Motilin- and ghrelin-induced gastric contractions in different parts of *Suncus* stomach *in vitro*

(スンクスの胃各部位でのモチリン及びグレリンによる収縮調節機構)

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DECLARATION

I hereby declare that this PhD thesis entitled "Motilin- and ghrelin-induced gastric contractions in different parts of *Suncus* stomach *in vitro*" was written by myself for the degree of Doctor of Philosophy under the guidance and supervision of Professor Takafumi Sakai, Graduate School of Science and Engineering, Saitama University, Japan.

Parts of this thesis have been published as research articles in Journal of Comparative Physiology B on April 9, 2016 (entitled "The proximal gastric corpus is the most responsive site of motilin-induced contractions in the stomach of the Asian house shrew").

For the present thesis, which I am submitting to Saitama University, no degree or diploma or distinction has been conferred on me before, either at this or any other University. No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

Amrita Dudani

Thesis dedicated to

My grandparents

For being my first teacher

My mother

A strong and gentle lady who taught me to trust in god, believe in hard work and that so much could be done with little

My father

For earning an honest living for us and for supporting and encouraging me to believe in myself

My brother and his wife

For being my backbone during my educational career

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Amrita Dudani

Saitama, Japan

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ABBREVIATIONS

%	Percent
° C	Degree Celsius
µm	Micrometer
µM	Micromolar
Ach	Acetylcholine Chloride
ANOVA	Analysis of variance
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
cDNA	Complementary Deoxyribonucleic acid
CNS	Central nervous system
Co.	Company
CO_2	Carbon dioxide gas
DAB	3,3-diaminobenzidinetetrachloride
DNase	Deoxyribonuclease
DW	Distilled water
e.g.	For example
EC-50	Half maximal effective concentration
et al.	And others
Fig.	Figure
g	Gram
GABA	Gamma-aminobutyric acid

GHS-R	Growth hormone secretagogue receptor
GI	Gastrointestinal
GPR38	G protein-coupled receptor 38 (motilin receptor)
gwt	Gram-weight
h	Hour
H_2O_2	hydrogen peroxide
ip	Immunopositive
IP	Intra-peritoneal
KAT	Kathmandu-strain
KCl	Potassium Chloride
Ltd.	Limited
M	Molar
$MgSO_4$	Magnesium sulphate
min	Minutes
mM	Millimolar
Mm^2	Millimeter square
MMC	Migrating motor complex
mRNA	Messenger Ribonucleic Acid
NaCl	Sodium Chloride
NaH_2PO_4	Sodium dihydrogen Phosphate
$NaHCO_3$	Sodium hydrogen carbonate/ Sodium bicarbonate
O_2	Oxygen gas
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction

pH	Potential of Hydrogen
pmol/L	Picomole per litre
qPCR	Quantitative polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
SEM	Standard error mean
St	Saint
TTX	Tetrodotoxin
UK	United Kingdom
USA	United States of America

ABSTRACT

Background and Aim: Motilin and ghrelin are released in the interdigestive state to initiate and regulate phase III contractions of migrating motor complex (MMC). The MMC is responsible for emptying the stomach during the interdigestive period to prepare for the next meal. Gastric phase III contractions of the MMC originate in the stomach and propagate downward in the alimentary canal. Previously, I found that motilin and ghrelin synergistically induced gastric contractions both *in vitro* and *in vivo*. Motilin-induced contractions are regulated by a ghrelin-mediated GABAergic pathway. Therefore, I hypothesized that some regions of the stomach are more responsive to motilin and ghrelin and propagate strong contractions. The present study determined active responsive sites for motilin- and ghrelin-induced contractions in the stomach and elucidated mechanisms underlying the induction of these contractions.

Methods: The stomachs of *Suncus murinus* or Asian house shrew, a small insectivorous mammal, were dissected, and the fundus, proximal corpus, distal corpus, and antrum were isolated to examine the effect of motilin- and/or ghrelin-induced contractions by using an organ bath system. The stomach segments were pretreated with tetrodotoxin, atropine, bicuculline, phaclofen, adenosine, and dopamine to determine the involvement of neural pathways. Quantitative PCR (qPCR) was performed to measure the mRNA expression of the motilin receptor GPR38. Distribution of ghrelin-immunopositive cells and mRNA expression of the GHSR in the different segments of the suncus stomach were examined by performing immunohistochemical analysis and RT-PCR, respectively.

Results: Results of this *in vitro* study showed that treatment with 10^{-10} M motilin induced contractions only in the proximal corpus. In contrast, treatment with 10^{-9} M motilin induced strong contractions in the other segments of the suncus stomach. Motilin-induced contractions in each dissected stomach segment were inhibited by tetrodotoxin and atropine pretreatment, suggesting that these contractions were mediated by a cholinergic neural pathway in the myenteric plexus. Treatment with ghrelin (10^{-11}–10^{-7} M) in the presence of low-dose motilin (10^{-10} M) induced gastric contractions in a dose-dependent manner in the fundus and proximal corpus but not in the distal corpus and antrum. In addition, pretreatment with ghrelin antagonist D-Lys3-GHRP6 blocked motilin-induced contractions in all the stomach segments. In contrast, treatment with GABA antagonists reversed this blockade in all the stomach segments. Treatment with adenosine A_{2A} receptor and dopamine D_2 receptor agonists also reversed ghrelin antagonist-induced inhibition of motilin-induced contractions. The mRNA expression of motilin receptor, GPR38 was highest in the proximal corpus and was the lowest in the antrum. The mRNA expression of GPR38 varied, with low expression in the mucosal layer and higher expression in the muscle layer. The mRNA expression of the ghrelin receptor, GHSR was detected in all the stomach segments. Density of ghrelin-immunopositive cells was significantly higher in the fundus and proximal corpus than in the other stomach segments.

Conclusions: These results suggest that each gastric segment shows a different response toward motilin and/or ghrelin. The fundus and proximal corpus, including the cardia, are the most sensitive and responsive to motilin- and/or ghrelin-induced synergistic gastric contractions, suggesting that the proximal part of the stomach along with fundus is the first contractile site for MMC onset. In addition, present results indicate that adenosine

(via A_{2A} receptor) and dopamine (via D_2 receptor) play vital roles in regulating motilin- and ghrelin-induced gastric contractions.

Chapter 1. General Introduction and Objectives

1.1. Research background

1.1.1. The migrating myoelectric complex (MMC) of the gastrointestinal (GI) motility

Fasting period (or the interdigestive state) is defined as the duration between two successive meals. In all monogastric species including humans, under normal conditions, ingested food material is digested by the stomach and upper intestine during the digestive period, and the upper alimentary canal is almost empty during interdigestive state [Romański 2009]. However, the undigested food remnants, bacteria, and cellular debris can remain in the gastrointestinal lumen. Therefore, the motor function in the interdigestive period ensures mixing, transport, digestion, and absorptive processes of the relatively small amounts of luminal content during this period. These movements maintain a homeostatic balance between the various secretions during the digestive period and absorption in the fasting state which in turn prevents accumulation of the digestive juices and also prevents from gastrointestinal motor abnormalities [Romański 2009]. Since these cycles move down along the bowel, they were called as the migrating myoelectric complexes (MMC) [Szurszewski 1969], and it has been considered that MMC plays an important role as ‘‘gastrointestinal housekeeper’’ [Code 1979].

In 1969, Szurszewski recorded intestinal migrating myoelectrical activity using dogs and was the first to describe that the motility of the stomach and small intestine in the interdigestive occurs cyclically [Szurszewski 1969]. In 1977, Vantrappen et al. reported the similar results in humans [Vantrappen et al. 1977]. In human and dog, MMC is cyclic

and occur between every 90-120 mins [Wingate 1981, Itoh 1997, Charles 1975]. It is subdivided into three phases in mammalian species: Phase I (period of motor quiescence), is the quiescence phase with substantially no contractions; Phase II (period of irregular and low-amplitude contractions) consists of intermittent, irregular low-amplitude contractions; Phase III (period of regular and high-amplitude contractions) is characterized by the most active and a short burst of phasic contractions at maximum amplitude and frequency [Husebye 1999, Nieuwenhuijs et al. 1998, Sarna 1985].

Maintaining MMC cycle in the fasting period is physiologically important because MMC phase III contributes to the interdigestive flow [Kerlin et al. 1982] and impaired gastric phase III activity may result in various GI disorders as it leads to various abnormalities like impediment of the gastric contents as well as small intestinal bacterial overgrowth (SIBO) [Nieuwenhuijs et al. 1998, Pimental et al. 2002, Takahashi 2013]. The regulation of MMC involves myogenic, hormonal and neural mechanisms [Aeberhard et al. 1980, Hall et al. 1986, Naslund et al. 1998, Ormsbee et al. 1979, Hakim et al. 1989, Harvey 1975]. Among those, several hormones have been reported to be involved in mediating MMC, but motilin and ghrelin are included to the most vital regulating factors for MMC [Itoh et al. 1976, Tack et al. 2006].

1.1.2. Motilin and its regulatory mechanism

Motilin known as a prokinetic hormone is released from the endocrine M-cells of the duodenojejunal mucosa during the fasting period [Brown et al. 1973, Dryburgh et al. 1975]. Motilin was given this name because of its ability to stimulate gastric motility [Brown et al. 1971]. MMC re-occurs every 90-120 minutes for the reason that plasma motilin levels increase cyclically and completely corresponds with the gastric phase III

peak in dogs [Hall et al. 1983; Itoh et al. 1976] and humans [Janssens et al. 1983; Vantrappen et al. 1979] and this cyclical release of motilin decreases after ingestion of food [Ohno et al. 2010]. Exogenous administration of motilin also propagates gastric phase III-like contractions in human, dogs, and suncus [Itoh et al. 1976; Janssens et al. 1983; Kuroda et al. 2015; Wingate et al. 1976; Sakahara et al. 2010]. From the *in vitro* studies, it was reported that motilin-induced a dose-dependent gastric contractions in monogastric animals [Broad et al. 2016; Kitazawa et al. 1994; Tsutsui et al. 2009; Mondal et al. 2011; Strunz et al. 1975]. Hence, endogenous motilin is considered as the physiologically most predominant to induce phase III contraction [Zietlow et al. 2010]. Mechanism of motilin-induced gastric contraction varies among the species. However, in humans and rabbits, gastric contraction induced by high doses of motilin is mediated through direct stimulation of smooth muscle while low doses of motilin exert its effects via a neural pathway [Broad et al. 2016; Coulie et al. 1998; Dass et al. 2003; De Smet et al. 2009; Depoortere et al. 2003; Jarvie et al. 2007; Sanger 2012; Van Assche et al. 1997]. Besides these, motilin induces gastric contractions through neural pathways in the dog and suncus [Mizumoto et al. 1993; Mondal et al. 2011]. Together these results suggest that regulatory mechanism of motilin-induced contraction varies not only among the species but also with concentration of motilin.

1.1.3. Ghrelin and GI motility

Ghrelin is a 28-amino-acid octanoylated peptide hormone that was first identified in the rat and human stomach in 1999 as the endogenous ligand for the growth hormone secretagogue receptor (GHS-R) [Kojima et al. 1999]. Plasma ghrelin levels have been found to intensify before and decrease after a meal in humans [Cummings et al. 2001]. Several previous studies have reported that ghrelin has gastroprokinetic effects

[Depoortere et al. 2005, Sallam 2010]. The major physiological actions of ghrelin include the regulation of growth hormone secretion [Kojima et al. 1999, Seoane et al. 2000, Tolle et al. 2001], food intake [Nakazato et al. 2001, Wren et al. 2000], energy metabolism [Tschop 2000, Perez-Tilve et al. 2011], GI motility [Nakamura 2010, Perboni 2010, Zheng J 2009], gastric acid secretion [Fukumoto et al. 2008, Masuda et al. 2000, Yakabi 2008], cardiovascular function [Okumura 2002], and cell proliferation [Duxbury et al. 2003, Maccarinelli et al. 2005]. The ghrelin-stimulated gastric contraction was observed in rats [Masuda et al. 2000] and mice [Zheng J 2009], suggesting that it acts as a substitute for motilin in motilin-lacking rodents [Peeters 2004] as for GI motility. Furthermore, peak plasma ghrelin levels are correlated with phase-III-like contractions in rats [Ariga et al. 2007]. Ghrelin-induced phase III-like gastric contractions are mediated via vagal cholinergic pathways in mice [Zheng J 2009]. Even though ghrelin is not able to stimulate gastric phase-III contractions in dogs [Ohno et al. 2006], high dose of ghrelin induced premature phase-III-like contractions in human stomachs [Tack et al. 2006]. However, it has been observed that endogenous ghrelin is necessary for phase II contractions, and a certain level of ghrelin is needed to initiate phase-III contractions in suncus [Kuroda et al. 2015].

1.1.4. Similar properties of motilin and ghrelin

In humans, 50% of the precursor mRNAs for motilin and ghrelin are homogenous, and their ligands also share about 21% amino acid identity [Peeters 2005]. The specific receptor for ghrelin is GHS-R [Howard et al. 1996] and for motilin is GPR38 [Feighner et al. 1999], both having similar structures belong to the class A rhodopsin-like G-protein-coupled seven-transmembrane receptor family [McKee et al. 1997]. The receptors for motilin and ghrelin also exhibit a substantial homology in sequence with an

overall identity of 44%, which is 87% in the transmembrane regions [Peeters 2005]. Recent reports on suncus clearly showed using quantitative RT-PCR that suncus ghrelin is highly expressed in the mucosal layer of gastric corpus, pylorus, and antrum whereas high expression of motilin was found in the small intestine [Ishida et al. 2009, Tsutsui et al. 2009]. It has also been shown that both GHS-R and GPR38 receptors in the central nervous system were expressed in the hypothalamus, medulla oblongata, pituitary gland and the nodose ganglion [Suzuki et al. 2012]. GHS-R mRNA expression has been found in both muscle and mucosa of the stomach and small intestine, but GPR38 was found to express in the gastric muscle layer, large intestine, lungs and heart [Suzuki et al. 2012]. These findings suggest that in suncus, ghrelin and motilin exert their functions through specific receptors expressed in the GI tract and in the central nervous system. Moreover, previous studies have demonstrated a synergistic effect of motilin and ghrelin on gastric contractions *in vitro* and in anesthetized suncus specimens *in vivo* [Mondal et al. 2012]. In suncus, the coordination of motilin and ghrelin is necessary to initiate phase III contraction of the MMC [Mondal et al. 2013]. Therefore, it is reasonable to assume an additional or synergistic relationship between these family peptides on various physiological functions. However, detailed studies are needed regarding physiological functions of motilin and ghrelin besides the stimulation of gastric contraction.

1.1.5. Asian house shrew (*Suncus murinus*)

In spite of the fact that motilin was recognized decades earlier as compared with ghrelin, there are lesser reports on its physiological roles besides its contribution in GI motility. The possible reason for this might be due the fact that studying the biological action of motilin is difficult. Also, one of the possible reasons for the delayed advancement of research concerning motilin is that the worldwide conveniently used rodents cannot be

utilized for motilin studies owing to the fact that since the common ancestor of these animals has the impotent motilin gene, rodents are the natural knockout for motilin and its receptors [He 2010]. Dogs and humans are most widely used to study the physiological effects of motilin *in vivo* [Itoh et al. 1978], which limits the study of the detailed mechanisms of action, e.g., the distribution of motilin and GPR38 mRNA expression and the neuronal signaling pathways involved in motilin stimulation. Ghrelin can induce gastric contraction in rats, mice, and humans but not in the dogs and rabbits [Ohno et al. 2006]. Therefore, a new animal model relevant to human physiology is necessary to study the motilin system. We used Asian house shrew (*S. murinus*, suncus used as a laboratory name), belonging to the order Insectivora and the family Soricidae. Order Insectivora has traditionally been regarded as one of the key links for studying the origin of mammals [Murphy 2007, Depoortere 2003]. In recent years, suncus has been used for anti-emetic research for identifying mechanisms of vomiting and in the development of anti-emetic drugs unlike mice and rats [Ito et al. 2002, Ito 2005, Ito et al. 2003, Matsuki 1996]. Suncus viscera resembles a lot to that of humans and therefore is an appropriately suitable model organism for studying the physiology and pathophysiology of humans [Yi et al. 2004, Yi et al. 2005]. The structure of the suncus gastric mucosa is identical to that of humans, but not hamsters, rats, and mice [Kanamori 1989]. For example, a glandular mucosa with well-developed luminal folds has been identified in the gastric mucosa of suncus, and it has no forestomach as observed in mice and hamsters. cDNA sequence of suncus motilin and ghrelin and their receptors have already been identified [Suzuki et al. 2012, Ishida et al. 2009, Tsutsui et al. 2009] and it was found that suncus has almost identical GI motility and motilin responses to those found in humans and dogs [Tsutsui et al. 2009, Sakahara et al. 2010], suggesting that

suncus is the suitable animal that can be conveniently used to study the effects of both ghrelin and motilin on gastric motility and their mechanisms of action for applications in human medicine and physiology.

1.2. Hypothesis and Objective

Suncus produce motilin and ghrelin and their receptors throughout the GI tract as discussed above. Taken together, I deliberated that some comparatively active site might be present in the stomach that respond differently towards motilin and ghrelin. Using suncus as a model animal, I examined the main active site of motilin by dividing the stomach into various parts. In the second experiment, I studied the synergistic effect of motilin and ghrelin on gastric motility in different parts and its underlying mechanism. Therefore, this study aimed at finding the most active and responsive site for motilin-induced contractions in suncus stomach and their regulatory mechanisms.

Chapter 2. The proximal gastric corpus is the most responsive site of motilin-induced contractions in the stomach of the Asian house shrew *in vitro*

2.1. Introduction

2.1.1. Motilin as an important regulator of MMC

During the fasting period, the upper gastrointestinal (GI) tract undergoes a temporally coordinated cyclic motor pattern known as the migrating motor complex (MMC) in both humans and dogs [Vantrappen et al. 1979, Szurszewski 1969]. MMC is believed to be physiologically important for the mechanical and chemical cleansing of the empty stomach and preparation for the next meal [Code 1979; Sarna et al. 1983; Sarna 1985; Wingate 1981]. Motilin was initially isolated from a side fraction produced during the purification of secretin [Brown et al. 1971], and later its complete amino acid sequence was determined, by J.C. Brown in 1973 from porcine duodenal mucosa [Brown et al. 1973]. Several physiological factors are needed to sustain motor function in the interdigestive period, but motilin is considered the most important in the regulation of MMC. Some previous studies have revealed that an increase in plasma motilin concentration results in simultaneous contractile activity in the stomach [Itoh et al. 1978; Janssens et al. 1983]. Also, exogenous motilin administration induced MMC phase III-like contraction in the stomach [Itoh et al. 1976] and gastric phase III contraction was completely eliminated by neutralizing circulating motilin with motilin antiserum or motilin antagonist [Lee et al. 1983; Lee et al. 1978; Ozaki et al. 2009; Sudo et al. 2008], indicating that endogenous motilin induces gastric phase III in the interdigestive period. These rhythmic motor patterns originate from the foregut and propagate downward in

the alimentary canal [Kellow et al. 1986; Sanger et al. 2010]. Since the plasma motilin peak is associated with the gastric phase III peak, I hypothesized that there could be a specific area in the stomach where motilin binds with its receptor and initiates gastric phase III contraction. There also may be a possibility that different areas of the stomach may respond differently towards motilin.

2.1.2. Advantages of suncus for studying gastrointestinal physiology including gastric motility

Previously, the contractile properties of the *S. murinus* stomach, in both conscious free-moving and in an organ bath experiment was studied, and found that *S. murinus* has almost the same GI motility and motilin response as that found in humans and dogs [Sakahara et al. 2010; Tsutsui et al. 2009], indicating that *S. murinus* can be used for GI motility studies. From *in vivo* and *in vitro* experiments using *S. murinus*, it was demonstrated that motilin-induced gastric contractions are mediated through the myenteric plexus [Mondal et al. 2011; Sakahara et al. 2010]. Similar results showing involvement of myenteric plexus in motilin-evoked gastric contractions have been reported in other species [De Smet et al. 2009; Kitazawa et al. 1995; Mizumoto et al. 1993; Ohshiro et al. 2008; Van Assche et al. 1997].

In this study, I investigated the active and most responsive site for motilin-induced gastric contractions in the suncus stomach. Thus, I examined the motilin-induced gastric contractile pattern in different parts of the stomach and its mechanism using an organ bath system. To confirm the functional results, I also measured the mRNA expression of

motilin receptor GPR38 in various parts of the *S. murinus* stomach using quantitative PCR analysis.

2.2. Materials and Methods

2.2.1. Ethical approval

All procedures were approved and performed in accordance with the Saitama University Committee on Animal Research. All efforts were made to minimize animal suffering and to minimize the number of animals used in the experiment.

2.2.2. Animals

Experiments were conducted with female *S. murinus*, aged 15-20 weeks old and weighing 50–75 g, of an outbred KAT strain, established from a wild population in Kathmandu, Nepal. Animals were housed individually in plastic cages equipped with an empty can as a nest box and were provided food (trout pellets; Nippon Formula Feed Manufacturing Co., Ltd., Yokohama, Japan) and water *ad libitum*. The metabolizable energy content of the pellets was 344 kcal 100 g^{-1}, consisting of 54.1% protein, 30.1% carbohydrate, and 15.8% fat. The animal room was maintained at 21–24°C and the light and dark cycle were controlled to change every 12 h (lights on from 8.00 to 20.00 h).

2.2.3. Drugs used

The administration volume of each drug was 1% of the bath volume. Acetylcholine chloride (ACh; Sigma-Aldrich Co. LLC., USA) was dissolved in distilled water (DW), and synthetic *S. murinus* motilin (Bex, Tokyo, Japan) was dissolved in 0.1% BSA/PBS. In antagonist or inhibitor experiments, the stomachs were equilibrated before the application of *S. murinus* motilin with the antagonist or inhibitor: atropine sulfate (10^{-6}

M; Merck, USA) and tetrodotoxin (TTX; 10^{-6} M; Wako Pure Chemical Industries, Ltd, Osaka, Japan) for 30 min. Concentrations of drugs are expressed as final molar concentrations in the bath solution. Both atropine sulfate and TTX were dissolved in DW before use. All reagents were prepared for each experiment according to the manufacturer's instructions.

2.2.4. Preparation of *S. murinus* isolated stomach

After the animals were fasted for 8 h, they were decapitated after being deeply anesthetized with pentobarbital sodium (100 mg/kg IP). The stomach was dissected out after laparotomy and immediately placed in freshly prepared Krebs' solution (NaCl 118 mM, KCl 4.75 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1.2 mM, NaH_2PO_4 1.8 mM, $NaHCO_3$ 25 mM, and glucose 11.5 mM; pH 7.2–7.4). The mesentery attachments and fatty tissues were removed, and the inside of the stomach was washed with Krebs' solution through a small incision in the gastric fundus. The stomachs were sectioned into four parts: the fundus, proximal corpus (includes the cardia), distal corpus, and antrum, as shown in Fig. 1. The cutting sites of the stomach were decided by two notches, the cardiac notch, and angular notch. Differences in tissue coloring were also used to distinguish between different parts. The whitish body above the cardiac notch was dissected as fundus. The proximal corpus was cut out along the lower part of the esophagus, and the cardiac region was included in the proximal corpus section. The remainder of the cylindrical body was considered distal corpus. The antrum is distinctly visible as a whitish pink body below the angular notch. Tissues with mucosa attached to the muscle layer were used instead of muscle layer strips. The longitudinal and circular muscle layers were not separated into strips because as previously shown, myenteric plexus is important for motilin-

induced suncus gastric contractions [Mondal et al. 2012], and keeping an intact anatomical structure of the myenteric neuron and smooth muscle is essential for studying motilin-induced contraction. The segmented stomach parts were mounted in 10-ml water-jacketed organ baths along the longitudinal muscle direction with the thread tied on cut edge surfaces. Contractility is hence obtained from longitudinal muscle. Tissues were initially loaded with approximately 0.5 g weight. The temperature of Krebs' solution was maintained at 37 ± 0.5°C, and the solution was aerated continuously with carbogen (a mixture of 95% O_2 and 5% CO_2).

2.2.5. Gastric Contractility study

Contractile activities of the stomach with motilin treatment were monitored using an isometric force transducer (UM-203, Iwashiya Kishimoto Medical Instruments, Kyoto, Japan) and software (PicoLog for Windows, Pico Technology Ltd., St. Neots, UK). To normalize the contractions of each segment, stomach sections were first treated with acetylcholine chloride (ACh 10^{-5} M) twice before the experiment, and at the end of the experiment, ACh (10^{-5} M) was introduced once again into the organ bath. The percentage of maximal contractions were then calculated by averaging the tonic response induced by these three ACh (10^{-5} M) administrations. To examine the effect of motilin, each stomach section was treated with the *S. murinus* motilin (10^{-10} to 10^{-7} M) in the absence or presence of antagonist or inhibitor, and motilin-induced contractions were measured, and contraction was measured in g weight tension. Then, the effects of *S. murinus* motilin in the absence or presence of antagonists or inhibitors were determined. The pH of the bath was checked before drug administration to ensure it was between 7.2 and 7.4.

2.2.6. GPR38 mRNA expression

Stomachs were dissected into four parts: fundus, proximal corpus, distal corpus, and antrum; then, the mucosal layers and the muscle layer were peeled off using a glass slide. Separated tissues were frozen with liquid nitrogen and broken using CRYO PLUS (Microtech Co., Ltd., Chiba, Japan) before being dipped in ISOGEN (Nippon Gene, Tokyo, Japan). The total RNA from the tissues was extracted using ISOGEN (Nippon Gene) according to the manufacturer's instructions and then subjected to DNase treatment. cDNA was synthesized from 1 μg total RNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems, USA) according to the manufacturer's instructions. The oligonucleotide-specific primers for *S. murinus* β-actin are forward 5′-TGCGTGACATCAGGAGAAG -3′; reverse 5′- TCCAGAGAGGAAGAGGATGC -3′, and those for GPR38 are forward 5′- ACAGGCAGACCATCCGC -3′; reverse 5′-TACATTGTCCGGGTGTCCTT -3′. The quantitative PCR (qPCR) reactions were performed using a Light Cycler (Roche Diagnostics, USA) with SYBR Premix Ex-Taq GC (Takara BIO, Shiga, Japan). The initial template denaturation was programmed for 30 s at 95°C. PCR was performed with 40 cycles of 10 s at 95°C, 20 s at 64°C and 30 s at 72°C, and a final cooling step was performed for 30 s at 40°C. The *S. murinus* β-actin mRNA was used as the invariant control. The expression of each mRNA is shown relative to β-actin mRNA expression. All reactions were performed in duplicate, and each transcript was quantitatively measured by establishing a linear amplification curve from serial dilutions of each plasmid containing the amplicon sequence. The amplicon size and specificity were confirmed using a melting curve analysis and 2% agarose gel electrophoresis.

2.2.7. Statistical analysis

The results are expressed as the mean ± SEM. Recording experiments were repeated individually at least three times, and similar results were obtained. The number of animals used for statistical analyses are represented in the figure legends. I used GraphPad Prism 5 software (GraphPad Software Inc., CA, USA) to analyze the data. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparison test. $P < 0.05$ was considered statistically significant.

2.3. Results

2.3.1. Spontaneous contractile pattern in the different segments of isolated stomach

The isolated *S. murinus* stomach in the organ bath showed spontaneous contraction activity under a basal 0.5-gram weight (gwt) resting tension, and contraction differed among all parts of the stomach. In the fundus, the spontaneous contractile activity was unclear and arrhythmic, and the amplitude of spontaneous contraction was about 0.2 gwt (Fig. 2A). The amplitude of the spontaneous contraction of the proximal corpus was approximately 0.4 gwt, and the contractile rate was 12–16 cycles per minute (Fig. 2B). In the distal corpus, the amplitude of the spontaneous contraction activity was approximately 0.3 gwt, and the rate was approximately 8–12 cycles per minute (Fig. 2C). In the antrum, the amplitude of the spontaneous contraction activity was approximately 0.5 gwt, and the rate was approximately 2–6 cycles per minute (Fig. 2D). The maximum tensions produced by ACh (10^{-5} M) were also different in each stomach segment (Fig. 2A–D). In fundus and proximal corpus, ACh-induced maximum tension was approximately 2.5 gwt, whereas it was 5 gwt in the distal corpus and 4 gwt in the antrum.

2.3.2. Responses to motilin in different segments of stomach

To examine the difference in response to motilin treatment in stomach sites, S. murinus motilin (10^{-10}–10^{-7} M concentrations) was introduced into the organ bath, and contractile amplitudes and frequencies were measured. Although motilin-induced contraction occurred in a concentration-dependent manner, the responses to motilin varied according to the stomach parts (Fig. 2A–D and Fig. 3). In the fundus, motilin-induced contractions in a dose-dependent manner were observed starting from a concentration of 10^{-9} M.

Maximum contractions at the 10^{-7} M concentration were about 64% of ACh-induced contractions (Fig. 2A, Fig. 3). In the proximal corpus, motilin-induced contractions were observed starting from a concentration of 10^{-10} M; 50% response of the ACh was observed in response to this concentration. Maximum contractions (89%) were observed in response to the 10^{-9} M concentration, and contractions were the same at higher doses of motilin, 10^{-8} M and 10^{-7} M (Fig. 2B, Fig. 3). In the distal corpus, the 10^{-10} M motilin concentration did not increase contractile activity. The contraction was induced by motilin concentrations of 10^{-9} M and higher; contraction responses were 25%, 54%, and 71% of the total ACh-induced contractions at 10^{-9} M, 10^{-8} M, and 10^{-7} M motilin concentrations, respectively (Fig. 2C, Fig. 3). In the antrum, the amplitude of contraction (gwt tension) was increased in a dose-dependent manner starting from the 10^{-9} M motilin concentration, and showed the maximum contraction response of 28% at 10^{-7} M (Fig. 3), although this was not significant. In addition, the frequency of contraction was increased significantly at 10^{-9} M, 10^{-8} M, and 10^{-7} M motilin concentrations and the rate of contractile activity changed to 8-9 cycles per minute (Fig. 2D). The EC50 values of motilin-induced contractions in different regions of the stomach are shown in Table-1. Since 10^{-11} M motilin concentration did not induce contraction in any stomach part, contractions at this concentration were considered to be zero. It was observed that the proximal corpus had higher potency because it had a low concentration of motilin-induced gastric contractions compared to other parts of the stomach. Comparing contraction responses for motilin in each stomach sections, the response in the proximal corpus was significantly higher than that in the other sections to the 10^{-9} M, 10^{-8} M and 10^{-7} M motilin concentrations (Fig. 3). Even at the lower dose (10^{-10} M), the proximal corpus showed high reactivity.

2.3.3. The cholinergic pathway of the motilin-induced contraction

Atropine, a muscarinic receptor antagonist, suppressed spontaneous contractile activity in all of the stomach sections (Fig. 4A–D). Under atropine pretreatment, motilin concentrations of 10^{-10} to 10^{-8} M did not induce contraction in any stomach parts (Fig. 4A–E). However, motilin concentration of 10^{-7} M induced contractions in all parts of the stomach, but they were not significant. (Fig. 4E).

2.3.4. The myenteric plexus pathway of the motilin-induced contraction

I also examined the involvement of TTX, a potent neurotoxin that blocks action potentials in nerves by binding to the voltage-gated Na^+ channel, and found that TTX did not affect the spontaneous contraction of the fundus, proximal corpus, and distal corpus (Fig. 5A–C). The spontaneous contraction of the antrum altered after TTX administration, as shown in Fig. 5D and the amplitude was slightly decreased. Under TTX pretreatment, motilin concentrations of 10^{-10} to 10^{-8} M did not evoke contraction in all of the stomach segments (Fig. 5A–E) and only the 10^{-7} M concentration slightly induced contractions, not significantly, in the fundus, proximal corpus, and distal corpus (Fig. 5E).

2.3.5. GPR38 mRNA expression in the stomach

To analyze the distribution of GPR38 in the stomach, I compared the GPR38 mRNA expression level in the fundus, proximal corpus, distal corpus, and antrum using qPCR. I found that GPR38 mRNA expression was low in the mucosal layer and high in the muscle layer in all areas of the stomach. In the muscle layer, the GPR38 mRNA

expression level differed among stomach parts, with the highest expression in the proximal corpus and lowest in the antrum (Fig. 6).

2.4. Discussion

2.4.1. Sensitivity towards motilin is region-specific

I observed that there are differences in response to motilin in various stomach parts *in vitro*. The response to motilin in the proximal corpus was higher than that in the other parts of the stomach, especially with the low motilin concentration, i.e., the 10^{-10} M motilin concentration, which slightly induced contractions and the contraction response at 10^{-9} M were significantly higher in the proximal corpus than that in the other parts. From previously published reports of concentration-dependent contractile effects of motilin on the isolated S. murinus whole stomach *in vitro*, it was reported that motilin-induced contraction occurred starting from the 10^{-9} M concentration [Mondal et al. 2011]. Even though the stomach was divided into four sections, each of sections retained the ability to react to motilin, similar to that observed *in vitro* in the whole stomach. Also, the speed of onset of contraction and the amplitude of contraction increased in all the parts with motilin in dose dependent manner. This delay might be caused by the permeation of the motilin into the tissue. Interestingly, the effect of the motilin in the antrum was different from that in the other sections, a dose-related increase in contractile frequency was observed in the antrum. However, both the fundus and antrum showed dose-dependent increases in contraction and the proximal corpus showed maximum contractile activity at much lower dose of motilin at 10^{-10} M concentration. These results suggest that the proximal corpus is the most sensitive to motilin treatment. In a study on humans, it has already been showed that different regions of the GI tract responded differently to electrical field stimulation-induced contractions under motilin pretreatment [Broad et al. 2012]. They clearly showed that in gastric fundus and antrum, EFS evoked

contraction were monophasic, and frequency-dependent which were caused by cholinergic activity dominating simultaneous activation of inhibitory nitrergic neurons, as well as this difference in region-specific response towards motilin, is attributed to various neural pathways like cholinergic, nitrergic and tachykinin-ergic being involved [Broad et al. 2012]. Likewise, in suncus, involvement of these neural pathways in the myenteric plexus has been reported by Mondal et al. to be involved in motilin-induced contraction responses [Mondal et al. 2011].

2.4.2. Physiological correlation of MMC and motilin

It has been found that plasma motilin concentration in dogs and humans shows cyclic changes during MMC, and its peak is correlated with phase III contractions. In humans and dogs, plasma motilin concentrations ranges approximately 100–500 pmol/L, corresponding to 10^{-10} M–10^{-8} M in the blood [Hall et al. 1983; Itoh et al. 1983; Itoh et al. 1978; Janssens et al. 1983; Vantrappen et al. 1979]. In the present study, only the proximal corpus responded to motilin at the concentration of 10^{-10} M, which is close to the physiological dose. Based on this result, the proximal corpus is thought to be the first target site of motilin, and it is probable that contractions begin at the proximal corpus and propagate downwards.

2.4.3 Regulatory mechanism of motilin-induced gastric contraction

I found that motilin-induced contractions in all gastric segments were inhibited by TTX and atropine pretreatment, suggesting that motilin-induced gastric contraction is mediated through the cholinergic neural pathway in the myenteric plexus in all regions

of the stomach. In the fundus and corpus, at high concentration, motilin 10^{-7} M slightly increased contraction even under atropine and TTX treatment but is almost negligible. It has been reported that plasma motilin concentration varies approximately between 10^{-10} M–10^{-8} M [Hall et al. 1983; Itoh et al. 1983; Janssens et al. 1983; Vantrappen et al. 1979]. The contractile action of motilin from 10^{-10} to 10^{-7} M concentration in all the segments of the suncus stomach was significantly eliminated by atropine and tetrodotoxin treatment and mediates through the neural pathway in all the parts of the suncus stomach. However, in previous reports, a high concentration of motilin that is thought to be pharmacological concentration stimulate gastric contraction by the myogenic effect. For example, motilin-induced myogenic contraction in rabbits at 10^{-6} M [Adachi et al. 1981; Depoortere et al. 1995] and 10^{-7} M for humans and chickens; [Coulie et al. 1998; Shim 2002; Kitazawa et al. 1995; Kitazawa et al. 1997]; however, this myogenic contraction was not evident in the suncus. Atropine or TTX treatment almost completely attenuated the motilin-evoked gastric contraction in each segment of stomach indicating that each gastric segment contracts with the similar mechanism i.e. cholinergic neural pathway.

2.4.4. Motilin receptor GPR38 mRNA expression

Previously, the *S. murinus* motilin receptor (GPR38) gene was identified and the fact that its expression was high in the stomach [Suzuki et al. 2012]. I analyzed this in more detail and showed that the distribution of GPR38 mRNA expression differs in different parts of the stomach by using quantitative PCR method. It was found that GPR38 mRNA expression was low in the mucosal layer and high in the muscle layer in every part of the stomach. Moreover, in the muscle layer, the GPR38 mRNA expression level differed in each stomach section, with the highest in the proximal corpus and the lowest in the

antrum. Therefore, the high reactivity for motilin in the proximal corpus may be caused by the high expression of GPR38, suggesting that the proximal corpus area, including the cardia, may be a site of onset of motilin-induced phase III contraction. In humans, motilin receptor immunoreactivity was identified in the muscle and myenteric plexus in the upper gut. Immunoreactivity studies indicate that the motilin receptor is co-expressed with choline acetyltransferase in neurons [Broad et al. 2012]. Together, these results suggest that GPR38 expressed in the neurons of the myenteric plexus is the first target of plasma motilin in MMC.

2.5. Summary

From the present study, it can be concluded that the proximal corpus and cardia was an important site for motilin-induced contractions, with high GPR38 mRNA expression. Motilin 10^{-9} M stimulates contraction in all the parts of the stomach, but the lower concentration of motilin 10^{-10} M stimulates contraction in the proximal corpus, suggesting that the proximal corpus is the first site in which contractions are induced by motilin stimulation, and hence the MMC propagates from the proximal corpus to the distal tract and downwards in the GI tract. These motilin-evoked gastric response is mediated by cholinergic neurons in the myenteric plexus.

Chapter 3. Synergistic effect of motilin and ghrelin induces different responses in different segments of the stomach of *Suncus murinus in vitro*

3.1 Introduction

3.1.1 Regulatory mechanism underlying motilin- and ghrelin-induced gastric contractions

An evident association between plasma motilin levels and gastric phase III contractions has been reported in dogs and humans [Itoh et al. 1976, Itoh et al. 1978, Janssens et al. 1983, Vantrappen et al. 1979, Hall et al., 1983]. Similar plasma ghrelin peaks and phase III-like contractions have been observed in rats [Ariga et al. 2007]. In *S. murinus*, the gastric migrating motor complex (MMC) is regulated synergistically by motilin and ghrelin both *in vitro* and *in vivo* [Mondal et al. 2012]. However, motilin-induced contractions vary among different stomach segments, as shown in Chapter 2. Therefore, I investigated the synergistic effect of motilin and ghrelin in different segments of the suncus stomach.

Endogenous ghrelin is important for inducing gastric phase II and phase III contractions [Mondal et al. 2013]. Moreover, ghrelin is an indispensable hormone for promoting motilin-induced gastric contractions in conscious suncus. Interestingly, GABAergic neurons seem to be involved in motilin-induced contractions [Kuroda et al. 2015]. Therefore, I examined the involvement of the GABAergic pathway in different stomach segments by using an *in vitro* organ bath system. To determine the complete mechanism underlying motilin-induced gastric contractions, I investigated the driving forces of

GABAergic neurons. Several studies on the central nervous system (CNS) indicate that adenosine and dopamine act as major neurotransmitters and neuromodulators of GABAergic neurons [Fuxe et al. 2003, Ferre et al. 1993].

3.1.2 Adenosine and dopamine as neurotransmitters and neuromodulators

Adenosine is a ubiquitous endogenous homeostatic modulator secreted by almost all cells, including neuronal and glial cells. Newby in 1984 named adenosine as a "retaliatory metabolite," and Englar in 1991 named it as a "signal of life" [Newby 1981, Englar 1991]. Adenosine performs different functions in the regulation of cardiopulmonary, renal, nervous, and gastrointestinal systems [Christofi et al. 2001, Riberio et al. 2003]. It also plays important roles in preventing and inducing apoptosis [Di Iorio et al. 2002]. Unlike other neurotransmitters, adenosine is not stored in synaptic vesicles but is released from cells in response to metabolic stress or after the breakdown of ATP, which is produced by both neuronal and non-neuronal cells [Ren et al. 2008, Abbracchio et al. 2006]. Under basal conditions, adenosine is nonspecifically released into the extracellular space by normal cells and neurons [Begg et. al 2002]. Hasko et al. reported that electrically induced longitudinal muscle preparations of the myenteric plexus release adenosine [Hasko et al. 2007] and that endogenous adenosine concentrations in the myenteric plexus of the gastrointestinal (GI) tract vary with pO_2 levels [Deshpande et al. 1999]. Dopamine is also an essential monoamine neurotransmitter abundantly found in the CNS and peripheral nervous system. It plays a major role in regulating emotion and cognition, pain, and reward system and in modulating GI motility [Nieoullon 2002, Wise 2004, Zhang et al. 2012, Carlino et al. 2016].

Endogenous adenosine and dopamine inhibit GABAergic neurons through A_{2A} and D_2 receptors, respectively [Hu et al. 1997, Jo et al. 1999, Sebastiao et al. 1996, Mayfield et al. 1994, Floran et al. 1997]. Although mechanisms underlying GABAergic signaling in the gut are unclear, recent data highlight the functional significance and effective role of enteric GABAergic signaling in motilin-induced gastric contractions. Mondal et al. showed that adenosine significantly suppressed ghrelin-induced contractions and did not inhibit motilin-induced contractions in the suncus stomach [Mondal et al. 2013]. Also, another study showed that dopamine D_2 receptor expressed by GABAergic neurons suppressed food intake and small intestinal transit [Kaneko et al. 2010]. These findings suggest that adenosine A_{2A} receptor and dopamine D_2 receptor play significant roles in regulating GABAergic signaling. Therefore, I investigated whether adenosine and/or dopamine can be used as substitutes for ghrelin and whether they can accelerate motilin-induced gastric contractions by inhibiting GABAergic pathway.

By using *S. murinus* as a model organism, I determined the synergistic effect of motilin and ghrelin on different stomach segments. In addition, I determined the involvement of adenosine and dopamine through the A_{2A} and D_2 receptors, respectively, on motilin-induced contractions in different segments of the suncus stomach and in the whole stomach by using specific agonists of these receptors.

3.2 Materials and Methods

3.2.1. Ethical approval

All procedures used in this study were approved by and were performed in accordance with the Saitama University Committee on Animal Research. All efforts were taken to minimize animal suffering and to minimize the number of animals used in the study.

3.2.2. Animals

Experiments were conducted with female *S. murinus* (age, at least 15–20 weeks; weight, 50–75 g) obtained from an outbred KAT strain established from a wild population in Kathmandu, Nepal. The animals were housed individually in plastic cages equipped with an empty can as a nest box and were provided food (trout pellets; Nippon Formula Feed Manufacturing Co., Ltd., Yokohama, Japan) and water *ad libitum*. Metabolizable energy content of the pellets was 344 kcal·100 g^{-1}, with 54.1% protein content, 30.1% carbohydrate content, and 15.8% fat content. The animal room was maintained at a temperature of 21°C–24°C and 12-/12-h light/dark cycle (lights on from 8.00 to 20.00 h). The animals were fasted for 8 h and were decapitated after being deeply anesthetized with pentobarbital sodium (100 mg/kg IP). Their stomachs were removed immediately by performing midline incision and were used for performing molecular, morphological, and organ bath studies. In addition, the stomachs were sectioned into four segments, namely, the fundus, proximal corpus (including the cardia), distal corpus, and antrum, as described in Chapter 2.

3.2.3. RT-PCR

The dissected stomach segments were frozen in liquid nitrogen and were ground using CRYO PLUS (Microtech Co., Ltd., Chiba, Japan) before dipping in ISOGEN (Nippon Gene, Tokyo, Japan). Total RNA from the tissues was extracted using ISOGEN, according to the manufacturer's instructions. Trace DNA contamination was removed by treating the RNA with DNase (Promega, Madison, WI, USA). Next, 1 μg DNase-treated total RNA was reverse transcribed using Superscript® III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and random primers (#48190-011; Invitrogen). The following primers were used for performing RT-PCR: (1) sense (5′-CTTCCTCCCAAGTCCTGCTG -3′) and antisense (5′-CTTCCTCCCAAGTCCTGCTG-3′) primers against the suncus ghrelin gene (fragment size, 165 bp) and (2) sense (5′-TGCGTGACATCAAGGAGAAG-3′) and antisense (5′-GACAGCACTGTGTTGGCCATA-3′) primers against the suncus β-actin gene (internal control; fragment size, 274 bp). PCR was performed using iCycler (Bio-Rad, Hercules, CA, USA).

3.2.4. Tissue preparation for morphological analysis

The stomach segments were immediately dissected, were opened along their longitudinal axes, and were immersed in 4% paraformaldehyde for 12 h. Tissues of different stomach segments were dehydrated in an ascending ethanol series, were immersed in xylene, and were embedded in paraplast (Oxford Labware, MO, USA). Next, tissue blocks produced were cut into 10-μm-thick sections by using a microtome and were mounted on slides coated with silane (Shin-Etsu Chemicals, Tokyo, Japan).

3.2.5. Immunohistochemical analysis

Immunohistochemical detection of ghrelin-immunopositive (ghrelin-ip) cells was performed using an anti-ghrelin serum (#603) and avidin–biotin–peroxidase complex (ABC) method. Production and specificity of the anti-ghrelin serum has been confirmed in previous studies [Date et al. 2000, Hosoda et al. 2000], which showed that the anti-ghrelin serum recognizes the N-terminal region of rat ghrelin. The stomach sections were deparaffinized with xylene and were rehydrated using a descending ethanol series. The sections were treated with 0.5% sodium metaperiodate for 10 min at room temperature to block endogenous peroxidase activity and were washed with distilled water (DW). Next, the sections were treated with 1% sodium thiosulfate for 10 min. After washing with DW, the sections were incubated with a blocking reagent (TNBS) for 1 h. Next, the sections were incubated overnight in a humid chamber with the anti-ghrelin serum diluted to 1:100,000 in the blocking reagent. After washing with phosphate-buffered saline (PBS), the sections were incubated for 1 h with biotin-conjugated anti-rabbit IgG serum (Vectastain ABC kit; Vector, Burlingame, CA, USA) diluted to 1:300 with the blocking reagent and were washed again with PBS. Next, the sections were incubated for 30 min with ABC solution (Vectastain ABC kit) prepared according to the manufacturer's instructions. After washing with PBS for 10 min, the sections were treated with 0.02% 3,3-diaminobenzidinetetrachloride (DAB) mixed with 0.006% hydrogen peroxide (H_2O_2) and 0.05 M Tris–HCl (pH 7.6) for 4–5 min to detect immunostaining. After washing with PBS and Millipore water, the sections were dehydrated using a graded ethanol series, cleared in xylene, mounted with Entellan (Merck, Darmstadt, Germany), and viewed under a light microscope (BX60; Olympus,

Tokyo, Japan). All the incubations were performed in a humid chamber at room temperature.

3.2.6. Morphometric analysis

Densities of ghrelin-ip cells in the different segments of the suncus stomach were estimated. Digital photographs were obtained using a light microscope equipped with a digital camera (DP70; Olympus). The number of ghrelin-ip cells in each section was counted, and the area of mucosal layer in each section was measured using a computerized image analysis program ImageJ (National Institutes of Health, Bethesda, MD). Density of ghrelin-ip cells was calculated as the number of immunopositive mucosal cells per unit area (cells/mm^2). All data are expressed as mean ± SEM.

3.2.7. Preparation of the isolated *S. murinus* stomach

The stomachs were dissected after performing laparotomy and were immediately placed in freshly prepared Krebs' solution (118 mM NaCl, 4.75 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.8 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11.5 mM glucose [pH 7.2–7.4]). Mesenteric attachments and fatty tissues were removed, and the insides of the stomachs were washed with Krebs' solution by creating a small incision in the fundus. Next, the stomachs were sectioned into four segments, i.e., the fundus, proximal corpus (including the cardia), distal corpus, and antrum. The gastroesophageal junction, i.e., the cardiac region, was included in the proximal corpus segment (criteria for dividing the stomach into segments and their preparation is mentioned in Chapter 2, section 2.2.4). The

stomach segments were mounted in 10 ml water-jacketed organ baths, with an initially loaded with approximately 0.5 gwt. The temperature of the Krebs' solution was maintained at 37°C ± 0.5°C, and the solution was aerated continuously with a mixture of 95% O_2 and 5% CO_2.

3.2.8. Analysis of *in vitro* gastric contractions

Mechanical activity of the stomach segments was monitored with an isometric force transducer (UM-203; Iwashiya Kishimoto Medical Industrials, Kyoto, Japan) and PicoLog for Windows (Pico Technology Ltd., St Neots, UK). The initial load was set at 0.5 g for each preparation. Experiments were initiated after stabilization of contraction pattern for 45 min. To normalize gastric contractions, the organ bath was treated with 10^{-5} M acetylcholine (ACh) twice before cumulatively administering motilin and once at the end of the experiment. The percentage of maximal contractions was calculated by averaging tonic response induced by the three treatments with 10^{-5} M ACh. Contraction amplitude was expressed as a relative contraction (%) of ACh-induced response. In each case, ACh administration induced almost the same tonic GI contractions in each stomach segment. To examine the synergistic effect of motilin and ghrelin, each stomach segment was cumulatively treated with suncus ghrelin (10^{-11}–10^{-7} M) with or without pretreatment with suncus motilin (10^{-10} M) for 30 s and responses induced were recorded. To elucidate mechanism underlying the cumulative effect of motilin and ghrelin, the stomach segments were pretreated with 10^{-6} M atropine for 30 min. To determine regulatory mechanism underlying the effect of motilin, the stomach segments were pretreated with D-Lys3-GHRP6 (a ghrelin receptor antagonist) 10 min before cumulatively administering motilin (10^{-11}–10^{-7} M). In addition, the stomach segments were treated

with 10^{-7} M adenosine (an adenosine receptor agonist) and 10^{-5} M 6-hydroxydopamine hydrochloride (a dopamine receptor agonist) 15 min before cumulatively administering motilin. The pH of the buffer was maintained between 7.2 and 7.4 before administering the drugs.

3.2.9. Drugs used

Administration volume of each drug was 1% of the bath volume. Atropine sulfate (Merck, USA), ACh, adenosine (a non-selective adenosine receptor agonist), 6-hydroxydopamine hydrochloride (a non-selective dopamine receptor agonist), PSB 0777 ammonium salt (an adenosine A_{2A} receptor agonist), B-HT 920 (a dopamine D_2 receptor agonist; Sigma-Aldrich Co. LLC., USA) were dissolved in DW. Synthetic *S. murinus* motilin (Bex, Tokyo, Japan), active human ghrelin (Asubio Pharma Co., Ltd. Hyogo, Japan), and D-Lys3-GHRP6 (ghrelin antagonist; Bachem, Torrance, CA, USA) were dissolved in 0.1% bovine serum albumin/PBS. Drug concentrations are expressed as final molar concentrations in the bath solution. Atropine sulfate was dissolved in DW before use. For each experiment, all the reagents were prepared according to the manufacturer's instructions.

3.2.10. Statistical analysis

Results are expressed as mean ± SEM from at least four separate animals. Recording experiments were repeated individually at least three times, and similar results were obtained. The number of animals used for statistical analyses are mentioned in figure legends. Data were analyzed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla,

CA, USA). Statistical analyses were performed using Student's *t*-test or two-way ANOVA followed by Tukey's multiple comparison test. $p < 0.05$ was considered statistically significant.

3.3 Results

3.3.1. Synergistic effect of motilin and ghrelin on the different segments of the suncus stomach

Stomach tissues were treated with 10^{-5} M ACh to stimulate the maximum contraction of smooth muscle cells. ACh induced strong contractions in all the stomach segments, with a maximum tension of approximately 2.2 gwt in the fundus (Fig. 7A [i]), 4.5 gwt in the proximal corpus (Fig. 7B [i]), 4.2 gwt in the distal corpus (Fig. 7C [i]), and 4.0 gwt in the antrum (Fig. 7D [i]). Cumulative administration of human ghrelin (10^{-11}–10^{-7} M) did not induce contractions in any stomach segment (Fig. 7A–D [iii, iv]; Fig. 1F). Next, I investigated ghrelin-induced contractions in the stomach segments in the presence of low-dose motilin (10^{-10} M). In the presence of 10^{-10} M motilin, ghrelin significantly induced contractions in both the fundus and proximal corpus (Fig 7A–B [ii, iv], 7E) in a dose-dependent manner but did not induce contractions in the distal corpus and antrum (Fig 7C–D [ii, iv], 7E).

3.3.2. Motilin and ghrelin synergistically induce gastric contractions through a cholinergic neural pathway

In the presence of low-dose motilin (10^{-10} M), ghrelin (10^{-11}–10^{-7} M) did not induce contractions in any stomach segment pretreated with atropine, a muscarinic receptor antagonist (Fig. 8A–E).

3.3.3. GHS-R mRNA expression in the different segments of the suncus stomach

mRNA transcripts of the GSHR were detected in all the four segments of the suncus stomach, i.e., the fundus, proximal corpus, distal corpus, and antrum (Fig. 9).

3.3.4. Localization of ghrelin-ip cells in the suncus stomach

Ghrelin-ip cells were detected in the mucosal layer of the fundus (Fig. 10A), proximal corpus (Fig. 10B), distal corpus (Fig. 10C), and antrum (Fig. 10D). The density of ghrelin-ip cells was the highest in the fundus (95.0 ± 7.6 cells/mm^2) and proximal corpus (44.3 ± 4.7 cells/mm^2) and was significantly different from that in the distal corpus (15.2 ± 0.8 cells/mm^2) and antrum (12.5 ± 1.7 cells/mm^2) (Fig. 10E).

3.3.5. Effect of D-Lys3-GHRP6 and GABA receptor antagonists on motilin-induced gastric contractions *in vitro*

In vitro organ bath experiments were performed to determine mechanisms underlying the effect of ghrelin on motilin-induced contractions in each segment of the suncus stomach. ACh response was obtained in all the dissected stomach segments (Fig. 11A–D [i]). Treatment with 10^{-9} M motilin induced strong contractions in the fundus, distal corpus, and antrum (Fig. 11A, C, D [ii, v]), whereas treatment with 10^{-10} M motilin induced contractions in the proximal corpus (Fig. 11B [ii, v]). In contrast, pretreatment with 10^{-5} M D-Lys3-GHRP6 almost completely inhibited motilin-induced contractions in all the stomach segments (Fig. 11A–D [iii, v]). However, pretreatment with bicuculline, a $GABA_A$ receptor antagonist, almost completely reversed D-Lys3-GHRP6-induced inhibition of motilin-induced contractions. Treatment with phaclofen, a $GABA_B$ receptor antagonist, also reversed D-Lys3-GHRP6-induced inhibition of motilin-induced gastric contractions, but, treatment with 10^{-9} M motilin induced contractions in the proximal

corpus (Fig. 12B [iv, v]), and treatment with 10^{-8} M motilin induced contractions in the fundus, distal corpus, and antrum (Fig. 12A, C, D [iv, v]).

3.3.6. Effect of non-selective adenosine and dopamine receptor agonists on motilin-induced gastric contractions

Cumulative administration of motilin (10^{-11}–10^{-7} M) induced contractions in the whole stomach (Fig. 13 [ii, v]) as well as in all the stomach segments (Fig. 14; Fig. 15A–D [ii, v]) in a dose-dependent manner. Pretreatment with 10^{-5} M ghrelin antagonist D-Lys3-GHRP6 completely abolished these contractions (Fig. 13–15 A–D [iii, v]). To determine whether adenosine and dopamine regulated motilin-induced gastric contractions, the whole stomach tissue was simultaneously treated with 10^{-8} M adenosine, the adenosine receptor agonist, and 10^{-5} M 6-hydroxydopamine hydrochloride, the dopamine receptor agonist, for 30 min. Treatment with adenosine and 6-hydroxydopamine hydrochloride partially reversed D-Lys3-GHRP6-induced inhibition of motilin-induced contractions in the whole stomach (Fig. 13 [iv, v]). Next, the effects of adenosine and 6-hydroxydopamine hydrochloride pretreatment on all the four segments of the suncus stomach were determined. Although individual pretreatment with these agonists recovered motilin-induced gastric contractions, the recovery was lower than that observed with simultaneous pretreatment with these agonists (Fig. 14; Fig. 15A–D [iv, v]).

3.3.7. Effect of adenosine A_{2A} receptor agonist and dopamine D_2 receptor agonist on motilin-induced gastric contractions

Next, effects of suspected candidate adenosine A_{2A} and dopamine D_2 receptors was examined after confirming the involvement of adenosine and dopamine. Individual pretreatment with adenosine A_{2A} receptor agonist PSB 0777-AS (10^{-6} M) and dopamine D_2 receptor agonist B-HT 920 (10^{-6} M) partially reversed D-Lys3-GHRP6-induced inhibition of motilin-induced contractions in all the stomach segments (Fig. 16; Fig. 17A–D [i–v]). Next, involvement of the A_{2A} and D_2 receptors was confirmed using the whole stomach tissue. Simultaneous pretreatment with adenosine A_{2A} receptor agonist PSB 0777-AS (10^{-6} M) and dopamine D_2 receptor agonist B-HT 920 (10^{-6} M) completely reversed ghrelin antagonist D-Lys3-GHRP6 (10^{-5} M)-induced inhibition of motilin-induced contractions (Fig. 18 [i–v]).

3.4 Discussion

3.4.1 Role of ghrelin in the MMC

Results presented in Chapter 2 clearly showed that cumulative administration of motilin (10^{-10}–10^{-7} M) induced contractions in all the segments of the suncus stomach in a dose-dependent manner. In the present study, I observed that these stomach segments responded differently to simultaneous motilin and ghrelin treatment *in vitro*. It was also observed that 10^{-10} M motilin treatment is sufficient to induce contraction in proximal corpus but, 10^{-9} M motilin treatment is required to induce contractions in the fundus, distal corpus, and antrum. However, cumulative administration of ghrelin (10^{-11}–10^{-7} M) induced contractions in the fundus and proximal corpus pretreated with a low dose (10^{-10} M) of motilin but not in the distal corpus and antrum, suggesting that administration of 10^{-9} M motilin with ghrelin was required to induce contractions in these stomach segments. Drug-mediated response is directly proportional to receptor concentration in the tissue. Thus, higher the receptor expression, higher the drug response in the tissue. Results of RT-PCR for the expression of motilin receptor GPR38 (presented in Chapter 2) indicated that this receptor was highly expressed in the proximal corpus. Results of the present study indicated that ghrelin receptor GHSR was also expressed in all the stomach segments. The density of ghrelin-ip cells was significantly high in the fundus, and proximal corpus, which may be the reason for the high sensitivity of these stomach segments to motilin and ghrelin Ghrelin-ip cells are present in the gastric mucosa, and the mucosa is inevitable for ghrelin-induced contractions in *S. murinus* stomach [Mondal et al. 2013].

3.4.2 Involvement of the GABAergic pathway in motilin-induced gastric contractions

I first examined the effect of D-Lys3-GHRP6 on motilin-induced gastric contractions and observed that blockade of the ghrelin receptor considerably inhibited motilin-induced contractions in all the stomach segments. These results indicate that motilin only induces strong phase III-like gastric contractions in the presence of ghrelin. Interestingly, I found that treatment with GABA antagonists reversed ghrelin antagonist-induced inhibition of motilin-induced gastric contractions. $GABA_A$ receptor antagonist bicuculline reversed ghrelin antagonist-induced inhibition of motilin-induced contractions more strongly than $GABA_B$ receptor antagonist phaclofen in all the stomach segments, suggesting that inhibitory GABAergic neurons suppressed motilin-induced contractions in all the segments of the suncus stomach. Previous immunohistochemical studies have reported heterogeneous distribution of $GABA_A$ and $GABA_B$ receptors in the enteric nervous system (ENS), with these receptors being localized on both submucosal and myenteric neurons [Poulter et al. 1999, Casanova et al., 2009]. Activation of $GABA_B$ receptors is mainly coupled with the presynaptic inhibition of voltage-dependent calcium channels, resulting in reduced ACh release from enteric neurons [Cherubini et al. 1984, Marcoli et al., 2000]. Conversely, activation of ionotropic $GABA_A$ receptors is usually associated with the stimulation of neurotransmitter release from cholinergic and NANC enteric neurons, resulting in contractile or relaxant response of GI smooth muscles [Zizzo et al. 2007, Frigo et al. 1987, Krantis et al. 1987, Kaputlu et al. 1996, Penchava 1997]. That is one possibility to explain that $GABA_A$ is more efficacious in motilin-induced contractions in all the segments of suncus stomach. In the ENS, GABAergic neurons strongly suppress motilin-induced smooth muscle contraction pathway. In contrast,

ghrelin stimulates this motilin pathway by inhibiting GABAergic neurons, which is consistent with the results obtained using the whole stomach of *S. murinus* [Kuroda et al. 2015].

3.4.3 Adenosine and dopamine are the key molecules for mediating motilin-induced gastric contractions

Results of the present study showed that both adenosine and dopamine agonists partially reversed ghrelin antagonist D-Lys3-GHRP6-induced inhibition of motilin-induced contractions in the whole stomach. Similar results were obtained when the different stomach segments were treated with adenosine or dopamine agonist alone. However, motilin-induced contractions were slightly stronger in stomach segments pretreated with the specific adenosine A_{2A} receptor agonist and dopamine D_2 receptor agonist than in stomach segments not pretreated with any selective agonist. Furthermore, pretreatment of the whole stomach with both the adenosine A_{2A} receptor agonist and dopamine D_2 receptor agonist almost completely reversed ghrelin antagonist D-Lys3-GHRP6-induced inhibition of motilin-induced contractions. These results suggest that both adenosine and dopamine partially suppress the inhibitory action of GABAergic neurons on the motilin pathway through the adenosine A_{2A} and dopamine D_2 receptors, thus promoting motilin-induced contractions both in the whole stomach and different stomach segments. Endogenous adenosine and dopamine are the key modulators of GABAergic inhibition [Regan et al. 1992, Ferre et al. 1997, Seamans et al. 2001, Olianas et al., 1978]. Some studies have clearly shown adenosine A_{2A} and dopamine D_2 receptor expression on enteric GABAergic neurons [Christofi et al., 2001 and Li et al., 2006]. Moreover, some studies have shown that endogenous adenosine and dopamine inhibit GABA through the

A_{2A} and D_2 receptors, respectively, in the myenteric plexus of the ENS [Sebastiao et al. 1996, Concas et al. 1993, Kaneko et al. 2010]. In the ENS of guinea pigs, adenosine acts as a presynaptic neuromodulator through the A_{2A} receptor to influence the release of excitatory neurotransmitter ACh [Gao et al. 2007, Christofi et al., 1993]. These findings clearly indicate that both adenosine and dopamine act through the A_{2A} and D_2 receptors, respectively, to promote GABA inhibition.

Adenosine is ubiquitously present in the synaptic and axonal regions of the ENS and CNS as a by-product of ATP consumption during various metabolic processes [Ren et al. 2008]. Dopamine is also an essential neurotransmitter secreted by almost all neurons of the ENS. Ghrelin receptors expressed on GABAergic and dopaminergic neurons are expected to stimulate dopamine secretion in the synaptic region [Naitou et al. 2016].
Results of the present study indicate that both adenosine and dopamine are the key driving forces of GABAergic pathway and regulate motilin-induced gastric contractions through the A_{2A} and D_2 receptors, respectively. However, these results have only been obtained by performing *in vitro* experiments. Therefore, it would be interesting to determine *in vivo* expression of adenosine and dopamine receptors in the stomach of *S. murinus*

4. Summary and Conclusion

Together, the results of this research indicate that the proximal corpus and cardia are important sites for motilin-induced contractions and show high mRNA expression of the motilin receptor GPR38. Treatment with 10^{-9} M motilin stimulates contractions in all the stomach segments. However, treatment with low motilin concentration (10^{-10} M) stimulates contractions only in the proximal corpus, suggesting that the MMC propagates from the proximal corpus to the distal region of the alimentary tract and that the proximal corpus is the first site of motilin-induced contractions.

A previous *in vivo* study showed that ghrelin is important for initiating phase III MMC contractions and that co-administration of motilin 10^{-10} M and ghrelin at concentrations ranging from 10^{-9} to 10^{-7} M induces a synergistic phasic response in prepared, isolated stomachs in a dose-dependent manner. The present *in vitro* study showed that ghrelin-induced contractions in the presence of motilin differed in different stomach segments and were mediated by the cholinergic neural pathway in the myenteric plexus. Treatment with 10^{-10} M motilin induced contractions only in the fundus and proximal corpus. The mRNA expression of ghrelin receptor GHSR was detected in all the stomach segments. Moreover, the density of ghrelin-ip cells was significantly higher in the fundus and proximal corpus than in the other stomach segments. These results suggest that the fundus and proximal corpus (constituting the proximal stomach) are the most sensitive and responsive to motilin- and ghrelin-induced synergistic contractions. Moreover, results of the present study showed that pretreatment with the $GABA_A$ antagonist bicuculline reversed ghrelin antagonist D-lys_3-$GHRP_6$-induced inhibition of motilin-induced gastric

contractions. Moreover, the $GABA_B$ antagonist phaclofen also reversed ghrelin antagonist D-lys_3-$GHRP_6$-induced inhibition of gastric contractions but less effectively than the $GABA_A$ antagonist bicuculline.

Adenosine and dopamine are well-known neurotransmitters and neuromodulators of the CNS and ENS. Several studies have shown that adenosine and dopamine regulate the inhibition of GABAergic neurons, which is the major turning point of this research. In addition to their numerous functions, adenosine and dopamine aid in modulating GI motility. Involvement of adenosine and dopamine in motilin-induced contractions was determined using the different stomach segments as well as the whole stomach. Results clearly showed that adenosine and dopamine partially reversed ghrelin antagonist-induced inhibition of motilin-induced contractions. Next, I examined the suspected receptor candidates adenosine A_{2A} receptor and dopamine D_2 receptor. Surprisingly, simultaneous pretreatment with the agonists of both these receptors almost completely recovered motilin-induced contractions in all the stomach segments as well as in the whole stomach, suggesting that adenosine and dopamine played key roles in motilin-induced gastric contractions in *S. murinus* through the A_{2A} and D_2 receptors, respectively.

5. Figures, Tables, and Annexure

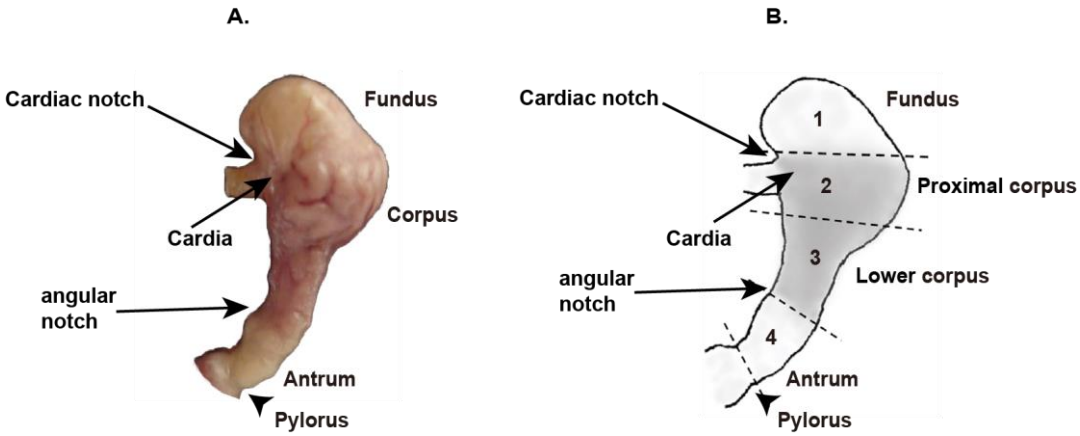


Fig 1. Stomach segments used in the study

(A) Photograph showing the different stomach segments, namely, the fundus, proximal corpus, distal corpus, and antrum. (B) For the organ bath experiment, the stomachs were dissected into four segments: (i) fundus, (ii) proximal corpus (including the cardia), (iii) distal corpus, and (iv) antrum. The arrows indicate the cardia (gastroesophageal junction), cardiac notch, and angular notch. The arrowheads indicate the pylorus.

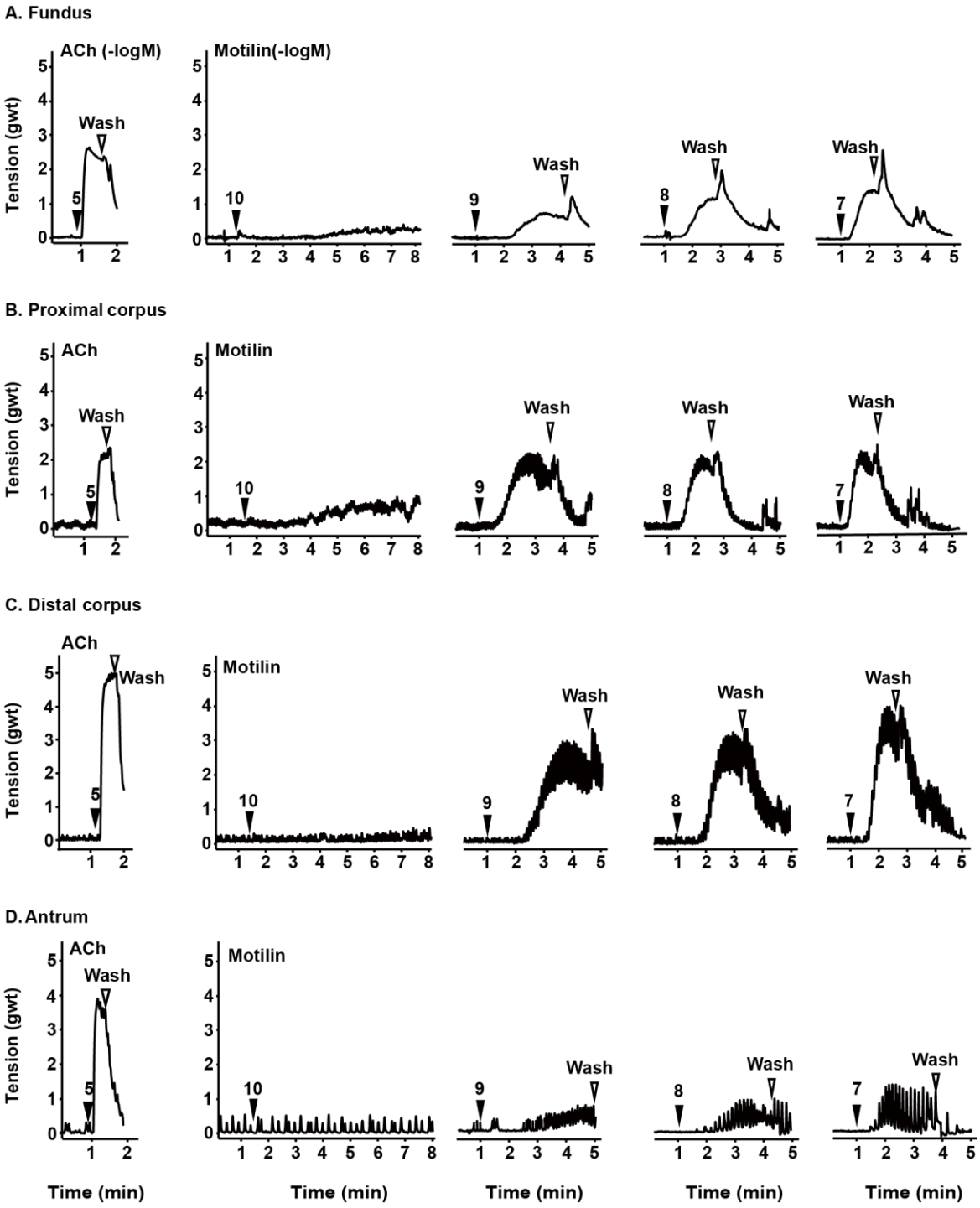


Fig 2. Motilin induced contractions in the different segments of the isolated stomach

ACh (10^{-5} M) was used to induce maximum contractions in the stomach tissue. ACh induced contractions in all the stomach segments, with a maximum tension of approximately 2.5 gwt in the fundus (A, left), 2.2 gwt in the proximal corpus (B, left), 5.0 gwt in the distal corpus (C, left), and 4.1 gwt in the antrum (D, left). Treatment with a low dose (10^{-10} M) of motilin induced contractions in the fundus (A, right) and proximal corpus (B, right), which showed 28% contraction, and in the distal corpus (C, right) and antrum (D, right). Contractile amplitudes were measured after serially treating the (A) fundus, (B) proximal corpus, (C) distal corpus, and (D) antrum with motilin (10^{-9}–10^{-7} M). Treatment with 10^{-9} M motilin induced dose-dependent contractions in the (A) fundus, (B) proximal corpus, and (C) distal corpus. (D) In the antrum, contraction amplitude changed with an increase in motilin dose. The arrowhead indicates the timing of reagent administration; the number indicates drug concentration used (-logM).

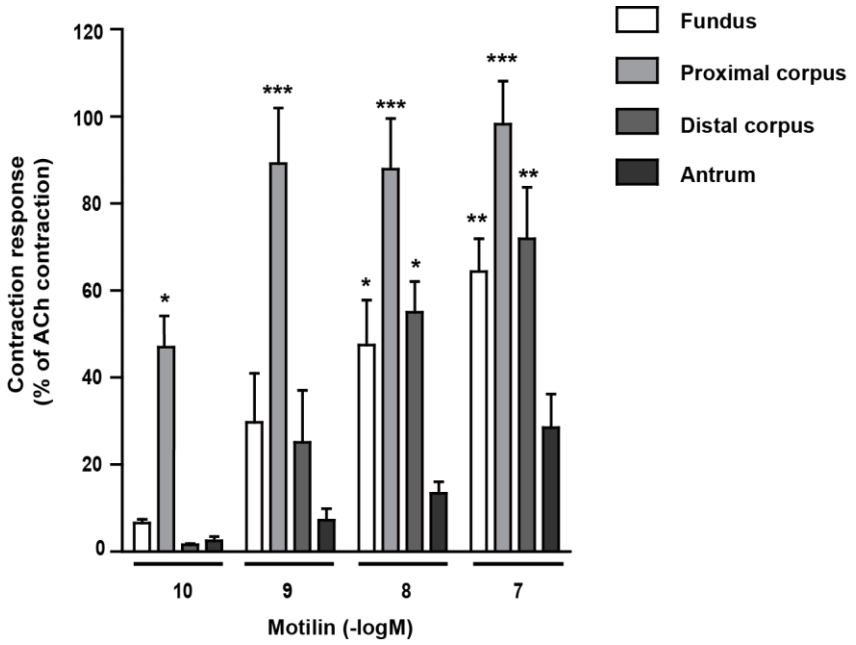


Fig 3. Motilin induced contractions in the stomach segments

Contraction responses were calculated as percentage maximum contractions induced by 10^{-5} M ACh. Treatment with a low dose (10^{-10} M) of motilin induced significantly stronger concentration response in the proximal corpus than in the other stomach segments. Each histogram represents mean ± SEM values (n = 5). Different letters denote significant difference ($P < 0.05$) among groups.

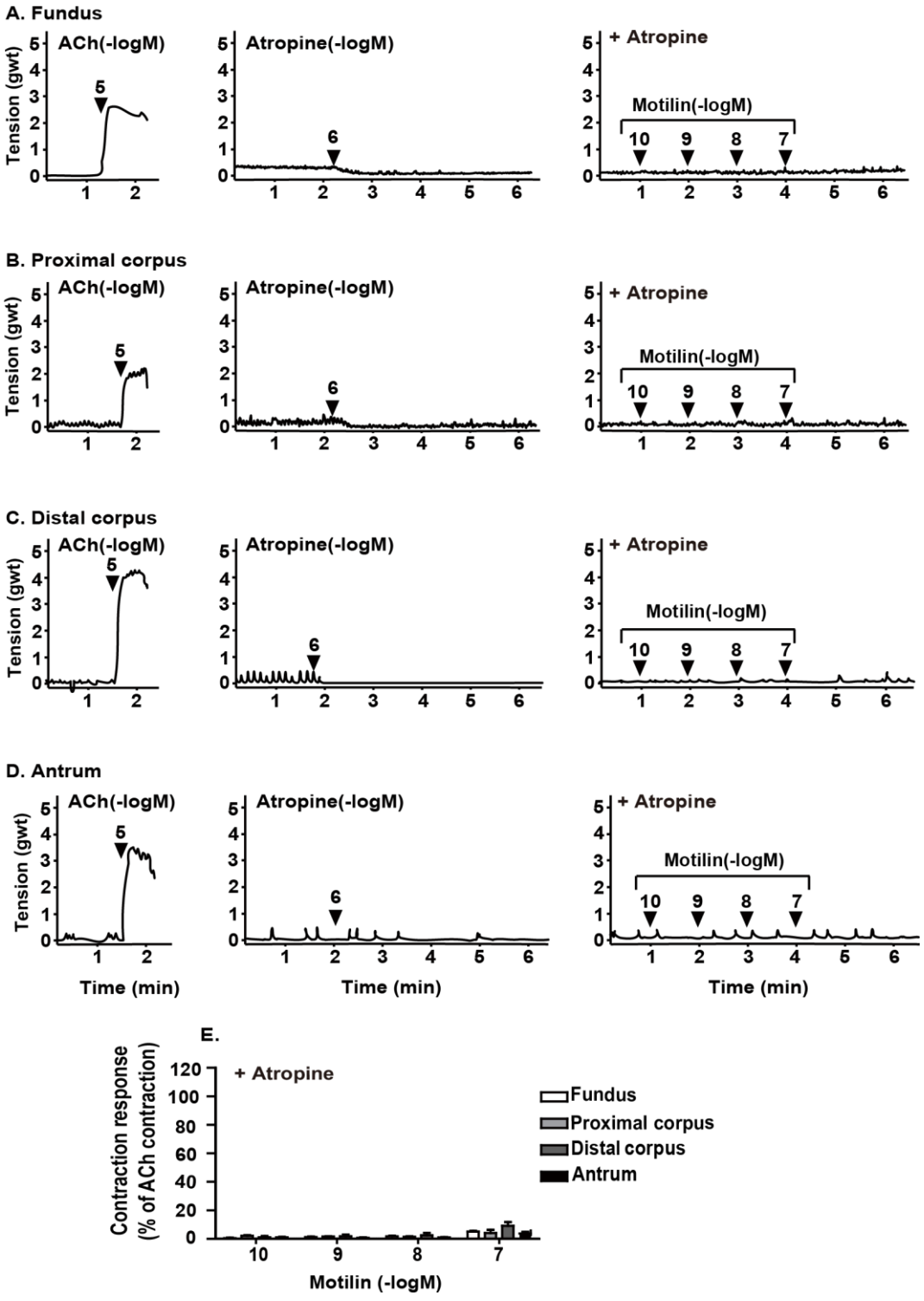


Fig 4. Atropine inhibits motilin-induced contractions in the different stomach segments

Pretreatment with 10^{-6} M atropine almost completely inhibited 10^{-10}–10^{-7} M motilin-induced contractions in all the stomach segments, i.e., the fundus (A, left), proximal corpus (B, left), distal corpus (C, left), and antrum (D, left). Results obtained by performing motilin treatment in the presence of atropine are summarized in panel E. ▼ Timing of reagent administration. The number indicates drug concentration (-logM). Each histogram represents mean ± SEM values (n = 4).

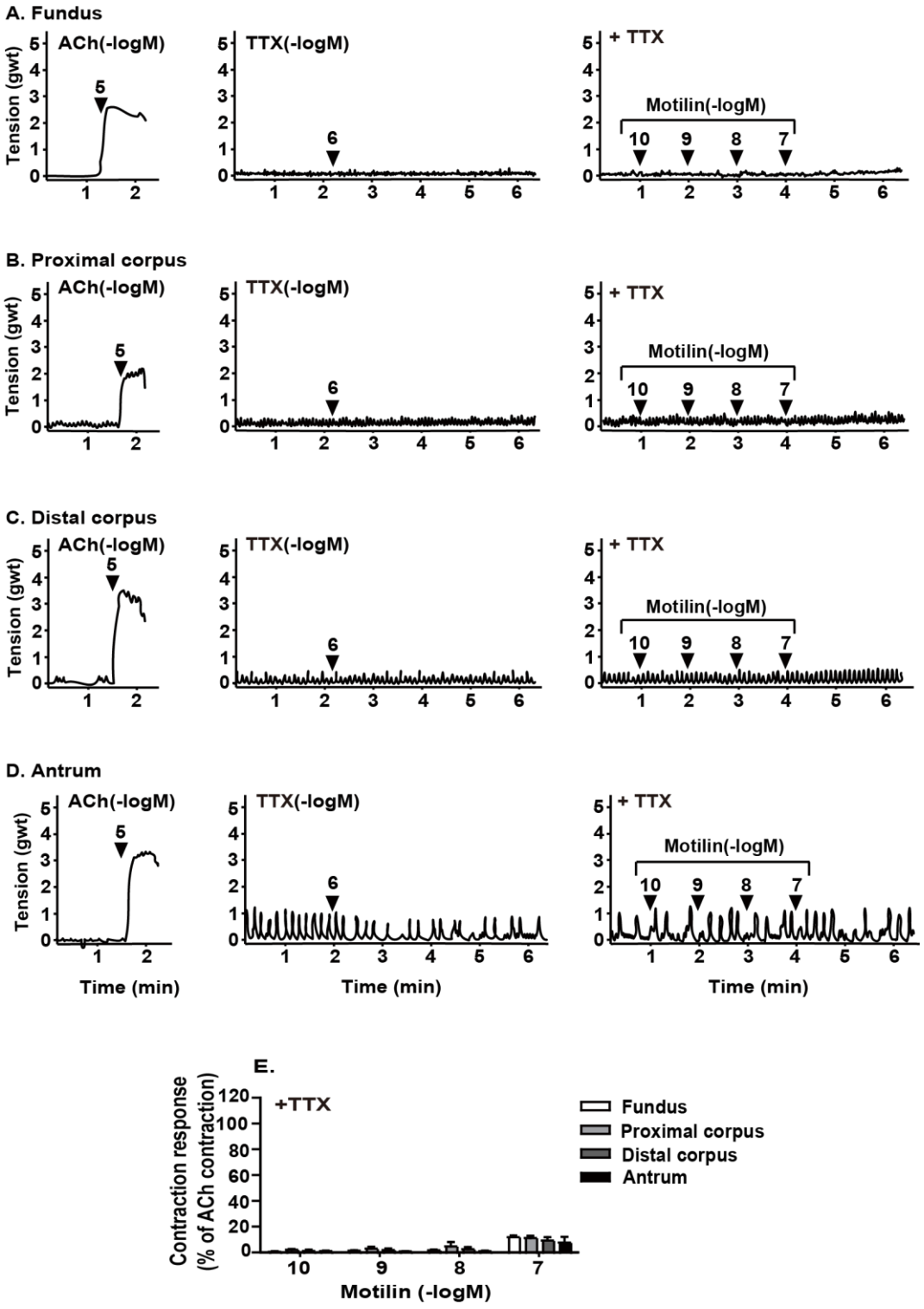


Fig 5. Tetrodotoxin inhibits motilin-induced contractions in the different stomach segments

Effects of tetrodotoxin (TTX) administration on contractions in all the stomach segments, i.e., the fundus (A, left), proximal corpus (B, left), distal corpus (C, left), and antrum (D, left). Pretreatment with 10^{-6} M TTX almost completely inhibited 10^{-10}–10^{-7} M motilin-induced contractions in all the stomach segments (A–D), with a slight reduction in contraction amplitude in the antrum (D). Results obtained by performing motilin treatment in the presence of TTX are summarized in panel E. ▼Timing of reagent administration. The number indicates drug concentration (-logM). Each histogram represents mean ± SEM values (n = 4).

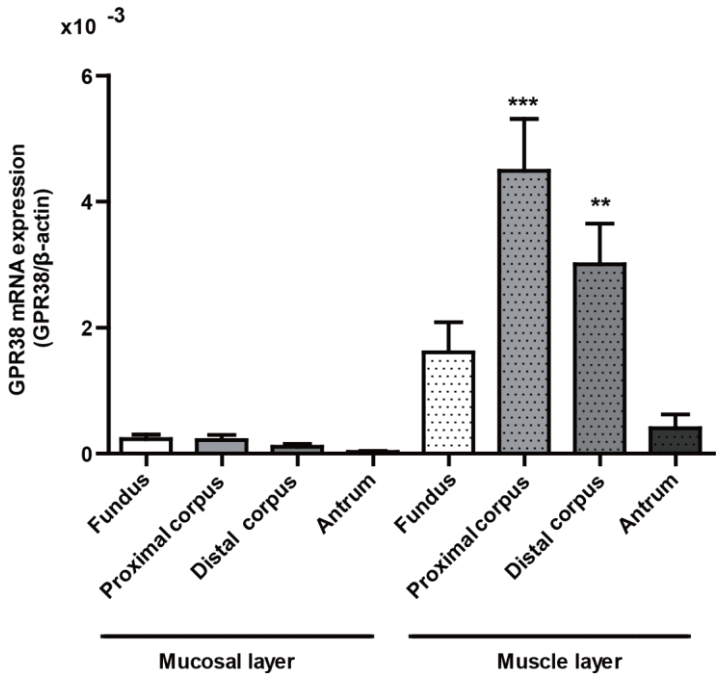


Fig 6. mRNA expression of the motilin receptor GPR38 in the suncus stomach

The mRNA expression level of the GPR38 was low in the mucosal layer and was high in the muscle layer. Moreover, the mRNA expression level differed among the muscle layer of different stomach segments, with the highest level being observed in the muscle layer of the proximal corpus and the lowest level being observed in the muscle layer of the antrum. Each histogram represents mean ± SEM values (n = 6). Different letters denote significant difference ($P < 0.05$) among groups.

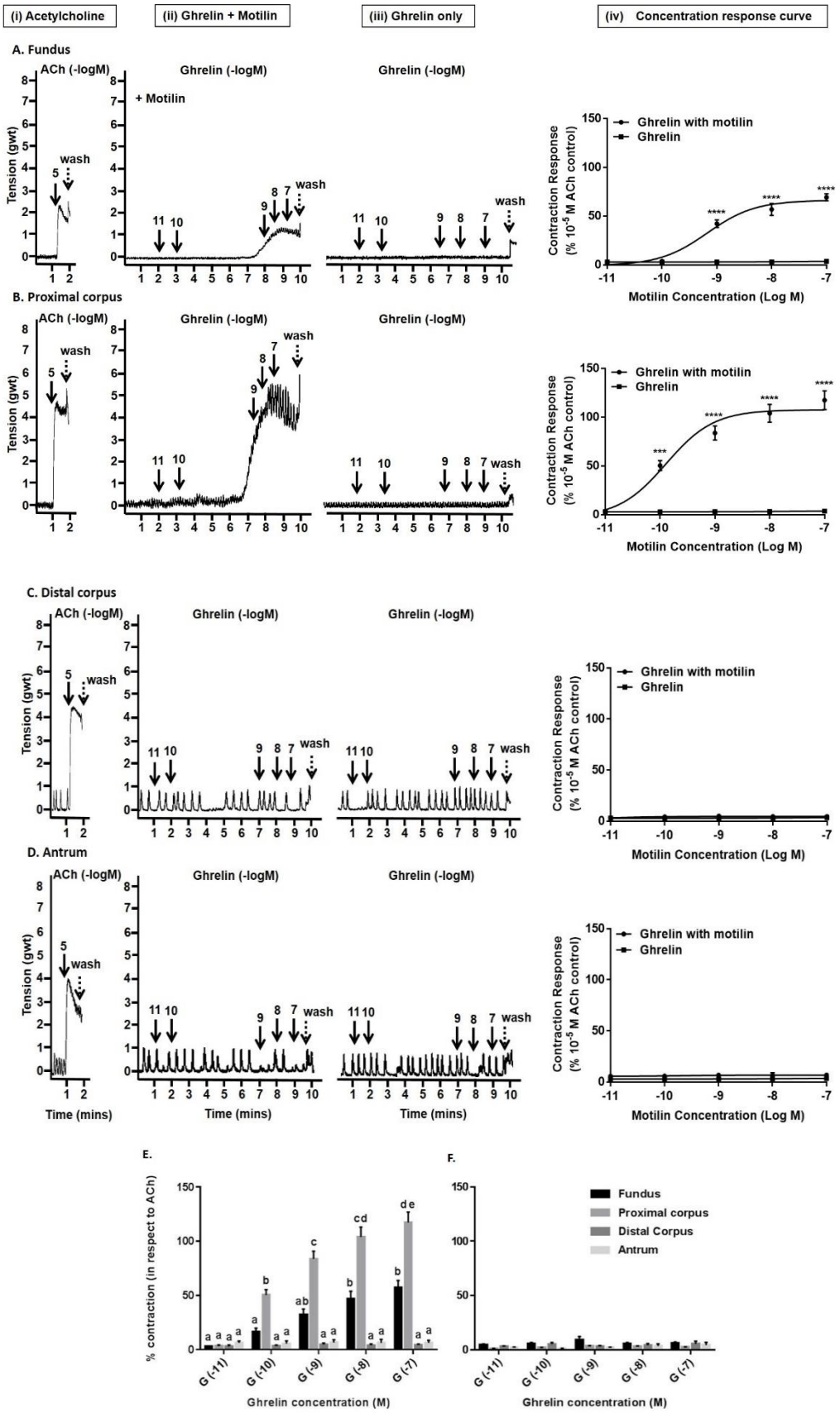

(i) Acetylcholine
(ii) Ghrelin + Motilin
(iii) Ghrelin only
(iv) Concentration response curve
A. Fundus
B. Proximal corpus
C. Distal corpus
D. Antrum
ACh (-logM)
Ghrelin (-logM)
+ Motilin
Tension (gwt)
wash
Time (mins)
Ghrelin with motilin
Ghrelin
Contraction Response (% 10-5 M ACh control)
Motilin Concentration (Log M)
E.
F.
% contraction (in respect to ACh)
Fundus
Proximal corpus
Distal Corpus
Antrum
G (-11)
G (-10)
G (-9)
G (-8)
G (-7)
Ghrelin concentration (M)

Fig 7. Motilin and ghrelin synergistically induce contractions in the different stomach segments

ACh response was assessed to determine the viability of the tissue in all the stomach segments, i.e., the fundus (A [i]), proximal corpus (B [i]), distal corpus (C [i]), and antrum (D [i]). The segments were pretreated with a low dose (10^{-10} M) of motilin for 30 s before cumulatively administering ghrelin (10^{-11}–10^{-7} M) (A–D [ii]). In addition, contractions were measured in all the stomach segments not pretreated with motilin but cumulatively treated with ghrelin (10^{-11}–10^{-7} M) (A–D [iii]). Concentration-response curve corresponding to percentage maximum contractions induced by ACh (10^{-5} M) is shown for all the stomach segments (A–D [iv]). Results of ghrelin treatment in the presence or absence of motilin pretreatment are summarized in panels E and F, respectively. Arrow represents the timing of reagent administration, and dotted arrow represents the timing of tissue washing. The number indicates drug concentration (-logM). Each value is expressed as mean ± SEM (n = 5). Contractions in the different segments of the suncus stomach were compared using two-way ANOVA followed by Tukey's post hoc test. ***$p < 0.001$ and ****$p < 0.0001$ indicate significant difference among groups.

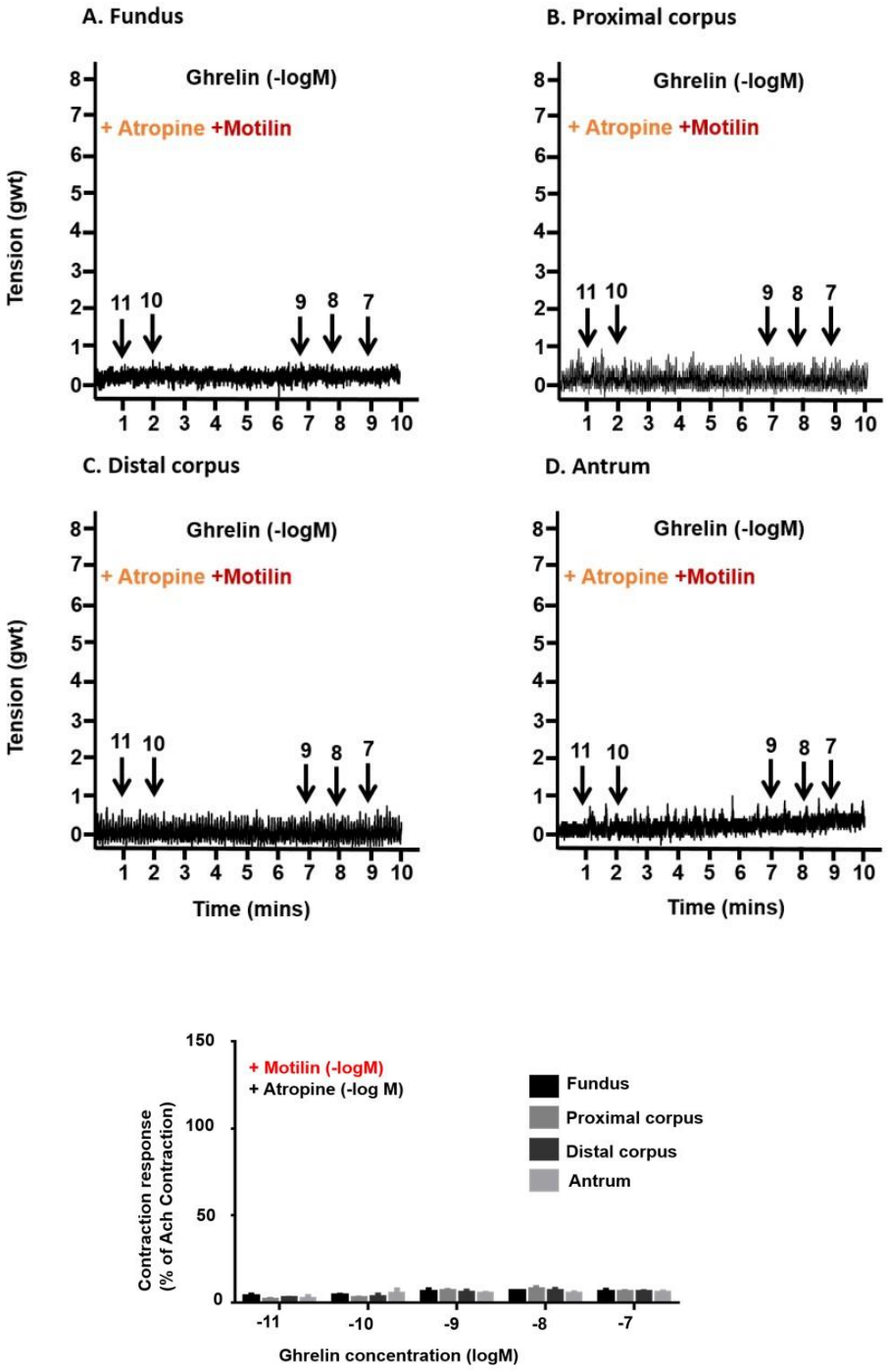


Fig 8. Atropine inhibits motilin- and ghrelin-induced contractions in the different stomach segments

Pretreatment with 10^{-6} M atropine almost completely inhibited 10^{-10} M motilin- and 10^{-11}–10^{-7} M ghrelin-induced contractions in all the stomach segments (A–D). Results of ghrelin and motilin treatment in the presence of atropine are summarized in panel E. Arrow represents the timing of reagent administration. The number indicates drug concentration (-logM). Each value is expressed as mean ± SEM (n = 4). Contractions in the different segments of the suncus stomach were compared using two-way ANOVA.

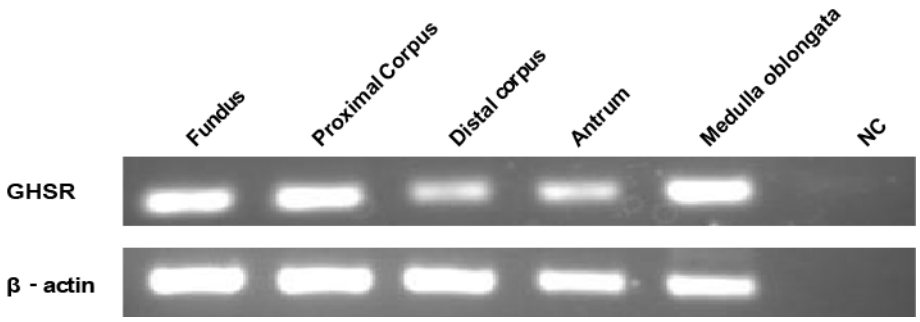
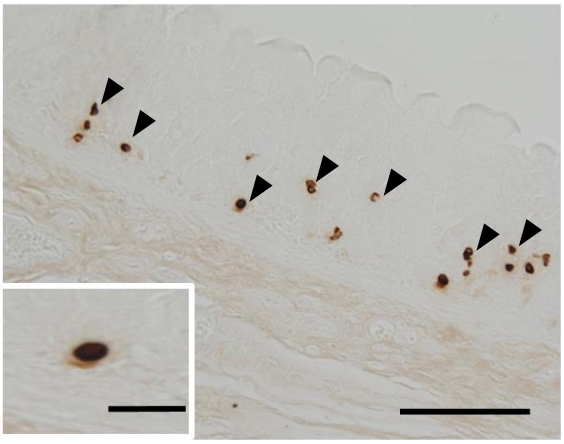


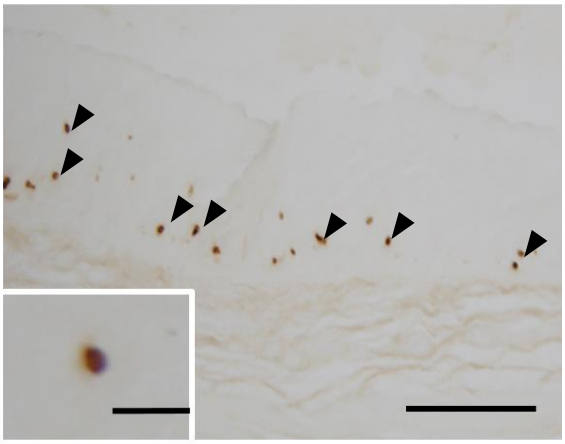
Fig 9. mRNA expression of the ghrelin receptor, GHSR

PCR products and specificity were confirmed by performing agarose gel electrophoresis on a 2% gel. The mRNA expression of the ghrelin receptor GHSR in all the stomach segments and the medulla oblongata (positive control). Distilled water (DW) was used as a negative control, and β-actin was used as an internal control.

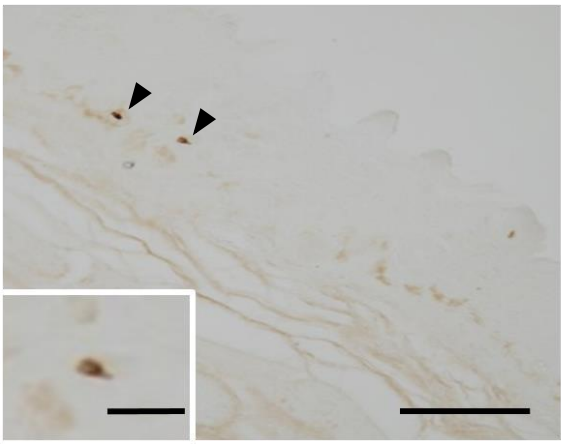
A. Fundus



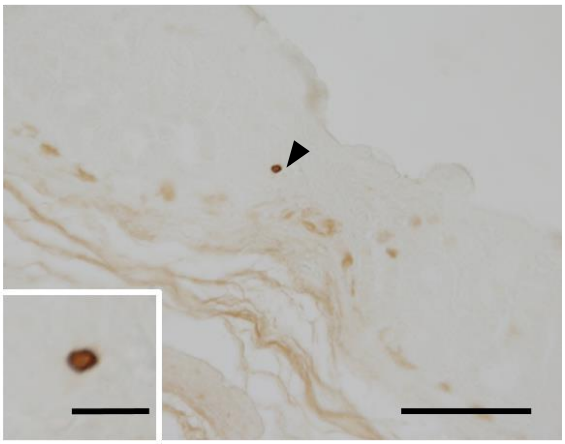
B. Proximal corpus



C. Distal corpus



D. Antrum



E.

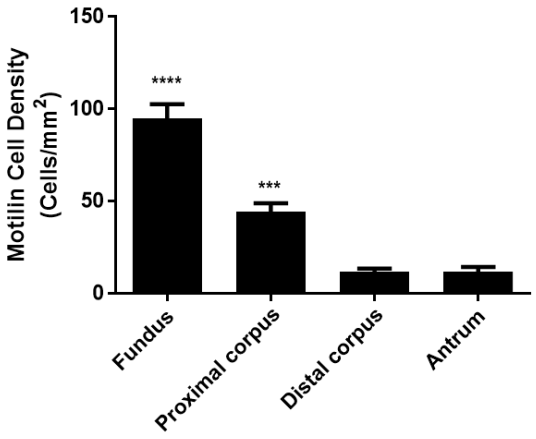


Fig 10. Microphotographs of ghrelin-immunopositive cells in the different stomach segments

Ghrelin-immunopositive (ghrelin-ip) cells in the (A) fundus, (B) proximal corpus, (C) distal corpus and (D) antrum were determined by performing immunohistochemical analysis. Insets show high magnification of ghrelin-ip cells. Ghrelin-ip cells are scattered in the epithelium of the mucosal layer of the suncus stomach. Bars in A–D correspond to 20 and 100 μm in the insets. (E) Histograms showing the densities (cells/mm^2) of ghrelin-ip cells in different stomach segments, as determined by performing immunohistochemical analysis. Arrowheads represent ghrelin-ip cells. Each value is expressed as mean ± SEM (n = 4). Densities of ghrelin-ip cells in the different segments of the suncus stomach were compared using one-way ANOVA followed by Tukey's post hoc test. ***$p < 0.001$ and ****$p < 0.0001$ indicate significant difference among groups.

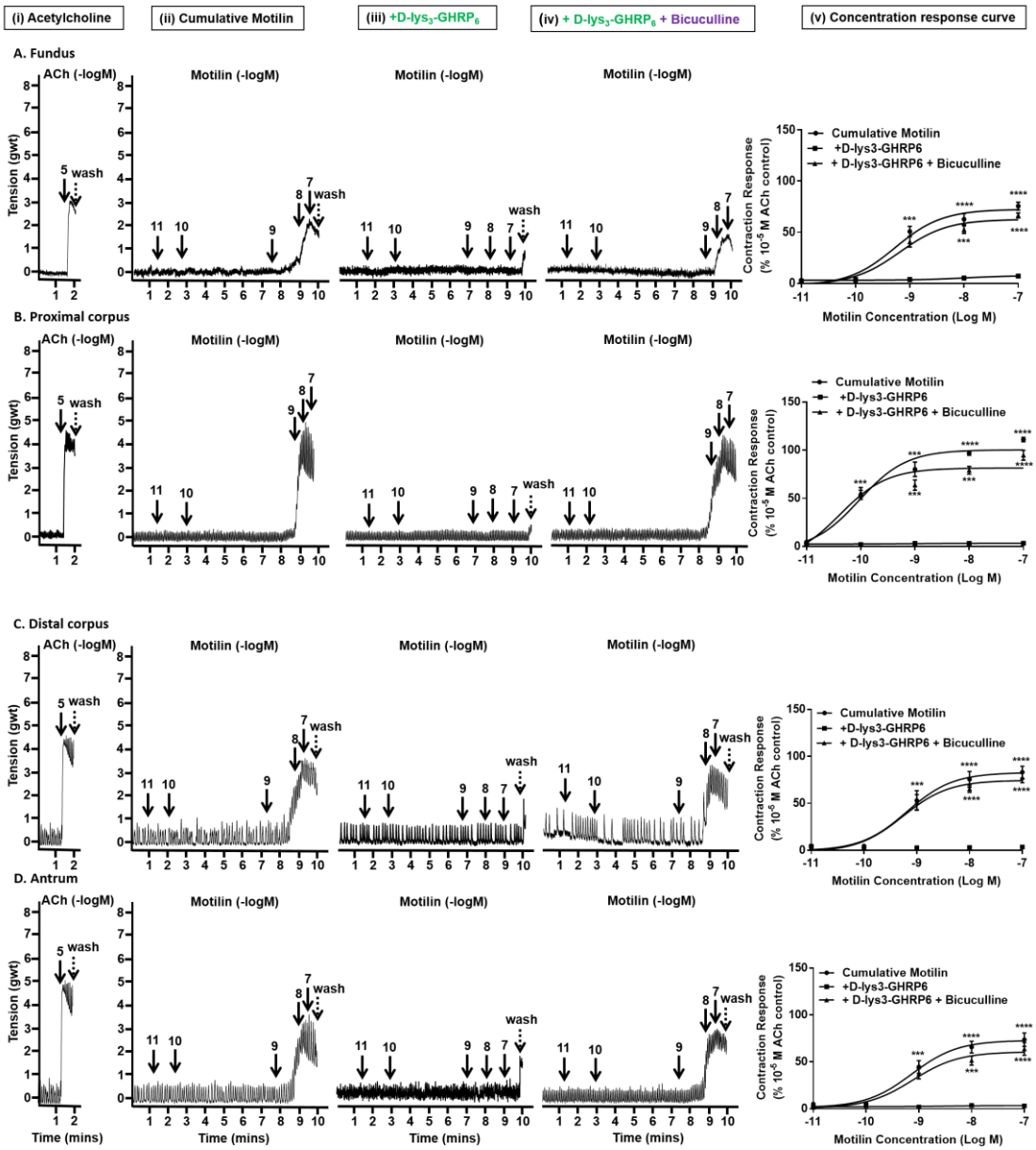


Fig 11. *In vitro* bicuculline pretreatment shows that the ghrelin-mediated GABAergic pathway is involved in motilin-induced gastric contractions

ACh treatment of all the stomach segments (A–D [i]). Treatment with 10^{-11}–10^{-7} M motilin induced gastric contractions in a dose-dependent manner (A–D [ii]). Pretreatment with the ghrelin antagonist D-Lys3-GHRP6 (10^{-5} M) completely inhibited motilin-

induced contractions in all the segments of the suncus stomach (A–D [iii]), which were completely reversed by pretreatment with 10^{-5} M bicuculline, a $GABA_A$ antagonist (A–D [iv]). Concentration-response curve corresponding to percentage maximum contractions induced by 10^{-5} M ACh is shown for all the stomach segments (A–D [v]). Arrow represents the timing of reagent administration, and dotted arrow represents the timing of tissue washing. The number indicates drug concentration (-logM). Each value is expressed as mean ± SEM (n = 4). Contractions in the different segments of the suncus stomach were compared using two-way ANOVA followed by Tukey's post hoc test. ***$p < 0.001$ and ****$p < 0.0001$ indicate significant difference among groups.

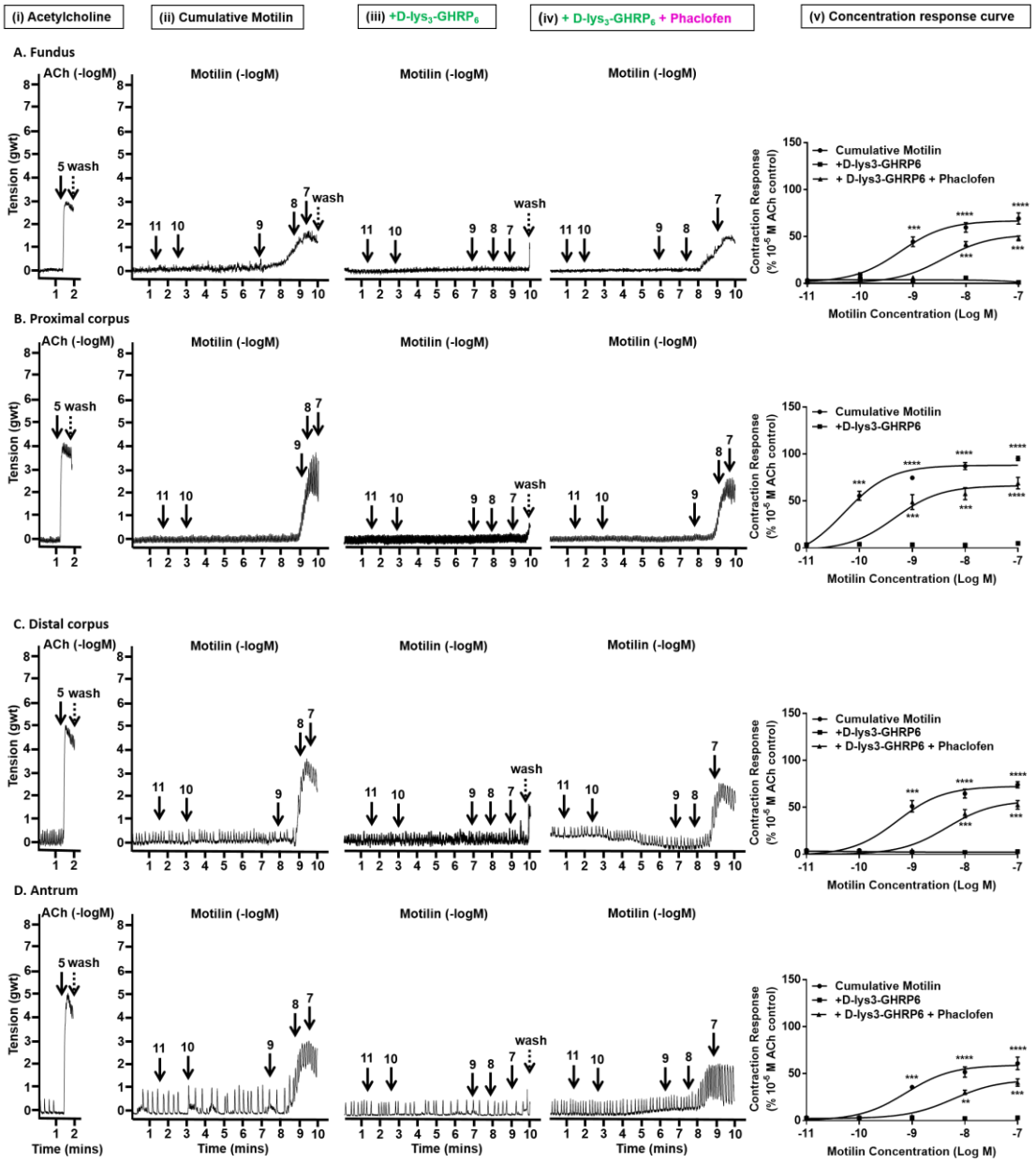


Fig 12. *In vitro* phaclofen pretreatment shows that the ghrelin-mediated GABAergic pathway is involved in motilin-induced gastric contractions

Concentration-response curve corresponding to percentage maximum contractions induced by 10^{-5} M ACh was constructed for all the stomach segments. Treatment with 10^{-11}–10^{-7} M motilin induced contractions in the (A) fundus, (B) proximal corpus, (C) distal corpus, and (D) antrum in a dose-dependent manner. Pretreatment with the ghrelin

antagonist D-Lys3-GHRP6 (10^{-5} M) completely inhibited motilin-induced contractions in all the stomach segments, which were partially reversed by pretreatment with 10^{-5} M phaclofen, a $GABA_B$ antagonist (A–D). Each value is expressed as mean ± SEM (n = 4). Contractions in the different segments of the suncus stomach were compared using two-way ANOVA followed by Tukey's post hoc test. **p < 0.01, ***p < 0.001, and ****p < 0.0001 indicate significant difference among groups.

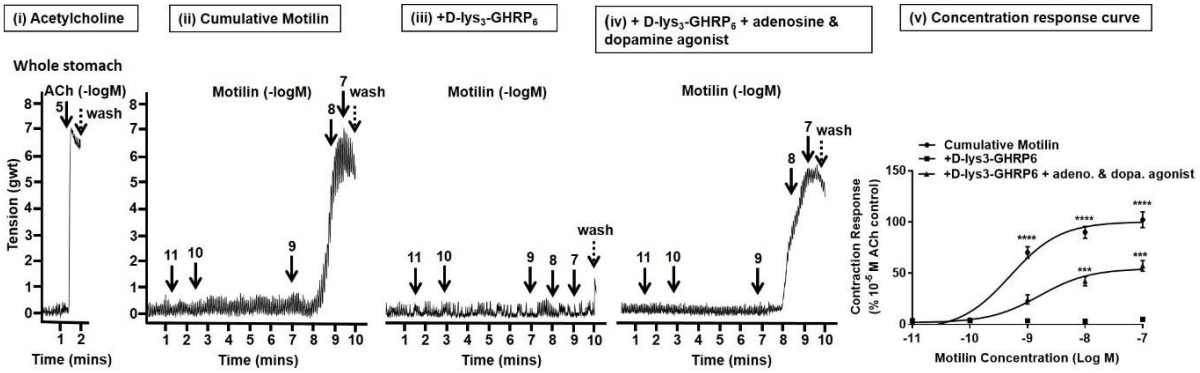


Fig 13. Adenosine and dopamine promote motilin-induced contractions in the whole stomach through the ghrelin-mediated GABAergic pathway

ACh response (i). Pretreatment with 10^{-8} M adenosine, the adenosine receptor agonist, and 10^{-5} M 6-hydroxydopamine hydrochloride, the dopamine receptor agonist, partially reversed 10^{-5} M ghrelin antagonist D-lys3-GHRP6-induced inhibition (iii) of motilin-induced gastric contractions (ii, iv, v). Results are presented as a concentration-response curve corresponding to percentage maximum contractions induced by 10^{-5} M ACh (v). Arrow represents the timing of reagent administration, and dotted arrow represents the timing of tissue washing. The number indicates drug concentration (-logM). Each value is expressed as mean ± SEM (n = 4). Contractions in the whole stomach were compared using two-way ANOVA followed by Tukey's post hoc test. ***$p < 0.001$ and ****$p < 0.0001$ indicate significant difference among groups.

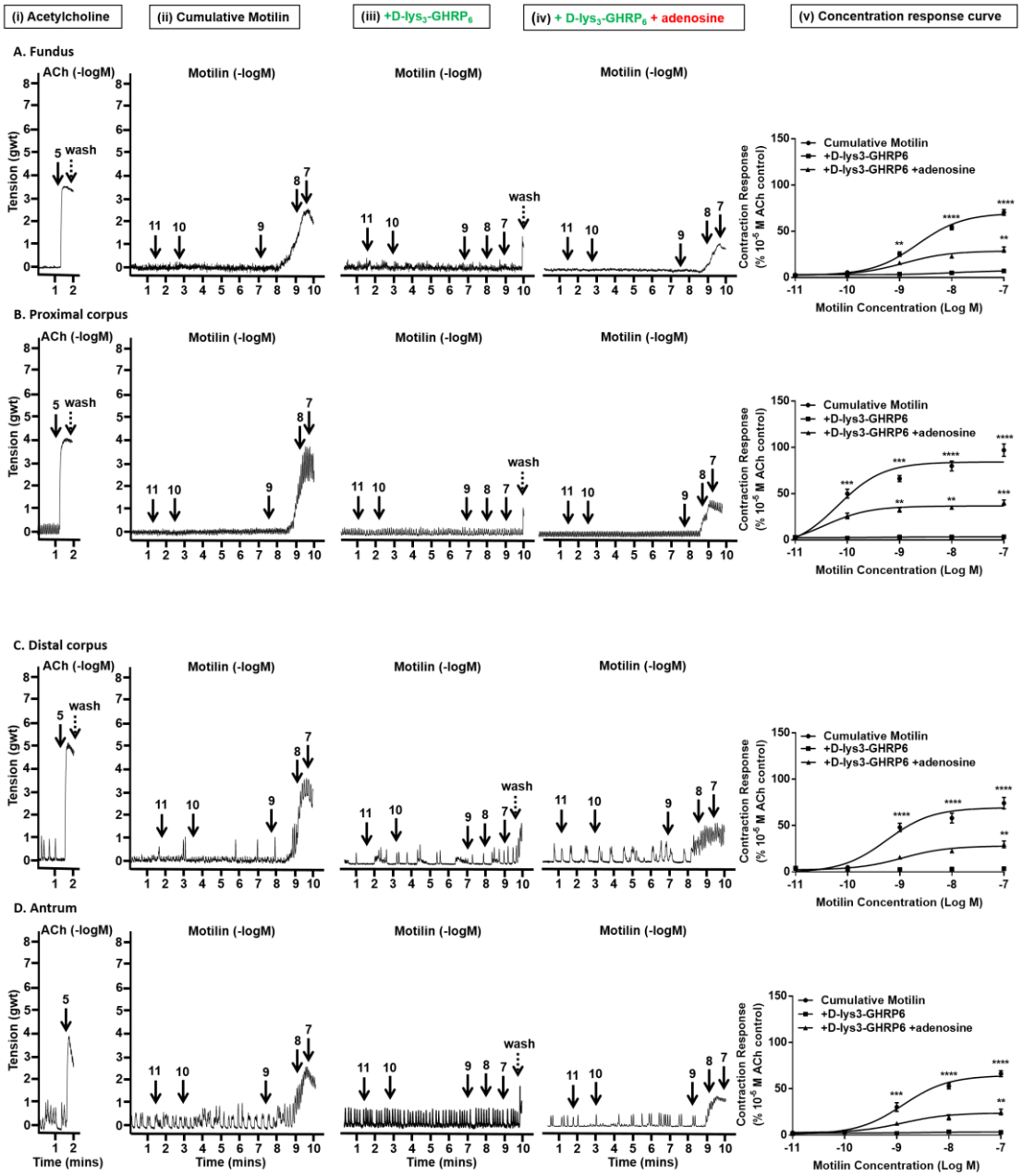


Fig 14. Adenosine promotes motilin-induced gastric contractions through the ghrelin-mediated GABAergic pathway

Concentration-response curve corresponding to percentage maximum contraction induced by 10^{-5} M ACh in all the stomach segments. Treatment with 10^{-11}–10^{-7} M motilin induced contractions in the (A) fundus, (B) proximal corpus, (C) distal corpus, and (D)

antrum in a dose-dependent manner. Pretreatment with the ghrelin antagonist D-Lys3-GHRP6 (10^{-5} M) completely inhibited motilin-induced gastric contractions, which were partially reversed by pretreatment with adenosine (10^{-8} M). Each value is expressed as mean ± SEM (n = 4). Contractions in the different segments of the suncus stomach were compared using two-way ANOVA followed by Tukey's post hoc test. **p < 0.01, ***p < 0.001, and ****p < 0.0001 indicate significant difference among groups.

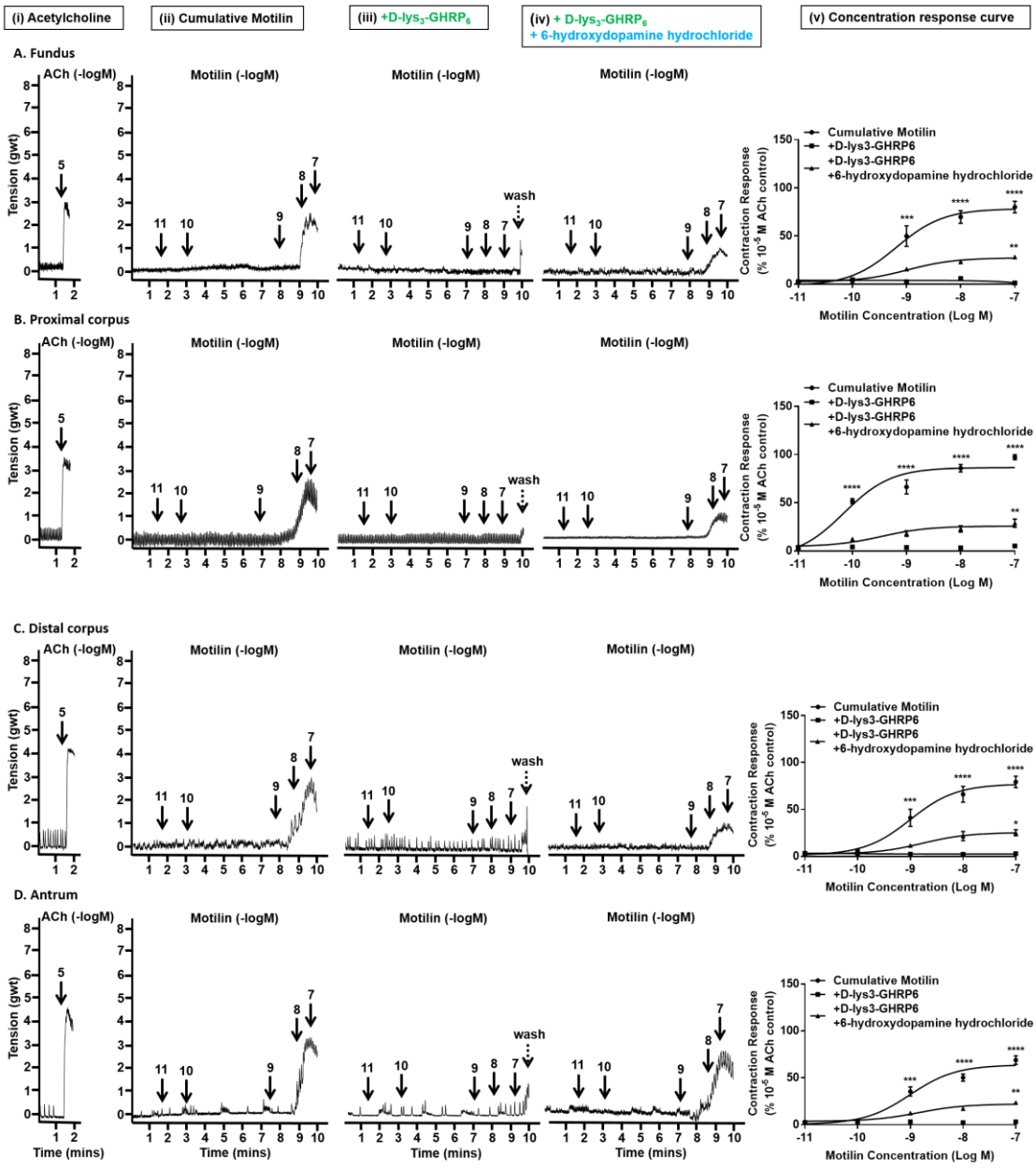


Fig 15. Dopamine promotes motilin-induced gastric contractions through the ghrelin-mediated GABAergic pathway

Concentration-response curve corresponding to percentage maximum contraction induced by 10^{-5} M ACh in all the stomach segments. Treatment with 10^{-11}–10^{-7} M motilin induced contractions in the (A) fundus, (B) proximal corpus, (C) distal corpus, and (D)

antrum in a dose-dependent manner. Pretreatment with the ghrelin antagonist D-Lys3-GHRP6 (10^{-5} M) completely inhibited motilin-induced gastric contractions, which were partially reversed by pretreatment with dopamine (10^{-6} M). Each value is expressed as mean ± SEM (n = 4). Contractions in the different segments of the suncus stomach were compared using two-way ANOVA followed by Tukey's post hoc test. **p < 0.01, ***p < 0.001, and ****p < 0.0001 indicate significant difference among groups.

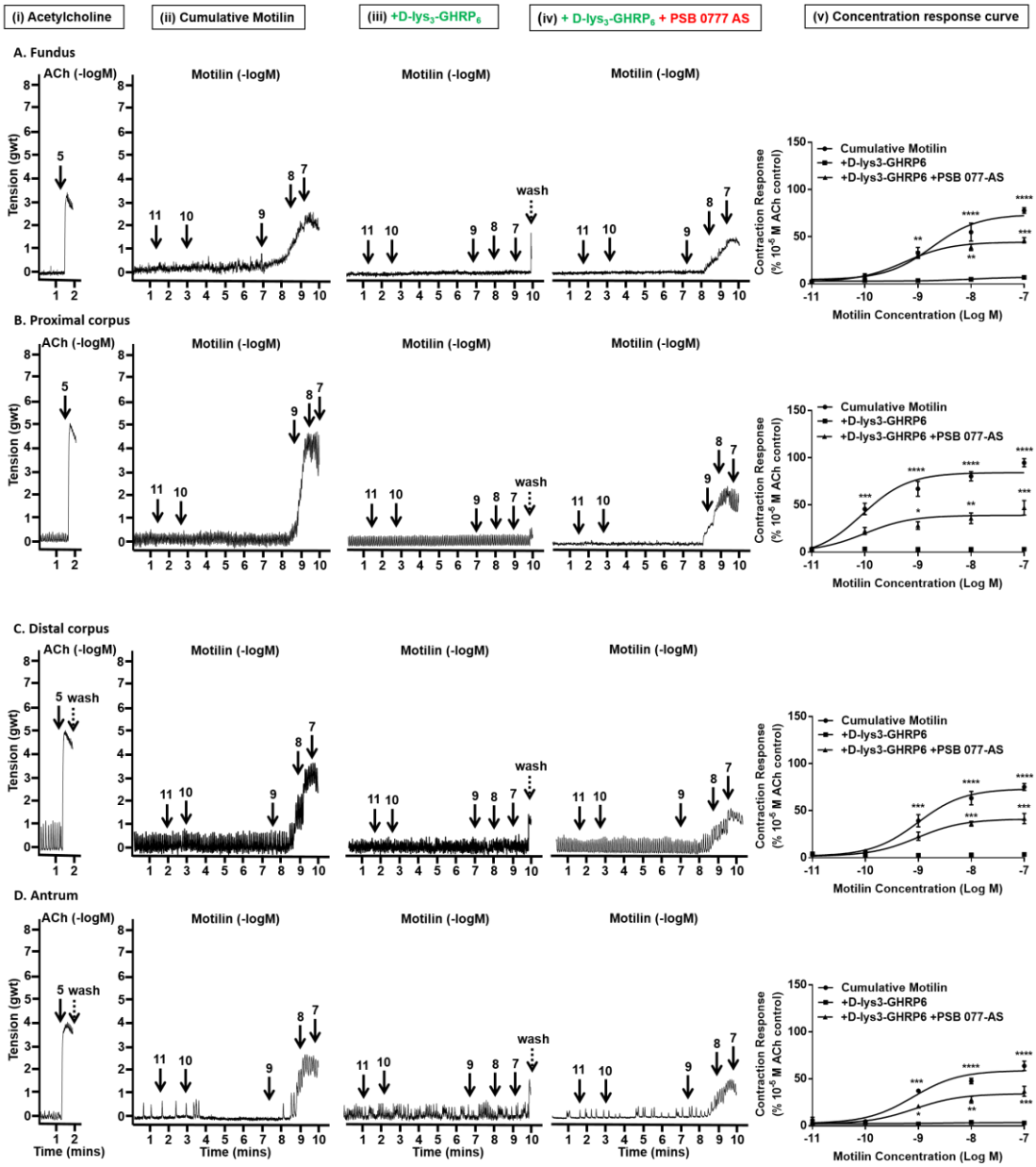


Fig 16 Adenosine A_{2A} receptor promotes motilin-induced gastric contractions through the ghrelin-mediated GABAergic pathway

Concentration-response curve corresponding to percentage maximum contraction induced by 10^{-5} M ACh in all the stomach segments. Treatment with 10^{-11}–10^{-7} M motilin induced contractions in the (A) fundus, (B) proximal corpus, (C) distal corpus, and (D) antrum in a dose-dependent manner. Pretreatment with the ghrelin antagonist D-Lys3-

GHRP6 (10^{-5} M) completely inhibited motilin-induced gastric contractions, which were reversed by pretreatment with the adenosine A_{2A} receptor agonist PSB 0777-AS (10^{-6} M). Each value is expressed as mean ± SEM (n = 4). Contractions in the different segments of the suncus stomach were compared using two-way ANOVA followed by Tukey's post hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ indicate significant difference among groups.

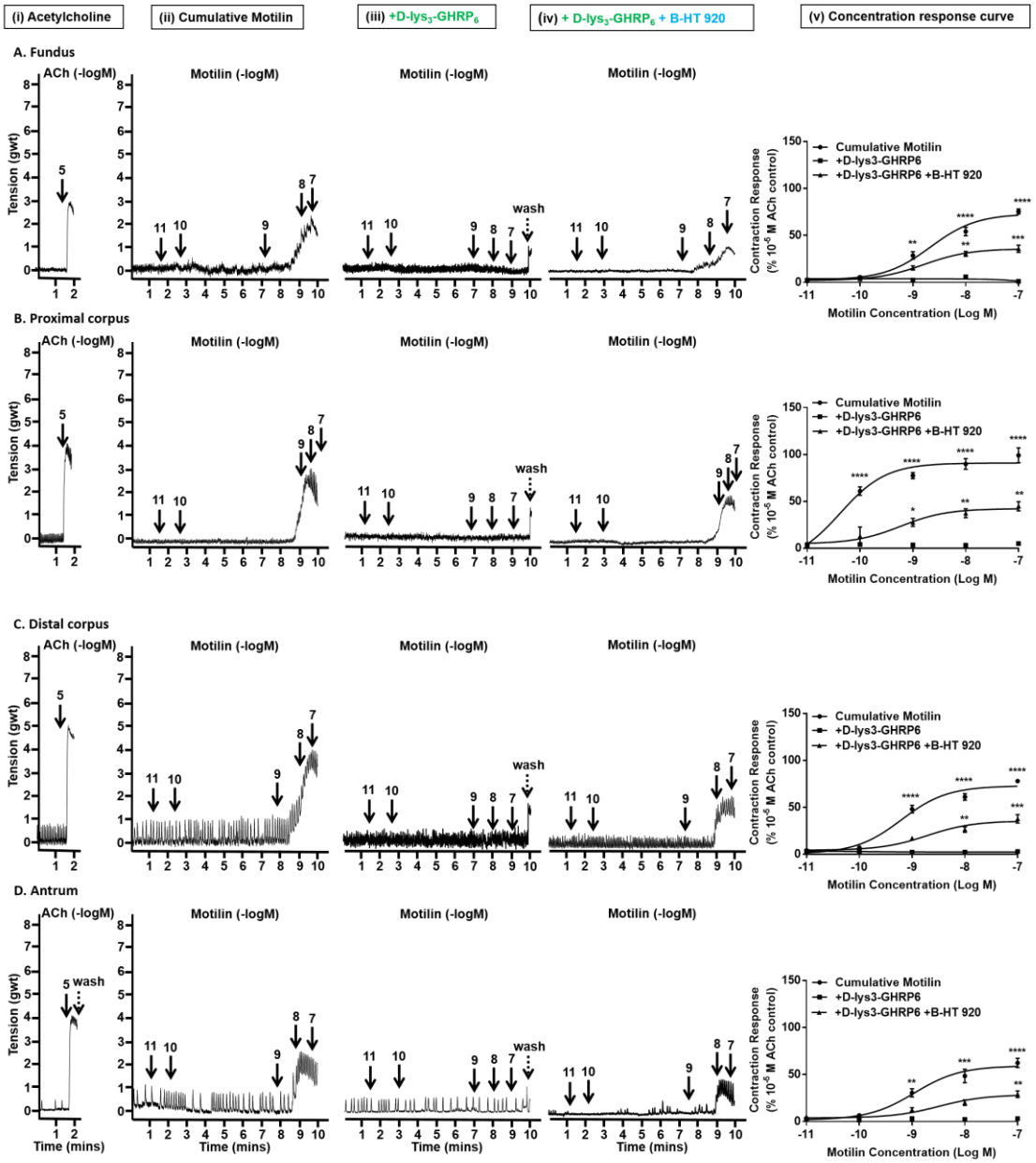


Fig 17. Dopamine D_2 receptor promotes motilin-induced gastric contractions through the ghrelin-mediated GABAergic pathway

Concentration-response curve corresponding to percentage maximum contraction induced by 10^{-5} M ACh in all the stomach segments. Treatment with 10^{-11}–10^{-7} M motilin induced contractions in the (A) fundus, (B) proximal corpus, (C) distal corpus, and (D) antrum in a dose-dependent manner. Pretreatment with the ghrelin antagonist D-Lys3-

GHRP6 (10^{-5} M) completely inhibited motilin-induced gastric contractions, which were reversed by pretreatment with the dopamine D_2 receptor agonist B-HT 920 (10^{-6} M). Each value is expressed as mean ± SEM (n = 4). Contractions in the different segments of the suncus stomach were compared using two-way ANOVA followed by Tukey's post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 indicate significant difference among groups.

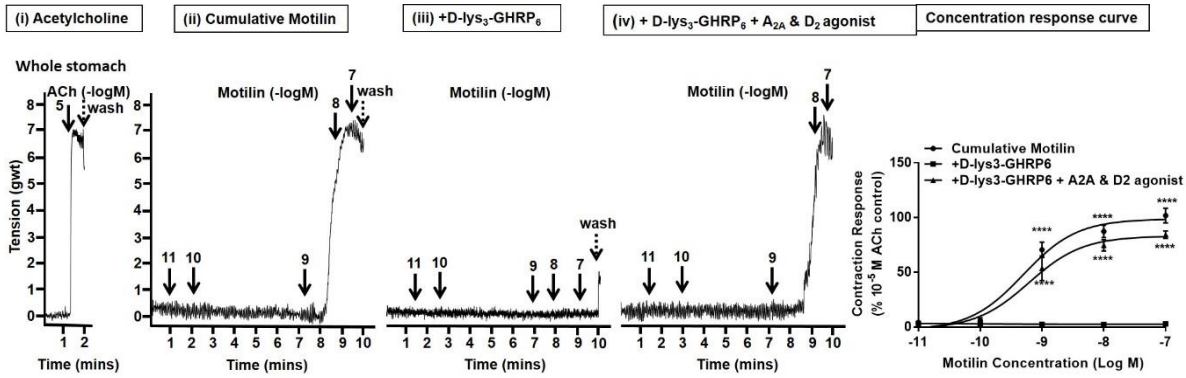


Fig 18. Both adenosine A_{2A} receptor and dopamine D_2 receptor promote motilin-induced contractions in the suncus stomach *in vitro* through the ghrelin-mediated GABAergic pathway

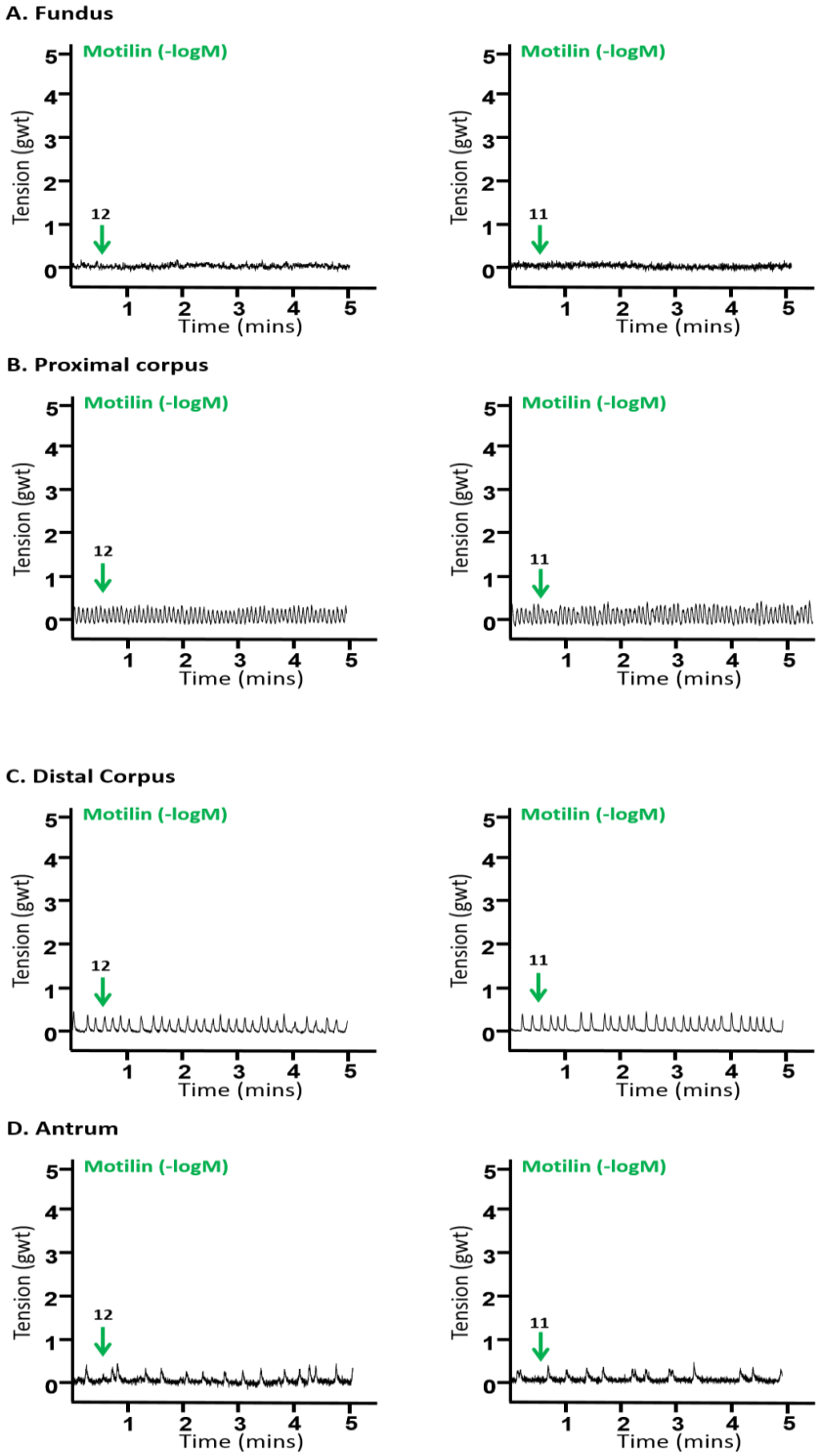
ACh response (i). Pretreatment with both PSB 0777-AS (10^{-6} M) and B-HT 920 (10^{-6} M) recovered motilin-induced gastric contractions (ii, iv, and v) abolished by treatment with the ghrelin antagonist D-lys3-GHRP6 (10^{-5} M) (iii). Results are represented as concentration-response curve corresponding to percentage maximum contractions induced by 10^{-5} M ACh (v). Arrow represents the timing of reagent administration, and dotted arrow represents the timing of tissue washing. The number indicates drug concentration (-logM). Each value is expressed as mean ± SEM (n = 4). Contractions in the whole stomach were compared using two-way ANOVA followed by Tukey's post hoc test. ****$p < 0.0001$ indicates significant difference among groups.

Table-1

Segments of stomach	EC_{50} (nmol L^{-1}) ± SE
Fundus	15.9 ± 9.2
Proximal corpus	0.9 ± 0.4
Distal corpus	9.6 ± 4.3
Antrum	314.7 ± 140.7

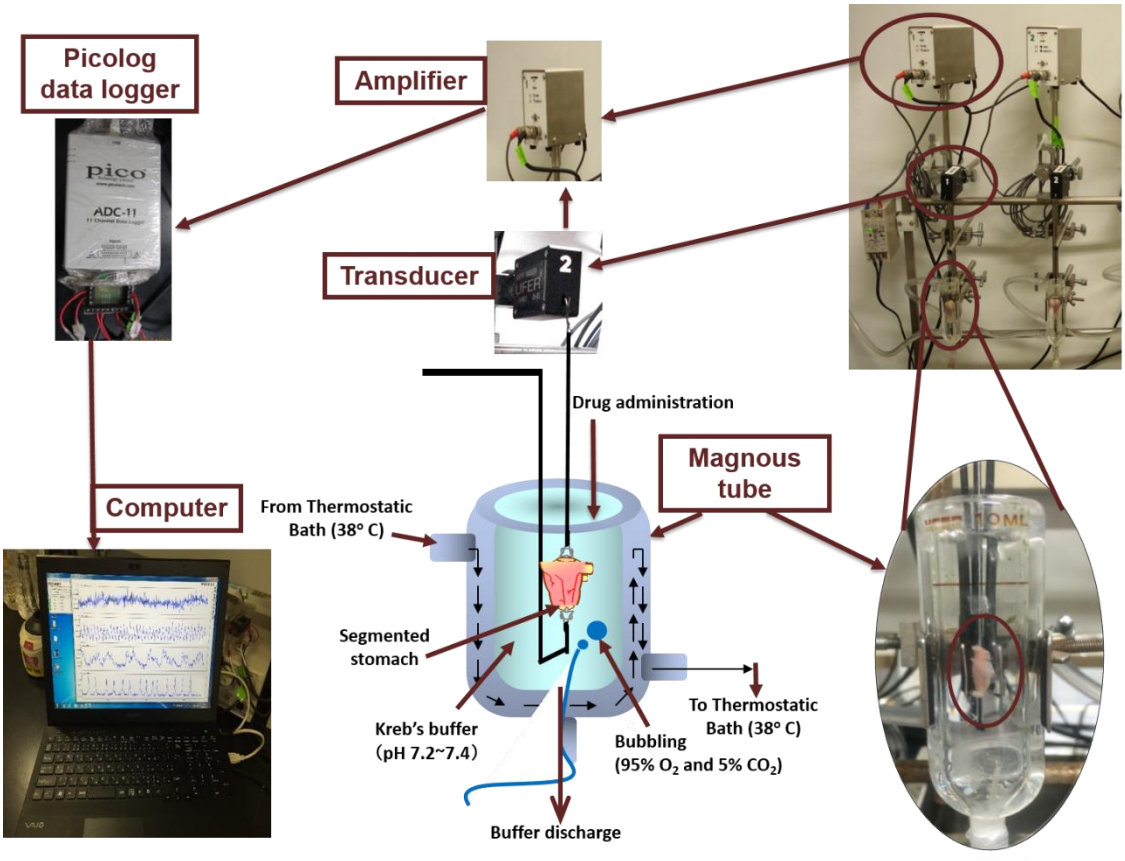
Table-1 Comparison of contractile activity in the different stomach segments based on EC_{50} values of concentration-response curves of motilin.

Appendix-I



Appendix-1 Effect of low-dose motilin on the different segments of the suncus stomach.

Appendix-II



Appendix-2 Organ bath system for monitoring GI contractions in *S. murinus*.

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