muscle strips to EFS (4 Hz) and ghrelin (0.01, 0.1, 0.5 and 1.0 $\mu$M) plus EFS (4 Hz) were observed in the control and model mice. The amplitude of contraction or relaxation of the strips was normalized using the responses evoked by EFS in the absence of ghrelin to evaluate the ghrelin-induced action. The EFS-induced action was normalized using the amplitude of spontaneous contraction or relaxation.

*Contractility measurements of isolated stomach*. Intragastric pressure levels were used to evaluate the reactive ability of all the gastric muscle layers to experimental substances as described in previous studies (26). The entire stomach was carefully isolated, removed and placed in Krebs solution. The stomach content (possible meal and gastric secretions) was flushed with Krebs solution. A soft polyethylene catheter (inner diameter, 1.7 mm; outer diameter, 2.2 mm) was implanted through the pylorus into the gastric cavity and connected to an external pressure transducer (Harvard Apparatus). Figures were recorded and stored on a computer for analysis using the SMUP-E biological signal processing system. The intragastric pressure was initially kept at 5 cm $H_2O$ and allowed to equilibrate for 1 h. The buffer was changed every 15 min. Measurements were taken when spontaneous pressure fluctuations were relatively stable.

The intragastric pressure responses to carbachol (50, 100, 200, 400 and 600 nM) were observed in the organ bath. The effect of carbachol on intragastric pressure was normalized by the mean of three maximal spontaneous pressure wave peak values.

*Immunofluorescence staining*

*GHS-R staining in gastric muscle layers*. Fluorescent staining of GHS-Rs in the gastric antrum muscle layers was studied in $Ghsr^{+/+}$ and $Ghsr^{-/-}$ mice. Gastric muscle layers, freed from mucous layers, were fixed on a platform and stretched to ~150%. The samples were subsequently fixed with 4˚C acetone for 15 min, after which the acetone was washed off with PBS. The samples were then incubated with 0.5% Triton X-100 for 30 min and flushed, after which they were incubated with 10% fetal bovine serum for 60 min. The primary antibody to GHS-R1a (F-16; goat anti-mouse) was diluted in PBS and added to the muscle tissues at a ratio of 1:100. The samples were incubated at 4˚C for 2 days. The secondary antibody (rabbit anti-goat) coupled with rhodamine (TRITC, red fluorescence) was diluted in PBS and added to the fixed samples at a ratio of 1:200. The tissues were then incubated for half a day in a dark room. DAPI was used as a counterstain for the cell nuclei. The samples were then coverslipped with 50% glycerol. Negative controls were prepared in the same manner as the samples, but without application of the primary antibody.

Double fluorescent staining of the nerve cells in the gastric antrum muscle layers was studied in the $Ghsr^{+/+}$ and $Ghsr^{-/-}$ mice. The GHS-R1a antibody (F-16) was used to label the GHS-Rs and the NF-H antibody (H-5), a neuron-specific marker, was used to label the nerve cells. The procedures of staining were as described above. The dilution rates of the primary antibodies GHS-R1a (F-16; goat anti-mouse) and NF-H (H-5; mouse anti-mouse) were 1:100. The dilution rates

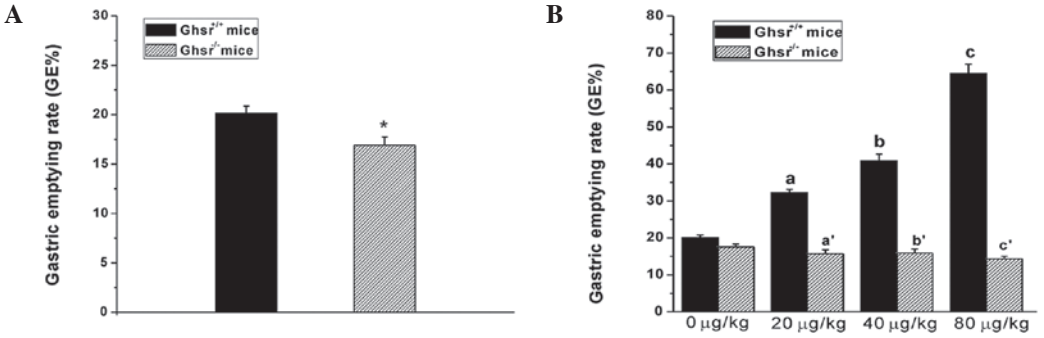


Figure 1. Gastric emptying rates (GE%) in Ghsr$^{+/+}$ and Ghsr$^{-/-}$ mice. (A) GE% for 20 min when no drugs were injected, $^{*}$P<0.05; (B) GE% for 20 min following the intraperitoneal administration of ghrelin. P-values indicate differences of gastric emptying rates between control and model mice when the same dose or different doses of drugs were administered. $^{a}$P<0.01, $^{a,b}$P<0.01, $^{b,c}$P<0.01; $^{a'}$P>0.05, $^{a',b'}$P>0.05, $^{b',c'}$P>0.05, $^{a',c'}$P>0.05; $^{a,a'}$P<0.01, $^{b,b'}$P<0.01, $^{c,c'}$P<0.01, n=4 per condition. Mean ± SEM.

of the secondary antibodies, coupled with rhodamine (TRITC, rabbit anti-goat) or fluorescein (FITC, goat anti-mouse), were 1:200. The samples were examined and scanned under a laser confocal microscope (Olympus, FV-1000, Tokyo, Japan).

*Statistical analysis*. Results were expressed as the mean ± SEM. Data were analyzed with Origin 8.0 software. Photoshop 8.0.1 and CorelDRAW X4 software were used to produce the figures. Data recordings were evaluated by one way analysis of variance (ANOVA) followed by Dunnett's test. P<0.05 was considered to indicate a statistically significant result.

## Results

*Gastric emptying in Ghsr$^{+/+}$ and Ghsr$^{-/-}$ mice*. The GE% values were significantly reduced in the Ghsr$^{-/-}$ mice when no drug was injected (Fig. 1A). Ghrelin increased the GE% in a dose-dependent manner in the Ghsr$^{+/+}$ mice when intraperitoneally administered, but had no effect on the GE% in the Ghsr$^{-/-}$ mice (Fig. 1B).

*Organ bath*

*Smooth muscle strips*. When EFS (4 Hz) was applied *in vitro*, the contractile response of the smooth muscle strips was lower in the strips from the Ghsr$^{-/-}$ mice than in those from the Ghsr$^{+/+}$ mice (Fig. 2A).

In the strips from the Ghsr$^{+/+}$ mice, ghrelin (0.01, 0.1, 0.5 and 1.0 $\mu$M) increased the amplitude of contraction or relaxation of the strips in a dose-dependent manner in the presence of EFS, while in the strips from the Ghsr$^{-/-}$ mice, this effect was not observed (Fig. 2A). There were statistically significant differences in the contractile amplitudes of the strips from the two types of mice when EFS and EFS plus ghrelin were applied (Fig. 2B and C).

*Isolated stomach*. The changes of the intragastric pressure levels were lower in the Ghsr$^{-/-}$ mice than in the Ghsr$^{+/+}$ mice when carbachol (50, 100, 200, 400 and 600 nM) was applied to the isolated stomach (Fig. 3A). There were statistically significant differences in the intragastric pressure levels when different concentrations of carbachol were administered (Fig. 3B).

*Immunofluorescent staining of gastric muscle layer nerve cells*. Immunofluorescent staining indicated that GHS-R1as (red fluorescence) were present in the Ghsr$^{+/+}$ mice (Fig. 4A) but not in the Ghsr$^{-/-}$ mice (data not shown). In the Ghsr$^{+/+}$ mice, GHS-R1as were mainly located on the membrane and the cytoplasms of the nerve cells in the gastric antrum muscle plexus (Fig. 4C).

Immunofluorescent staining of the nerve cells in the gastric antrum muscle layer in the Ghsr$^{+/+}$ and Ghsr$^{-/-}$ mice is shown in Fig. 5A. The number of nerve cells in the gastric antrum muscle layer was decreased in the Ghsr$^{-/-}$ mice (Fig. 5B).

## Discussion

In previous studies, the administration of ghrelin via the central nervous system has been demonstrated to have a pronounced effect on appetite and the motility of the gastrointestinal tract (27-29). Centrally, ghrelin acts through activation of GHS-Rs in the hypothalamus. These effects are mediated by the vagal nerve (30). When the vagal nerve is severed, central effects are eliminated (31). The peripheral administration of ghrelin also enhances the motility of the gastrointestinal tract (32-34). The peripheral effects of ghrelin may be caused by the activation of GHS-Rs on the vagal nerve (14) and gastrointestinal enteric plexus (35). Ghrelin exerts its effects by activating GHS-Rs in central or peripheral tissues (2-5).

In Ghsr$^{-/-}$ mice, GHS-Rs are defective due to the knockout of GHS-R genomic DNA. This is likely to influence a number of the effects mediated by ghrelin, including the promotion of gastrointestinal tract motility. Sun *et al* reported that the body weights of Ghsr$^{-/-}$ mice, regardless of high fat or regular diet, were slightly lower than those of their wild-type littermates (P<0.05) (18), and food intake following fasting was identical in Ghsr$^{+/+}$ and Ghsr$^{-/-}$ mice, indicating that the absence of the Ghsr in obese mice does not prevent weight gain following weight loss. Some of these results were obtained from obese rather than non-obese mice. Thus, there may be differences in food intake and gastric motility in non-obese mice, and further studies should be conducted.

Our experimental results *in vivo* demonstrated that gastric emptying rates were reduced in Ghsr$^{-/-}$ mice. Ghrelin promoted gastric emptying rates in a dose-dependent manner in Ghsr$^{+/+}$ mice. In Ghsr$^{-/-}$ mice, no effect of ghrelin on gastric emptying rates was observed. In Ghsr$^{+/+}$ mice, GHS-Rs are expressed normally, allowing ghrelin to exert its biological functions by activating GHS-Rs in a dose-dependent manner, while in Ghsr$^{-/-}$ mice,

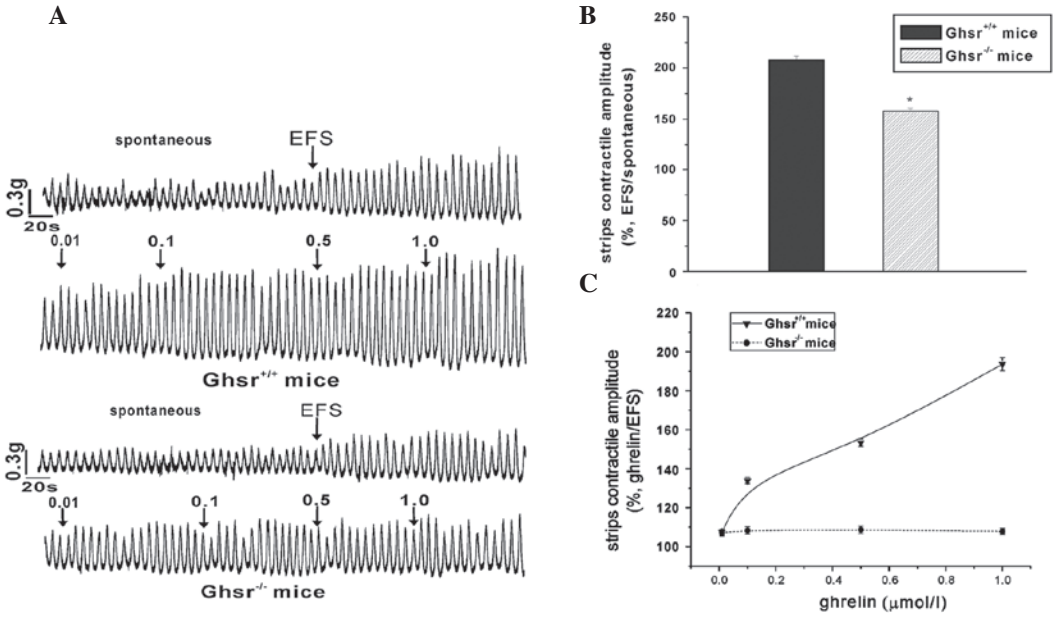


Figure 2. Contractility data for smooth muscle strips. (A) Contractile waveforms of smooth muscle strips under the following conditions: spontaneous, electrical field stimulation (EFS) and EFS plus ghrelin. (B) Comparison of the contractile amplitudes of spontaneous and EFS-treated strips, $^{*}$P<0.01. (C) Comparison of the contractile amplitudes of ghrelin- and EFS-treated strips. P>0.05 for the 0.01 $\mu$mol/l group; P<0.01 for the other dose groups compared to the spontaneous contractions, n=6 per condition. Mean ± SEM.

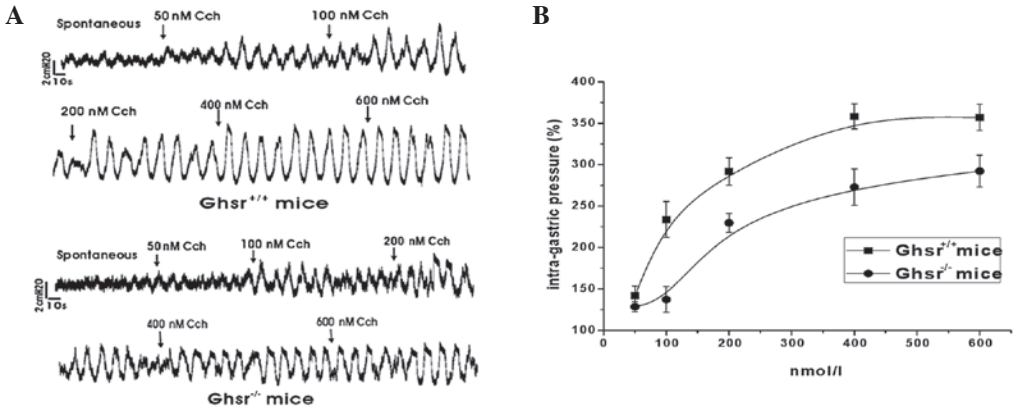


Figure 3. Intragastric pressure responses to carbachol (Cch). (A) Intragastric pressure waveforms with or without application of Cch; (B) Comparison of intragastric pressure (%) curves in the presence of Cch *in vitro*. P-values indicate the differences of intragastric pressures between control and model mice when the same dose or different doses of drugs were administered. P>0.05 for the 50 nmol/l group; P<0.05 for the other dose groups, n=6 per condition. Mean ± SEM.

the gastric emptying induced by ghrelin was eliminated, which may involve changes to the central and peripheral channels. The absence of the GHS-Rs in the central system may attenuate the effect of the hypothalamus on gastric motility, while the absence of GHS-Rs in the stomach may eliminate the peripheral effect of ghrelin on gastric motility. Ghrelin was administered at physiological stimulus doses of 20, 40 and 80 $\mu$g/kg. Under physiological conditions, the plasma levels of ghrelin in rats are 500-2000 pmol/l (36). Fujino *et al* reported that the injection of 1.5 nmol ghrelin in rats resulted in an ~600 pmol/l increase in plasma ghrelin concentrations (14). This result is supported by our finding that the plasma concentrations of ghrelin were ~2400 pmol/l following the administration of 80 $\mu$g/kg ghrelin, which approached physiological concentrations.

*In vitro*, EFS induced a contractile response in smooth muscle strips, and the amplitudes of contraction or relaxation of the strips differed between the Ghsr$^{+/+}$ and Ghsr$^{-/-}$ mice. The different contractile responses of the smooth muscle strips, we hypothesize, may relate to the functions and states of the smooth muscle cells or nerve cells in the muscle layers. The effects of ghrelin on the isolated strips were the same as those observed *in vivo*. These results indicate that ghrelin is able to induce a contractile response in smooth muscle strips from Ghsr$^{+/+}$ mice only when the strips are stimulated through excitatory nerves. *In vivo*, ghrelin is not able to induce a contractile response directly, but enhances the amplitudes of contraction or relaxation of the strips induced by EFS. In Ghsr$^{-/-}$ mice, the absence of the Ghsr in the strips may negate the effect of ghrelin on the contractile response of the smooth muscle strips.

*In vitro*, ghrelin played a gastroprokinetic-like role when smooth muscle strips were stimulated by an electrical field (24).

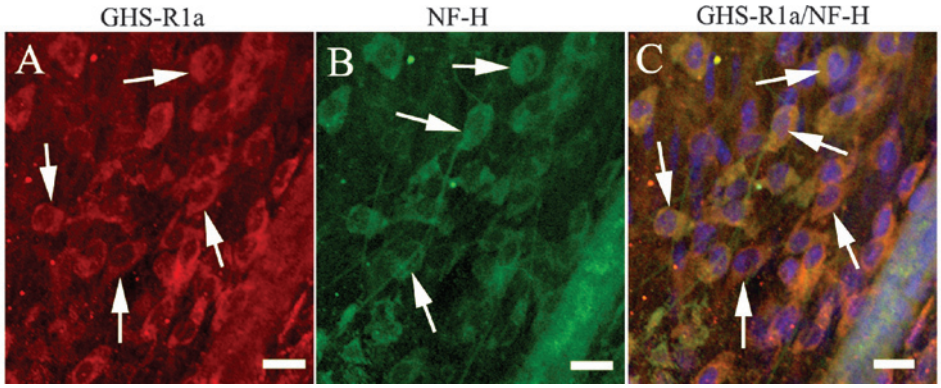


Figure 4. Ghrelin receptor (GHS-R1a) and neurofilament heavy polypeptide (NF-H) immunofluorescent staining in Ghsr$^{+/+}$ mice are shown. (A) GHS-R1a staining (red fluorescence, arrow); (B) NF-H staining (green fluorescence, arrow); (C) GHS-R1a/NF-H staining (tan fluorescence, arrow). Cell nuclei, DAPI staining. Bar, 15 $\mu$m.

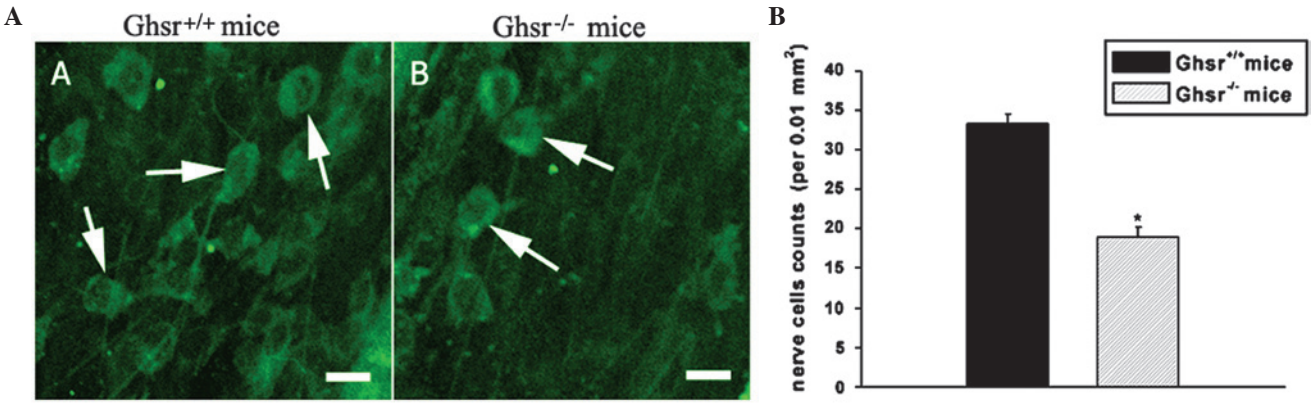


Figure 5. Nerve cell staining and counts in gastric antrum muscle layers are shown. (A) Nerve cells in gastric antrum muscle layers were labeled with neurofilament heavy polypeptide (NF-H); (B) Comparison of nerve cell counts (per 0.01 mm$^2$) in gastric antrum muscle layers, $^*$P<0.01, n=6 per condition. Mean ± SEM.

This finding suggests that ghrelin exerts its effect only in response to excitatory nerve impulses or changes in membrane potential, i.e., it is able to enhance but not directly induce smooth muscle cell contraction. In the current study, we found that a single administration of ghrelin did not affect intragastric pressure levels. No effects were observed even though the increased dose of ghrelin exceeded physiological concentrations 50-fold (data not shown), which is in accordance with results previously obtained from gastric smooth muscle strips (24). This may be as a result of the effect of ghrelin on gastric motility being mediated by the activation of GHS-Rs located on nerve cells in the gastric plexus, i.e., when the stomach was isolated, the motor nerves were cut off and the functions of the gastric plexus downstream from the motor nerves were suppressed. Intragastric pressure levels were lower in the Ghsr$^{-/-}$ mice than in the Ghsr$^{+/+}$ mice when the same dose of carbachol was administered, suggesting that the reactive ability of gastric muscle layers to carbachol was altered in the Ghsr$^{-/-}$ mice. This may be related to the deficiency of GHS-Rs and the functions and states of the smooth muscle cells or nerve cells in the gastric plexus. In Ghsr$^{+/+}$ mice, the effect of carbachol was likely to be enhanced by ghrelin (37,38), which is secreted by mucous X/A-like cells (9).

Fluorescent staining results supported the results obtained from the *in vivo* and *in vitro* studies. The fluorescent staining of gastric muscle tissues indicated that GHS-Rs were mainly located on nerve cells in the gastric plexus. This result morphologically supports the previous theory that ghrelin acts through the nerve cells in the gastrointestinal plexus (24,39). GHS-Rs were not detected in muscle layers in the Ghsr$^{-/-}$ mice, which demonstrated the reliability of the knockout of GHS-R genomic DNA. Thus, loss of GHS-Rs eliminated the gastroprokinetic-like role mediated by ghrelin in the Ghsr$^{-/-}$ mice, and the central and peripheral effects of ghrelin were also eliminated. The number of nerve cells in the gastric antrum muscle layers was decreased in the Ghsr$^{-/-}$ mice, which may affect the release of excitatory neurotransmitters, i.e., the quantity of neurotransmitters released from nerve cells in the gastric plexus may be decreased in Ghsr$^{-/-}$ mice when the same intensity of EFS and concentration of carbachol are applied. The decrease in the number of nerve cells in the gastric plexus and the loss of GHS-Rs in muscle layers may together lead to the decrease in the contractility of gastric smooth muscle to EFS and carbachol *in vitro*. Since the functions and states of the smooth muscle cells themselves are unknown, further studies should be performed.

In summary, GHS-R-deficient mice were a platform to study the effects of the ghrelin receptors. In the present study, the ghrelin receptor deficiency weakened gastric motility. The knockout of ghrelin receptor genomic DNA may affect the development of nerve cells in the gastric plexus and lead to

a reduction in the number of nerve cells. The loss of ghrelin receptors in muscle layers and of nerve cells in the gastric plexus may together attenuate gastric motility. However, more studies should be carried out to clarify the mechanisms.

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