Regulatory mechanism of motilin-induced gastrointestinal motility in Japanese quail

(ウズラにおけるモチリン誘導性消化管運動調節機構の研究)

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ABSTRACT

Motilin is a peptide hormone that plays an important role in the regulation of gastrointestinal (GI) motility. In the current study, we determined the cDNA and amino acid sequences of motilin and its receptor (GPR38) in Japanese quail, and studied the distribution of motilin-producing cells and the expression of GPR38 mRNA in the quail GI tract. Using an *in vitro* organ bath, we examined the motilin-induced contractile properties of different regions of the quail GI tract. We also determined the age-dependent changes and mechanisms of motilin-induced contraction in the proventriculus and duodenum of the quail. Mature quail motilin was composed of 22 amino acids, and showed high homology to chicken (95.4%), human (72.7%), and dog (72.7%) motilin. Immunohistochemical analysis revealed that motilin-immunopositive cells were present in the mucosal layer of the duodenum (23.4 ± 4.6 cells/mm^2), jejunum (15.2 ± 0.8 cells/mm^2), and ileum (2.5 ± 0.7 cells/mm^2), but were not observed in the crop, proventriculus, and colon. We also cloned quail GPR38-encoding cDNA from the medulla oblongata and obtained a partial sequence of 183 bp. Real-time PCR analysis showed that quail GPR38 mRNA was expressed in the crop, proventriculus, duodenum, jejunum, ileum, colon, liver, heart, and muscle. In the organ bath study, chicken motilin evoked dose-dependent contraction of the proventriculus, duodenum, jejunum, and ileum. In contrast, chicken ghrelin had no effect on contraction in the quail GI tract. Motilin-induced contraction in the duodenum was not inhibited by atropine, hexamethonium, ritanserin, ondansetron, GR125487, or tetrodotoxin. However, motilin-induced contractions in the proventriculus were significantly inhibited by atropine and tetrodotoxin. In addition, motilin-induced contraction was significantly decreased with age in the proventriculus, but not

in the duodenum. These results suggest that motilin stimulates gastrointestinal motility, and that the site of action for motilin is different between the small intestine and proventriculus of Japanese quail.

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Chapter 1. General introduction and objectives

1.1. Research background

1.1.1. The migrating motor complex of gastrointestinal (GI) motility

The contractile activity of the upper gastrointestinal tract differs between interdigestive and postprandial states in humans and dogs (Itoh, 1997). Rhythmic contractions in the postprandial state facilitate the mixing of food in the stomach and the subsequent migration of the food into the intestines (Houghton *et al.*, 1988). In contrast, gastrointestinal motility in the interdigestive state occurs in a regular cycle, called the migrating motor complex (MMC) (Takahashi, 2013). There are three phases of the MMC cycle in mammalian species; Phase I is the quiescent period with virtually no contractions, Phase II includes intermittent, irregular, low-amplitude contractions, and Phase III is a short burst of regular high-amplitude contractions (Vantrappen *et al.*, 1977). Phase III contractions at the interdigestive state occur every 90–120 minutes in humans, dogs, and shrews (Itoh *et al.*, 1976; Vantrappen *et al.*, 1979; Sakahara *et al.*, 2010). The physiological role of the MMC is to prepare the stomach to receive the next meal through mechanical and chemical cleansing (Vantrappen *et al.*, 1977). Motilin and ghrelin hormones are secreted in the GI tract during the interdigestive state and are known to be involved in the regulation of the MMC cycle (Itoh *et al.*, 1976; Tack *et al.*, 2006). The mechanism of MMC regulation is complex. It has been reported that phase II of the MMC is regulated by the ghrelin-mediated vagal pathway, whereas phase III is regulated via both motilin and ghrelin, independent of the vagal pathway in *Suncus murinus* (Miyano *et al.*, 2013). Subsequently, the periodic pattern of interdigestive gastric contractions changes to

irregular and continuous motor activity after the ingestion of food (Itoh *et al.*, 1976). A previous report has demonstrated that the effect of motilin on gastric phase III-like contractions is strongly inhibited by food ingestion (Itoh *et al.*, 1976). Moreover, exogenous administration of a high dose of motilin was shown to elicit a strong contraction in the fasted state but failed to induce contraction in the postprandial state (Kuroda *et al.*, 2015). The inhibition of motilin-evoked contraction in the postprandial state is mediated through the vagal pathway (Miyano *et al.*, 2013). It has also been reported that vagus nerve plays an important role in the initiation and maintenance of postprandial contractions (Miyano *et al.*, 2013).

1.1.2. Motilin

Motilin was first isolated from the mucosa of the porcine small intestine (Brown *et al.*, 1971) and the molecular structure of mature motilin was determined to be a 22-amino acid (aa) polypeptide with a molecular weight of 2698 Da (Brown *et al.*, 1973; Schubert & Brown, 1974). Motilin-immunoreactive cells were predominantly found in the upper small intestine of humans (Bloom *et al.*, 1976), pigs (Kishimoto *et al.*, 1981), dogs (Polak *et al.*, 1975), monkeys (Pearse *et al.*, 1974), and shrews (Tsutsui *et al.*, 2009). In humans, the motilin gene was mapped to the p21.2-21.3 region of chromosome 6, is composed of five exons, and spans approximately 9 kb (Yano *et al.*, 1989). The 22-aa motilin peptide was found to be encoded by exons 2 and 3 of the gene (Daikh *et al.*, 1989; Yano *et al.*, 1989). The biological response to motilin is elicited through binding to its specific receptor (GPR38) in the GI tract (Miller *et al.*, 2000b).

During the interdigestive state, motilin is periodically released at ~100-min intervals in humans and in dogs (Itoh *et al.*, 1978; Janssens *et al.*, 1983) and the fluctuation in plasma motilin levels coincides with the cyclical interdigestive contractions of the stomach (Itoh *et al.*, 1978). Plasma motilin levels reach their peak during the period of gastric phase III in humans (Vantrappen *et al.*, 1979; Janssens *et al.*, 1983) and dogs (Itoh *et al.*, 1976; Hall *et al.*, 1983), and after ingestion of a meal, this cyclic release of motilin ceases (Ohno *et al.*, 2010). Hence, endogenous motilin has a close association with phase III contractions (Zietlow *et al.*, 2010). Moreover, exogenous administration of motilin elicited gastric phase III-like contractions in humans, dogs, and shrews during the interdigestive state (Itoh *et al.*, 1976; Wingate *et al.*, 1976; Janssens *et al.*, 1983; Kuroda *et al.*, 2015). *In vitro* studies also demonstrated dose-dependent gastric contractions after motilin administration in monogastric animals (Strunz *et al.*, 1975; Kitazawa *et al.*, 1994; Mondal *et al.*, 2011; Broad *et al.*, 2016). In addition, plasma motilin concentrations decline with food ingestion and this lower concentration is sustained as the gastric motor activity is in the digestive pattern (Itoh *et al.*, 1978). Moreover, exogenous motilin administration did not stimulate gastric contractions in the postprandial state in *Suncus murinus* (Kuroda *et al.*, 2015). Therefore, motilin is considered an important endocrine regulator for the stimulation of interdigestive gastrointestinal motility.

1.1.3 Ghrelin

Ghrelin, a 28 aa peptide hormone, was first isolated from the stomach in 1999 as the endogenous ligand for the growth hormone secretagogue receptor (GHS-R) (Kojima *et al.*, 1999). The octanoylation of the third serine residue is essential for ligand-receptor binding (to GHS-R) (Kojima *et al.*, 1999). Ghrelin is mainly produced in the oxyntic mucosa of the

stomach (Ariyasu *et al.*, 2001). In humans, the ghrelin-encoding gene was mapped to the short arm of chromosome 3 (3p25-26) and contains six exons (two are noncoding) and four introns (Kanamoto *et al.*, 2004; Nakai *et al.*, 2004; Seim *et al.*, 2007). Preproghrelin is composed of 117 aa, and contains a 23-aa signal peptide; the remaining 94-aa segment corresponds to proghrelin (Korbonits *et al.*, 2004). Proghrelin consists of the 28-aa ghrelin peptide and a 66-aa carboxyterminal peptide called C-ghrelin (Pemberton *et al.*, 2003; Bang *et al.*, 2007; Seim *et al.*, 2007). C-ghrelin is then processed to a 23-aa peptide called obestatin (Zhang *et al.*, 2005). The physiological functions of ghrelin include regulation of growth hormone (GH) secretion (Kojima *et al.*, 1999; Seoane *et al.*, 2000; Tolle *et al.*, 2001), food intake (Wren *et al.*, 2000; Nakazato *et al.*, 2001), energy metabolism (Tschop *et al.*, 2000; Perez-Tilve *et al.*, 2011), gastrointestinal motility (Zheng *et al.*, 2009; Nakamura *et al.*, 2010; Perboni & Inui, 2010), gastric acid secretion (Masuda *et al.*, 2000; Fukumoto *et al.*, 2008; Yakabi *et al.*, 2008), cardiovascular function (Okumura *et al.*, 2002), and cell proliferation (Duxbury *et al.*, 2003; Maccarinelli *et al.*, 2005). In rodents, the MMC pattern is completely different than that of mammals (Fujino *et al.*, 2003; Tatewaki *et al.*, 2003; Ariga *et al.*, 2007). Phase III-like contractions of gastric motility and peak plasma ghrelin levels were found to be closely related in rats (Ariga *et al.*, 2007). Exogenous administration of ghrelin stimulated gastric contractions in rats and mice (Masuda *et al.*, 2000; Zheng *et al.*, 2009). Among mammals, plasma ghrelin levels do not significantly fluctuate with the MMC in humans (Deloose *et al.*, 2015), whereas plasma ghrelin concentrations fluctuate in dogs and reach their peak during gastric phase I of the MMC (Ohno *et al.*, 2006; Zietlow *et al.*, 2010; Ogawa *et al.*, 2012). However, exogenous administration of ghrelin does not stimulate GI motility in conscious dogs (Ohno *et al.*, 2006).

1.1.4. Co-ordinated action of motilin and ghrelin in the GI tract

Motilin and ghrelin are released by the gut during the interdigestive state and are regarded as peptides of the same family (Tomasetto *et al.*, 2000). Ghrelin and motilin ligands share approximately 27% amino acid identity (Kojima *et al.*, 1999). Specific receptors for motilin and ghrelin are GPR38 (Feighner *et al.*, 1999) and growth hormone secretagogue receptor (GHS-R) (Howard *et al.*, 1996), respectively. Both receptors belong to the class A rhodopsin-like G protein-coupled seven-transmembrane receptor family and display marked sequence homology, with the 52% overall identity in humans (McKee *et al.*, 1997; Takeshita *et al.*, 2006), and are 86% identical in their transmembrane regions (McKee *et al.*, 1997). Moreover, these peptides have shown common gastroprokinetic effects on human and dog GI tracts (Itoh, 1997). In the presence of a low dose of ghrelin, motilin was shown to induce phase III-like gastric contractions, both in interdigestive and postprandial states, in *Suncus murinus* (Kuroda *et al.*, 2015). Moreover, *in vitro* studies showed that in the presence of a low dose of either motilin or ghrelin, both peptides significantly stimulated gastric contractions (Mondal *et al.*, 2012), and pre-treatment with a ghrelin antagonist (d-Lys3-GHRP6) completely suppressed motilin-induced gastric contraction *in vivo* and *in vitro* (Kuroda *et al.*, 2015).

1.1.5. Regulatory mechanism of motilin-induced GI contraction

The regulatory mechanism of motilin-induced contraction varies among organs and species. However, the absence of a functional motilin receptor in the rodent GI tract has hampered studies on the underlying mechanism of motilin-mediated actions (Aerssens, 2004). *In vitro* studies demonstrated the neurally mediated effects of motilin-induced contraction in strips from the rabbit antrum (Van Assche *et al.*, 1997) and chicken proventriculus (Kitazawa *et al.*,

1995). In the rabbit antrum, electrical field stimulation studies showed that motilin enhanced contractions through cholinergic neurotransmission at low doses, whereas motilin interacted directly with antral smooth muscle receptors at high doses (Van Assche *et al.*, 1997). Similar concentration-dependent effects on cholinergic motor nerves or smooth muscle by a motilin receptor agonist was reported in the human gastric antrum (Broad *et al.*, 2016). In contrast, the contractile effects of motilin in human, rabbit, cat, dog, and chicken small intestines were mediated through direct action on smooth muscle cells (Strunz *et al.*, 1975; Adachi *et al.*, 1981; Poitras *et al.*, 1987; Depoortere *et al.*, 1990; Depoortere *et al.*, 1993; Kitazawa *et al.*, 1994; Boivin *et al.*, 1997).

1.1.6. Japanese quail *(Coturnix japonica)*

Japanese quail, a domesticated form of the common quail, belongs to the order Galliformes, the family Phasianidae, and the genus *Coturnix* (Vali, 2008). The earliest records of domestication of the Japanese quail were from the 12th century in Japan, though some evidence showed that this species was originally domesticated in the 11th century (Kayang *et al.*, 2004). Due to the close relationship and phenotypic similarities, the Japanese quail is often crossed with the common quail to create hybrids for restocking declining wild quail populations (Barilani *et al*., 2005; Puigcerver *et al*., 2007). The relatively short life span, early sexual maturity, small size, and ease of handing, combined with its physiological similarity to humans have made this bird an ideal model for studies on immunology, endocrinology, reproductive biology, and circadian control of brain function (Follett *et al.*, 1992; Holmes & Ottinger, 2003; Ottinger *et al.*, 2004).

Most motilin-related GI studies have been conducted using dogs, rabbits, and humans due to the absence of motilin and GPR38 in commonly used laboratory animals such as rats and mice (He *et al.*, 2010). Recently, *Suncus murinus* has been used and is considered a suitable model animal for studies on motilin (Tsutsui *et al.*, 2009). In contrast, motilin-related GI studies in avian species are infrequent compared to those using mammals. In avian species, it was reported that the frequency of MMCs in chickens, quails, owls, and *Strix* spp. are similar to that of mammals (Clench *et al.*, 1989). Previously, motilin and GPR38 have been identified in chicken, and *in vitro* studies also showed that motilin induced dose-dependent contractions in different regions of the chicken GI tract (Kitazawa *et al.*, 1995; De Clercq *et al.*, 1996; Kitazawa *et al.*, 1997) suggesting that motilin has a critical role in the regulation of GI motility in avian species. In addition, chicken ghrelin evoked contraction in a region-specific manner in isolated chicken GI tracts, which was mediated by neural and/or muscular GHS-R (Kitazawa *et al.*, 2007). Recently, ghrelin and GHS-R were identified in Japanese quail, and were shown to share 88% and 98% identity to chicken ghrelin and ghrelin receptor, respectively (Kitazawa *et al.*, 2009). However, ghrelin-mediated contraction was different in quail and chicken GI tracts (Kitazawa *et al.*, 2009), suggesting species-specific effects of ghrelin on the regulation of gastrointestinal motility. However, the motilin response in the GI tract of Japanese quail is still unknown.

1.2. Purpose of the study

The aim of the present study was to determine the nucleotide sequence of motilin- and GPR38-encoding genes, and to assess the motilin distribution and GPR38 mRNA expression profiles throughout the GI tract. In addition, this study was performed to determine the effect of motilin and its control mechanism on the GI tract of Japanese quail.

Chapter 2. Molecular cloning of motilin and mechanism of motilin-induced gastrointestinal motility in Japanese quail

2.1. Introduction

Motilin, a 22-aa polypeptide that stimulates gastrointestinal (GI) motility (Poitras *et al.*, 1994), was originally purified from pigs in the 1970s (Brown *et al.*, 1972, 1973; Schubert & Brown, 1974). Motilin mRNA and amino acid sequences have been identified mainly in mammals including humans, cows, dogs, rabbits, and shrews (Banfield *et al.*, 1992; Huang *et al.*, 1999; Strausberg *et al.*, 2002; Tsutsui *et al.*, 2009). Structural analysis demonstrated that the N-terminal amino acid of motilin is important for its biological activity (Poitras *et al.*, 1992). Motilin is produced in the upper small intestine and motilin-producing cells are localized to the crypts and villi of the mucosal layer, but are not present in the muscular layer (Tsutsui *et al.*, 2009). In mammals, motilin-producing cells exist as two types, open-type cells in the villi and closed-type cells in the crypts (Satoh *et al.*, 1995). Several studies have reported that the specific receptor for motilin, GPR38, is expressed in the brain, pituitary gland, lung, stomach, and small intestine (Takeshita *et al.*, 2006; Ohshiro *et al.*, 2008; Ter Beek *et al.*, 2008; Yamamoto *et al.*, 2008; Suzuki *et al.*, 2012), indicating that motilin has many physiological functions related to GI motility. Moreover, ghrelin, a stomach derived peptide belongs to the motilin family of peptides, and GPR38 and GHS-R are both highly conserved (Poitras & Peeters, 2008; Ohno *et al.*, 2010).

Patterns of GI motility differ between the fasted and postprandial states. Contractions observed during the fasted state are referred to as the MMC, which consists of phase I (motor quiescent period), phase II (irregular and low amplitude contraction period), and phase III (regular and high amplitude contraction period) in humans, dogs, and shrews (Itoh *et al.*, 1976; Vantrappen *et al.*, 1977; Janssens *et al.*, 1983; Sakahara *et al.*, 2010). Gastric phase III of the MMC is closely associated with peak plasma motilin concentrations (Itoh *et al.*, 1976; Vantrappen *et al.*, 1979; Hall *et al.*, 1983; Janssens *et al.*, 1983). Intravenous administration of motilin was shown to elicit gastric phase III-like contractions (Itoh *et al.*, 1976; Wingate *et al.*, 1976; Janssens *et al.*, 1983; Kuroda *et al.*, 2015) *in vivo* and motilin was shown to induce dose-dependent gastric contractions in monogastric animals including humans, rabbits, and shrews *in vitro* (Strunz *et al.*, 1975; Kitazawa *et al.*, 1994; Mondal *et al.*, 2011; Broad *et al.*, 2016). Recent studies have shown that ghrelin is involved in phase II via the vagal afferent nerve, and that phase III contractions are regulated by the coordination of motilin and ghrelin (Mondal *et al.*, 2012; Miyano *et al.*, 2013). Moreover, motilin-induced gastric contractions are mediated via cholinergic, adrenergic, serotonergic, opioidergic, and nitric oxide (NO) neurons (Mondal *et al.*, 2011).

In avian species, it was reported that the frequency of chicken MMCs is 77–122 min and the duration of phase III is 5–8 min (Clench *et al.*, 1989), indicating that the MMC in chickens is similar to that in mammals. Moreover, *in vitro* studies have revealed that motilin induces contractions in a dose-dependent manner in the chicken GI tract through both neural and direct smooth muscle pathways (Kitazawa *et al.*, 1995; De Clercq *et al.*, 1996; Kitazawa *et al.*, 1997). However, studies on motilin and GI motility in avian species are lacking.

Therefore, we determined the cDNA and amino acid sequence of motilin in Japanese quail and assessed the distribution of motilin-producing cells. We also examined the effects of motilin on gastrointestinal contractions, and its regulatory mechanisms *in vitro.*

2.2. Materials and Methods

2.2.1. Drugs used

Chicken motilin (Peptide Institute Inc., Osaka, Japan), chicken ghrelin (provided by the Asubio Pharma Co., Ltd., Japan), synthesized motilin from *Suncus murinus* (Scrum, Tokyo, Japan), active human ghrelin (Asubio Pharma, Hyogo, Japan), acetylcholine chloride (ACh) (Sigma-Aldrich Co. LLC., USA), atropine sulfate (Merck, San Diego, CA, USA), hexamethonium chloride (Wako, Osaka, Japan), tetrodotoxin (TTX) (Wako), ondansetron (Hikari Pharmaceutical, Imado, Japan), GR125487, and ritanserin (Tocris Bioscience, Ellisville, USA) were all used in the present study. Ritanserin was dissolved in ethanol (final concentration, 0.01% v/v); GR125487 was dissolved in 0.9% NaCl, suncus motilin and human ghrelin were dissolved in 0.1% BSA/PBS and other drugs were dissolved in distilled water.

2.2.2. Animals

The experiments were performed using male Japanese quails weighing 70–90 g (at 5 weeks of age), purchased from a commercial source (Motoki Co. Ltd., Honcho, Tokorozawa city, Saitama, Japan). All procedures were performed in accordance with the guidelines of the Saitama University Committee on Animal Research.

2.2.3. Cloning of quail motilin cDNA

Total RNA was extracted from the duodenum using Isogen (NIPPON GENE Co. Ltd., Tokyo, Japan) based on the manufacturer's instructions. Trace DNA contamination was removed by DNase digestion (Promega, Madison, WI, USA) and cDNA was synthesized from 1 μg of DNase-treated total RNA using Thermoscript® Reverse Transcriptase (Invitrogen, CA, USA),

with Oligo-dT Anchor primer (#12577-011, Invitrogen, Carlsbad, CA, USA). PCR primers, used for cloning of quail motilin mRNA, were designed based on the predicted turkey motilin mRNA sequence (GenBank RefSeq record XM_010724334.1) with NCBI/Primer-BLAST. The following primers were designed to amplify quail motilin (fragment size, 335 bp): sense primer, 5′-CCGGTTTGCTCCTGGTGTA-3′, and antisense primer, 5′-CTGCTGGTATCAGTCAGCGT-3′. PCR amplifications were performed using AmpliTaq Gold (Roche Molecular Systems, NJ, USA). Amplification reactions were performed using a Thermal Cycler (Bio-Rad, Hercules, California, USA). Initial template denaturation was performed for 10 min at 95 °C, and the cycle profile was then programmed as follows: 30 sec at 94 °C (denaturation) and 1 min at 57 °C (annealing and extension), for 40 cycles, with a final extension for 10 min at 60 °C. Amplicon size and specificity were confirmed by 2% agarose gel electrophoresis. The PCR product was cloned into pGEM-T Easy vector (Promega, Madison, WI) and sequencing was performed by Eurofins Genomics K.K. (Tokyo, Japan).

2.2.4. Tissue preparation

Animals were sacrificed by anesthesia with sodium pentobarbital (100 mg/kg, i.p.), after which approximately 1.5 cm of crop, proventriculus duodenum, jejunum, ileum, and colon tissues were removed. Each part of the GI part was opened along its longitudinal axis in Bouin's-Hollande fixative solution and incubated for 16 h. The tissue blocks were dehydrated with a series of increasing ethanol concentrations and xylene, and embedded in Paraplast Plus (McCormick Scientific, St Louis, MO). Serial sections (10 μm thick) were produced using a microtome and mounted on slides coated with silane (ShinEtsu Chemicals, Tokyo, Japan).

2.2.4.1. Immunohistochemistry

Immunohistochemical detection of quail motilin cells using rabbit anti-porcine motilin serum was performed by the avidin-biotin-peroxidase complex (ABC) method. The sections were deparaffinized with xylene and rehydrated with decreasing ethanol concentrations. They were then treated with 0.5% sodium metaperiodate for 15 min at room temperature, to block endogenous peroxidase and washed with distilled water. The sections were further treated with 1% sodium thiosulfate for 10 min. After washing with distilled water, the sections were incubated with TNBS (0.4% Triton X-100 and 1% bovine serum albumin in PBS) for 2 h. Incubation was conducted for 16 h in a humidity chamber with anti-porcine motilin serum (Tsutsui *et al.*, 2009) diluted 1:8000 in TNBS. After washing with PBS, the sections were incubated for 30 min with biotin-conjugated anti-rabbit IgG serum (Vector, Burlingame, CA, USA), diluted 1:300 in TNBS, and again washed with PBS. Finally, the sections were incubated for 30 min with ABC solution (Vectastain ABC kit, Burlingame, CA, USA) prepared according to the manufacturer's instructions. After washing with PBS for 10 min, the sections were reacted with 0.02% 3,3′-diaminobenzidine tetrachloride (DAB) mixed with 0.006% hydrogen peroxide (H_2O_2) in 0.05 M Tris-HCl, pH 7.6, for 4–5 min to detect immunostaining. Sections were washed with PBS and Millipore water (Millipore, Tokyo, Japan), dehydrated with a graded ethanol series, cleared in xylene, and mounted with Entellan (Merck, Darmstadt, Germany). Thereafter, the sections were viewed under a light microscope (BX60, Olympus, Tokyo, Japan). All incubations were performed at room temperature in a humidity chamber. For the antigen adsorption test, anti-porcine motilin serum (1:8000 dilution) was incubated with chicken motilin (0, 5, 10, and 20 μg/ml) overnight at room

temperature. After centrifugation at 15000 rpm for 10 min, the supernatant was used for immunohistochemistry.

2.2.4.2. Morphometric analysis

Digital photographs were taken under a light microscope (BX60, Olympus, Japan) with a digital camera (DP70, Olympus, Japan), and the number of motilin-positive cells was counted in each section. Thereafter, the area of the mucosal layer was also measured in each section using the image analysis program, Image J (National Institutes of Health, Bethesda, MD). Motilin cell density was expressed as the number of immunopositive mucosal cells per unit area (cells/mm^2).

2.2.5. Organ bath study

Quails were anesthetized with pentobarbital sodium (100 mg/kg BW) and different sections of the GI tract (crop, proventriculus, duodenum, jejunum, ileum, and colon) were removed through a midline incision, and immediately placed into freshly prepared Krebs solution. The mesenteric attachments and fatty tissues were removed, and luminal contents were flushed out using Krebs solution. Each tissue was cut into segments of 15–20 mm in length. The crop and proventriculus were also removed and mounted along their longitudinal axes in the organ bath tubes (10 ml), containing warm (37 °C) Krebs solution, to measure longitudinal muscle contraction. The composition of the Krebs solution was as follows (mM): NaCl, 118; KCl, 4.75; $MgSO_4$, 1.2; NaH_2PO_4, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25; glucose, 11.5; pH 7.2. The temperature of the Krebs solution was maintained at 37 ± 0.5 °C and the solution was aerated continuously with a mixture of 95% O_2 and 5% CO_2. Mechanical activity of the GI

preparations was monitored with an isometric force transducer (UM-203, Iwashiya Kishimoto Medical Industrials, Kyoto, Japan) using appropriate software (PicoLog for Windows, Pico Technology Ltd., St. Neots, UK) (Appendix I). The initial load was set at 1.0 gwt for each preparation. The experiments commenced after stabilization for 45 min. To normalize contractions, 10^{-4} M ACh was added twice to the organ bath, before the cumulative administration of motilin. To examine the effects of motilin, each GI section was treated with chicken motilin (10^{-11} to 10^{-6} M, cumulatively) in the organ bath and the evoked responses were recorded. Similarly, GI contractions were recorded following administration of cumulative doses of 10^{-11} to 10^{-6} M chicken ghrelin. It was reported that even though a high dose of ghrelin did not stimulate contraction of stomach preparations, ghrelin administration of 10^{-11} to 10^{-7} M, following pretreatment with a low dose of motilin (10^{-10} M), induced gastric contraction in a dose-dependent manner (Mondal *et al.*, 2012). Therefore, we selected motilin and ghrelin concentrations, based on the previously published report, to determine the coordinated effects of motilin and ghrelin in the quail GI tract. In our study, to determine the coordinated effects, chicken ghrelin, at doses of 10^{-11} to 10^{-6} M, was also cumulatively administered to the organ bath following pretreatment with 10^{-10} M chicken motilin. The amplitude of contraction for all preparations was normalized to that of a standard contraction induced by ACh (10^{-4} M), and expressed as the relative contraction (%); subsequently, concentration response curves were constructed. To elucidate the mechanism of motilin action, chicken motilin (10^{-11} to 10^{-7} M) was administered in the absence or presence of antagonists in quail proventriculus and duodenum tissues, and expressed as a percentage of the ACh (10^{-4} M)-induced contractions.

2.2.6. Statistical analysis

All values were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Significant differences between the values were determined at $p < 0.05$, based on a one-way analysis of variance (ANOVA) followed by a Tukey's *post-hoc* test for multiple comparisons.

2.3. Results

2.3.1. Cloning of quail motilin cDNA

Quail motilin cDNA was cloned from mRNA from the duodenum and its sequence was determined (Figure 1). The deduced amino acid sequence of quail mature motilin was 22 amino acids. Similar to motilin precursors in mammals, an endoproteinase cleavage dibasic site (KK) was present in quail motilin (Figure 2). Mature quail motilin showed high sequence homology to that of other avian species, including chicken (95.4%) and turkey (90.9%), and moderate homology to that of mammalian species, including humans (72.7%), dogs (72.7%), and suncus (68.1%). Mature quail motilin differs from chicken motilin by one amino acid (position 10), from turkey motilin by two amino acids (position 10 and 19), from suncus motilin by seven amino acids (position 2, 4, 7, 8, 9, 10, and 18), from dog motilin by six amino acids (position 4, 7, 9, 10, 13, and 14), and from human motilin by six amino acids (position 4, 7, 8, 9, 10, and 12). Nine amino acids (1–9) of the N-terminal region (FVPFFTQSD) of quail motilin were fully conserved compared to that of chicken and turkey (Figure 2).

2.3.2. Localization of motilin immunopositive (motilin-ip) cells in the GI tract of quail

Motilin-ip cells were observed in the mucosal layer of the duodenum (Figure 3A), jejunum (Figure 3B), and ileum (Figure 3C), but were not observed in the crop, proventriculus, and colon. The motilin-ip cell density was highest in the duodenum (23.4 ± 4.6 cells/mm^2), and gradually decreased in the jejunum (15.2 ± 0.8 cells/mm^2) and ileum (2.5 ± 0.7 cells/mm^2) (Figure 3D). Open- and closed-type motilin-ip cells were also observed in these regions and the percentage of open-type motilin-ip cells among all immunopositive cells was 69.3% in the

duodenum, 67.1% in the jejunum, and 72.5% in the ileum. Immunoreactivity completely disappeared after application of antiserum that adsorbed chicken motilin (Appendix II).

2.3.3. Effects of motilin on the GI tract of quail

Chicken motilin-induced contractions in the crop and colon were weak (relative to those induced by ACh), and were 27.3 ± 5.8% and 35.1 ± 6.0%, respectively (Figure 4A and B). In the duodenum, chicken motilin-induced contractions at a concentration of 10^{-10} M, which were shown to increase in intensity in a dose-dependent manner, reaching a maximum contraction level of 71.0 ± 9.7% (Figure 4C). In the jejunum and ileum, motilin-induced contraction was also observed at 10^{-10} M, and reached maximum levels of 81.7 ± 11.6% and 74.1 ± 13.7%, respectively (Figure 4D and E). In contrast, contractile activity in the proventriculus reached a maximum level of 46.2 ±13.6% (Figure 4F). Suncus (shrew) motilin induced no contractions in the proventriculus or intestines (Appendix III).

2.3.4. Effects of ghrelin on the GI tract of quail

Cumulative administration of chicken ghrelin (10^{-11} to 10^{-6} M) resulted in mild contractile responses in the duodenum and proventriculus; however, these were not significant (Figure 5A and B). Administration of ghrelin induced no marked contractile responses in the crop, jejunum, ileum, or colon (data not shown). In addition, human ghrelin (10^{-11} to 10^{-7} M) did not elicit any contractile responses in the duodenum or proventriculus of quail (Figure 6A and B).

2.3.5. Effects of co-administration of motilin and ghrelin on the GI tract of quail

Pretreatment with chicken motilin (10^{-10} M) with subsequent cumulative administration of chicken ghrelin (10^{-11} to 10^{-6} M) did not result in additive effects in the duodenum or proventriculus (Figure 7A and B). Co-administration of motilin and ghrelin also did not result in synergistic or additive effects in the crop, jejunum, ileum, or colon (data not shown). Similarly, pretreatment with chicken motilin (10^{-10} M), with cumulative administration of human ghrelin (10^{-11} to 10^{-7} M), also did not result in synergistic effects in the duodenum or proventriculus of Japanese quail (Figure 8A and B).

2.3.6. Mechanism of chicken motilin-induced contraction in the duodenum of quail

To elucidate the neural pathways of motilin-induced contraction in the duodenum, atropine (a muscarinic receptor antagonist), hexamethonium (a nicotinic receptor antagonist) (Figure 9 and 10), TTX (a selective Na^+ channel inhibitor) (Figure 11), ritanserin (a 5-HT2A receptor antagonist), ondansetron (a 5-HT_3 receptor antagonist), and GR125487 (a 5-HT_4 receptor antagonist) (Figure 12, 13, and 14) were used for pretreatment, before motilin administration. None of the antagonists or inhibitors blocked motilin-induced duodenal contractions.

2.3.7. Mechanism of chicken motilin-induced contraction in the proventriculus of quail

In the proventriculus, treatment with atropine changed the baseline contractions, but did not affect spontaneous contractions (data not shown). Pretreatment with atropine (10^{-6} M) significantly inhibited motilin-induced contractions at motilin concentrations of 10^{-8} and 10^{-7} M (Figure 15). Pretreatment with TTX (10^{-6} M) significantly blocked motilin-induced contraction in the proventriculus at motilin concentrations of 10^{-8} and 10^{-7} M (Figure 16). In

contrast, pretreatment with ritanserin, ondansetron, and GR125487 did not inhibit motilin-induced contractions in the proventriculus (Figures 17, 18, and 19).

2.4. Discussion

2.4.1. cDNA and amino acid sequences of quail motilin

In the present study, we first determined the sequence of the mRNA coding region of quail motilin and subsequently assessed the localization of motilin-producing cells in the GI tract of quails. The N-terminal amino acid of motilin is very important and capable of affecting the full activity of the peptide, based on studies using cell lines that over-express the GPR38 (Poitras *et al.*, 1992). The deduced amino acid sequence revealed that the first nine amino acids were identical to those of chicken and turkey motilin, but showed low homology to N-terminal amino acids of mammalian motilin. In the present study, we observed that chicken motilin stimulated contractions in the proventriculus and duodenum; however, suncus motilin did not induce contractions in those tissues, suggesting that the N-terminal region of quail motilin is also important for specific binding of quail GPR38.

2.4.2. Immunohistochemical analysis of quail motilin

Immunohistochemical analysis showed that motilin-ip cells were scattered along the mucosal layer of the duodenum, jejunum, and ileum and existed as open- and closed-type cells. Motilin-producing cells have been observed in abundance in the upper intestine of humans (Polak *et al.*, 1975; Kishimoto *et al.*, 1981; Sjolund *et al.*, 1983), pigs (Pearse *et al.*, 1974; Polak *et al.*, 1975), rabbits (Satoh *et al.*, 1995), and suncus (Kitamura *et al.*, 1990; Tsutsui *et al.*, 2009). In addition, both open- and closed-type motilin-producing cells exist in mammals. These results suggest that tissue production of motilin in the quail occurs mainly in the upper small intestine, as it does in mammals, and that luminal conditions in the small intestine are important for the release of motilin in quails.

2.4.3. Effects of motilin and ghrelin, alone or together, on gastrointestinal motility in Japanese quail

The present study demonstrated that motilin stimulates contractions in the duodenum and proventriculus of quail. Similar region-specific variations in motilin-induced contractions have also been reported in chickens, with the strongest responses being observed in the small intestine (Kitazawa *et al.*, 1995; De Clercq *et al.*, 1996). In addition to motilin, we examined the effects of ghrelin on GI contraction and observed that ghrelin had no significant effects in quails. In the quail GI tract, ghrelin did not induce significant contractions (Kitazawa *et al.*, 2009), which was consistent with our results. On the other hand, ghrelin was shown to induce gastric contractions in rodents, which are genetically motilin-knockout animals (Masuda *et al.*, 2000; Depoortere *et al.*, 2005; Zheng *et al.*, 2009). Ghrelin also stimulates gastric contractions in humans (Tack *et al.*, 2006). Recent studies in suncus (which are suitable motilin-producing animals for GI motility research) revealed that ghrelin induces gastric contractions *in vitro* in combination with low doses of motilin, and that motilin also induces strong gastric contractions that are influenced by ghrelin *in vivo* (Mondal *et al.*, 2012; Kuroda *et al.*, 2015). Moreover, motilin is not involved in the contraction of the small intestines (Janssens *et al.*, 1983). Altogether, the present results suggest that the sites of action for motilin and the mechanisms of motilin-induced gastric contractions are different between mammals and birds.

2.4.4. Mechanism of motilin-induced contraction in quail proventriculus and duodenum

The site of motilin-induced contractions in the stomach is different even among mammalian species (Mizumoto *et al.*, 1993; Boivin *et al.*, 1997; Van Assche *et al.*, 1997; Mondal *et al.*, 2011). In the dog and in suncus, motilin induces gastric contractions through neural pathways

(Mizumoto *et al.*, 1993; Mondal *et al.*, 2011). In humans and rabbits, gastric contraction induced by high doses of motilin is mediated through direct stimulation of smooth muscle, whereas low doses of motilin exert their effects via neural pathways (Van Assche *et al.*, 1997; Coulie *et al.*, 1998; Dass *et al.*, 2003; Depoortere *et al.*, 2003; Jarvie *et al.*, 2007; De Smet *et al.*, 2009; Sanger, 2012; Broad *et al.*, 2016). In contrast, motilin-induced contractile activity in the small intestine of rabbits, cats, dogs, and humans has been reported to be via direct interactions with smooth muscle (Strunz *et al.*, 1975; Adachi *et al.*, 1981; Poitras *et al.*, 1987; Depoortere *et al.*, 1990; Depoortere *et al.*, 1993; Kitazawa *et al.*, 1994; Boivin *et al.*, 1997). We found that atropine and TTX pretreatments failed to suppress motilin-induced contractions in the duodenum but decreased contractions in the proventriculus of quails, by approximately 50%. This finding suggests that motilin induces proventricular contractions via direct neural and smooth muscle pathways. In contrast, motilin-induced duodenal contractions appeared to be via direct stimulation of smooth muscles. These results indicate that the mechanisms of motilin-induced contractions differ among regions of the GI tract in quails. In chickens, it was shown that GPR38 mRNA was differentially expressed in different regions of the GI tract (Kitazawa *et al.*, 2013). Moreover, GPR38 expression is reportedly localized to the myenteric plexus and muscle layers of the human stomach and small intestine (Takeshita *et al.*, 2006; Ter Beek *et al.*, 2008; Broad *et al.*, 2012). Our recent study in suncus showed that GPR38 mRNA expression is also higher in the upper corpus than in other regions of the GI tract, and that the expression levels of GPR38 mRNA are consistent with responses to motilin (Dudani *et al.*, 2016). Our findings therefore suggest that differential responses in the quail GI tract might be linked to the expression and/or localization of GPR38 mRNA.

2.4.5. Physiological significance of MMC in relation to motilin

The MMC occurs in the fasted state in mammals, and phase III of the MMC has been observed to occur at approximately 90–120 min intervals (Itoh *et al.*, 1976; Vantrappen *et al.*, 1979; Sakahara *et al.*, 2010). The biological and physiological relevance of the MMC is thought to be the mechanical clearance of remnants of indigestible food (Vantrappen *et al.*, 1977; Itoh, 1997); dysfunction of the MMC can lead to functional dyspepsia or other GI motility disorders (Kusano *et al.*, 1997; Gu *et al.*, 1998; Takahashi, 2013). Interestingly, the MMC cycle is highly conserved in humans, dogs, and suncus even though body weight and length of GI tract vary widely (Itoh, 1997; Kuroda *et al.*, 2015). This suggests that the 90–120 min cycle of the MMC is a primitive phenomenon. In chickens, similar MMC cycles have been observed using a myoelectric recording system (Clench *et al.*, 1989), indicating that an ultradian MMC cycle might be conserved in mammals and birds. For a deeper understanding of the biological and physiological significance of motilin, *in vivo* observations of quail GI contractions associated with the MMC would be necessary to further examine the effects of motilin.

2.5. Summary

The quail motilin mRNA sequence and the distribution of motilin-producing cells have been demonstrated in the present study. Motilin stimulated contractions in the proventriculus and duodenum occurred in a dose-dependent manner *in vitro*. In addition, we demonstrated the motilin-induced contractions in the proventriculus occur via both neural pathways and direct stimulation of smooth muscle, whereas motilin-elicited duodenal contractions occur only through direct stimulation of smooth muscle. Further studies are necessary to determine the expression levels and distribution of GPR38 mRNA within the GI tract, which will ultimately lead to a better understanding of the mechanisms of motilin-induced GI contraction in quails.

Chapter 3: Molecular identification and expression of GPR38 in the gastrointestinal tract of Japanese quail

3.1. Introduction

Feighner *et al.* first identified GPR38 in the human gastrointestinal tract in 1999, and it was shown to exist as two alternatively spliced forms (Feighner *et al.*, 1999). The active form of the receptor, GPR38A (splice variant 1a) encodes a 412 amino acid polypeptide with seven predicted transmembrane domains, whereas GPR38B (variant 1b) mRNA encodes a 386 amino acid polypeptide with only five of the seven predicted transmembrane domains, and is the pharmacologically inactive form of the receptor (Feighner *et al.*, 1999). It has been reported that GPR38 and GHS-R are members of G protein coupled receptor (GPCRs) family (Folwaczny *et al.*, 2001) with the amino terminus located on the extracellular side and the carboxyl terminus on the intracellular side (Howard *et al.*, 1996; Feighner *et al.*, 1999). In humans, these receptors have 52% sequence homology to each other (Takeshita *et al.*, 2006), with 86% homology in the transmembrane region (Folwaczny *et al.*, 2001; Ohno *et al.*, 2010). Tissue distribution of GPR38 has been examined via binding assays or through mRNA analysis (Bormans *et al.*, 1986; Takeshita *et al.*, 2006). Binding sites for motilin have been reported in the smooth muscle layer of the gastric antrum, duodenum, and colon in rabbits (Bormans *et al.*, 1986; Depoortere *et al.*, 1991; Sakai *et al.*, 1994). In addition, specific binding sites for motilin are predominantly observed in the circular muscle layers, whereas comparatively low levels are observed in the longitudinal muscle layers of the gastric antrum, duodenum, and colon in rabbits (Sakai *et al.*, 1994). In contrast, no motilin binding sites are found in the mucosa of the human and rabbit GI tract (Peeters *et al.*, 1988; Sakai *et al.*, 1994).

mRNA analysis showed that GPR38 is expressed in the upper and lower GI tract of humans (Takeshita *et al.*, 2006; Ter Beek *et al.*, 2008), dogs (Ohshiro *et al.*, 2008), and chickens (Kitazawa *et al.*, 2013). In addition, GPR38 immunoreactivity was reported in both the muscle layer and myenteric plexus, but not in the mucosal or submucosal cells of the GI tract in humans (Takeshita *et al.*, 2006). In dogs, GPR38 immunoreactivity was observed in the muscle fibers on both longitudinal and circular muscle layers (Ohshiro *et al.*, 2008).

The functions and expression of GPR38 in the GI tract of several animal species have been reported in previous studies, which have demonstrated differential distribution of GPR38 along the GI tract (Miller *et al.*, 2000a; Miller *et al.*, 2000b; Takeshita *et al.*, 2006; Ohshiro *et al.*, 2008). It was previously reported that the GPR38 density is higher in the gastroduodenal region, and decreases distally in the small intestine and towards the colon in humans and dogs (Peeters *et al.*, 1988; Ter Beek *et al.*, 2008). In contrast, GPR38 density is highest in the colon in rabbits (Depoortere *et al.*, 1991). Therefore, it is important to assess the regional expression of GPR38 to understand the mechanism wherein motilin controls GI motility. Recently, GPR38 was identified in the chicken and was shown to have high overall sequence homology to GPR38 of rabbits (65%), humans (59%), and pufferfish (55%) (Yamamoto *et al.*, 2008). Based on the amino acid sequence, seven transmembrane regions were found to be conserved in chicken GPR38, with relatively high identity to the corresponding regions of rabbit and pufferfish GPR38 (Yamamoto *et al.*, 2008).

We hypothesized that motilin-induced contractions in different regions of the GI tract depends on the tissue distribution of GPR38. Therefore, we studied the nucleotide sequence of GPR38 and examined its expression in different tissues to clarify the role of motilin and its regulatory mechanism in the GI tract of Japanese quail.

3.2. Materials and methods

3.2.1. Animals

The experiments were performed using male Japanese quails at 1, 3, and 5 weeks of ages, purchased from a commercial source (Motoki Co. Ltd., Honcho, Tokorozawa city, Saitama, Japan). All procedures were performed in accordance with the guidelines of the Saitama University Committee on Animal Research.

3.2.2. Cloning of quail GPR38

Total RNA was extracted from the medulla oblongata using Isogen (NIPPON GENE Co. Ltd., Tokyo, Japan) based on the manufacturer's instructions. Trace DNA contamination was removed by DNase digestion (Promega, Madison, WI, USA) and cDNA was synthesized from 1 μg of DNase-treated total RNA using Primescript II® Reverse Transcriptase (Takara Bio, Japan), using random primer (Invitrogen, Carlsbad, CA, USA).

To obtain the partial quail GPR38 sequence, we designed the degenerate primer qGPR38 FWD #2669, qGPR38 BWD#2670, and the nested primer qGPR38 FWD #2811 and qGPR38 BWD #2812 with NCBI/Primer-BLAST based on the human, rabbit, pig, dog, and chicken GPR38 sequence from the NCBI database (Appendix IV). PCR was performed using quail medulla oblongata cDNA with qGPR38 FWD#2669 and qGPR38 BWD#2670 primers and Ex Taq polymerase **(**Takara Bio, Japan). Amplification reactions were performed using a Thermal Cycler (Bio-Rad**,** Hercules, California, USA). Initial template denaturation was performed for 5 min at 94 °C, and the cycle profile was programmed as follows: 1 min at 94 °C (denaturation), 1 min at 60 °C, 1 min at 72 °C (annealing and extension), for 40 cycles, with a

final extension at 72 °C for 10 min. Amplicon size and specificity were confirmed by 2% agarose gel electrophoresis. The second PCR was performed using diluted products from the primary PCR as template with qGPR38 FWD #2811 and qGPR38 BWD#2812 primers with Ex Taq polymerase. The amplification reaction was performed under the following conditions: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 30 sec, and final extension for 10 min at 72 °C. The amplified fragments were run on 2% agarose gels to confirm amplicon size and specificity. The amplified fragments from the second PCR were then cloned into pGEM T-easy vector (Promega, Madison, WI) and sequencing was performed by Eurofins Genomics K.K. (Tokyo, Japan). Sequence homology of the obtained sequences was verified by a BLAST search (NCBI).

3.2.3. Reverse transcription (RT) and quantitative real-time PCR (Q-PCR) analysis

Total RNA was extracted from hypothalamus, liver, heart, and muscle tissue, and from seven regions of the gastrointestinal tract (crop, proventriculus, gizzard, duodenum, jejunum, ileum, and colon) from male quails 5 weeks of age, using Isogen, and in accordance with the manufacturer's instructions. Total RNA was treated by DNase digestion (Promega, Madison, WI, USA) to eliminate trace DNA contamination. cDNA was synthesized from 1 μg of DNAse-treated total RNA with random primers and Primescript II Reverse Transcriptase (Takara Bio, Japan). According to the manufacturer's instructions, real-time quantitative PCR was performed with SYBR Premix Ex Taq (Takara BIO, Shiga, Japan) using a Light Cycler (Roche Diagnostics). Each sample was run in duplicate with the following PCR conditions to minimize sample variability: 95 °C for 30 sec, 40 cycles of 95 °C for 5 sec, and 60 °C for 30

sec. To evaluate the specificity of amplification, melting curves were obtained and analyzed. Quantitative measurement for each mRNA target was performed by establishing a linear amplification curve from serial dilutions of each plasmid for quail GPR38.

3.2.4. Organ bath study

Quails of three different ages (1, 3, and 5 weeks) were anesthetized by pentobarbital sodium (100 mg/kg BW). Through a midline incision, the proventriculus and duodenum of the GI tract were collected and immediately placed in freshly prepared Krebs solution. The mesenteric attachments and fatty tissues were removed, and luminal contents were flushed out using Krebs solution. Each tissue was cut into segments of 15–20 mm in length. The proventriculus and duodenum were mounted along their longitudinal axes in organ baths (10 ml), containing warm (37 °C) Krebs solution, to measure longitudinal muscle contraction. The composition of the Krebs solution was as follows (mM): NaCl, 118; KCl, 4.75; $MgSO_4$, 1.2; NaH_2PO_4, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25; and glucose, 11.5; pH 7.2. The temperature of the Krebs solution was maintained at 37 ± 0.5 °C and the solution was aerated continuously with a mixture of 95% O_2 and 5% CO_2. Mechanical activity of the GI preparations was monitored with an isometric force transducer (UM-203, Iwashiya Kishimoto Medical Industrials, Kyoto, Japan) and software (PicoLog for Windows, Pico Technology Ltd., St. Neots, UK) (Appendix I). The initial load was set at 1.0 gwt for each preparation. The experiments commenced after stabilization for 45 min. To normalize contractions, 10^{-4} M ACh was added to the organ bath twice, before the cumulative administration of motilin. To examine the effects of motilin, the proventriculus and duodenum were treated with chicken motilin (10^{-11} to 10^{-7} M) cumulatively in the organ bath and the responses were recorded. The amplitudes of contractions among all

preparations were normalized to that of a standard contraction induced by ACh (10^{-4} M), and expressed as the relative contraction (%); concentration response curves were then constructed.

3.2.5. Statistical analysis

All values were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Significance between values was determined at a level of $p < 0.05$, by performing a one-way analysis of variance (ANOVA), followed by Tukey's *post-hoc* test for multiple comparisons.

3.3. Results

3.3.1. Cloning of quail GPR38

Quail GPR38 was cloned from cDNA derived from the medulla oblongata. Using the PCR cloning method, we obtained a partial sequence of 183 bp. Comparison of the isolated partial sequence of quail GPR38 to the known human, pig, dog, rabbit, chicken, and turkey GPR38 sequences is shown in Figure 20. The partial quail GPR38 sequence showed homology to that of other species, including chicken (95%), turkey (92%), human (76%), rabbit (77%), pig (79%), and dog (69%). The ORF of the partial GPR38 cDNA encoded a 52-amino acid polypeptide. The deduced amino acid sequence of quail GPR38 was aligned with known GPR38 amino acid sequences of human, pig, dog, rabbit, chicken, and turkey (Figure 21). The deduced partial amino acid sequence of quail GPR38 showed sequence homology to that of chicken (89%), turkey (85%), human (63%), pig (58%), rabbit (58%), and dog (47%) GPR38.

3.3.2. Expression of GPR38 mRNA in quail tissues

The expression of GPR38 mRNA in the quail tissues was examined by Q-PCR. In 5-week-old quails, expression of GPR38 mRNA was observed in the crop, proventriculus, duodenum, jejunum, ileum, colon, gizzard, liver, heart, and muscle (Figure 22). GPR38 mRNA expression was similar in all GI tissues.

3.3.3. Age-dependent changes in motilin-induced contraction in the GI tract of Japanese quail

In vitro studies revealed age-dependent changes in motilin-induced contraction in the GI tract of Japanese quail. Chicken motilin-induced contraction in the proventriculus was significantly

decreased at ages 3 and 5 weeks compared to that at 1 week (Figure 23A). In contrast, motilin-induced duodenal contractions were not dependent on age in the Japanese quails (Figure 23B).

3.4. Discussion

In the present study, we found that GPR38 was expressed throughout the GI tract of Japanese quails. In humans, it was reported that GPR38 is expressed in both the upper and lower GI tracts and expression does not differ significantly among the tissues (Takeshita *et al.*, 2006); this is similar to results of the present study. However, in dogs, the expression of GPR38 varies depending on the GI tract region (He *et al.*, 2015). GPR38 mRNA is predominantly expressed in the duodenum, followed by ileum, jejunum, proximal colon, antrum, middle colon, and distal colon of the dog (He *et al.*, 2015). In the chicken, GPR38 is primarily expressed in the proventriculus, duodenum, and ileum (Yamamoto *et al.*, 2008; Kitazawa *et al.*, 2013). In addition, previous reports have shown that the expression of GPR38 varies with age and that age-related decreases in GPR38 expression lead to decreases in motilin-induced contractile activity in the proventriculus, but not in the ileum, of chickens (Kitazawa *et al.*, 2013). In the present study, we reported that motilin-induced contraction decreased in the quail proventriculus, but not in the duodenum, with age. GPR38 mRNA expression in the chicken crop, proventriculus, ileum, and colon were shown to decrease with age; however, this decrease was more remarkable in the proventriculus than in the ileum (Kitazawa *et al.*, 2013). Moreover, other investigators reported that in chickens, GPR38 mRNA is highly expressed in the pre-hatch period when compared to that of the post-hatch period, and gradually declines with age (Yamamoto *et al.*, 2008).

It has been reported that motilin-induced contractions in the GI tract are mediated by direct stimulation of GPR38 located on smooth muscle cells or in the enteric nervous system (Miller *et al.*, 2000b). In rabbits and humans, GPR38 is distributed on both the myenteric plexus and

smooth muscle cells of the GI tract (Peeters *et al.*, 1988; Miller *et al.*, 2000b; Takeshita *et al.*, 2006). *In vitro* human and rabbit studies revealed that motilin-induced contraction in the stomach is mediated by the neural pathway at low doses and directly by smooth muscle cells at high doses, whereas in the intestine it is only mediated by direct action on smooth muscle cells (Van Assche *et al.*, 1997; Broad *et al.*, 2016). These results of motilin-induced contraction are in agreement with the GPR38 distribution in the GI tract. In this study, we also found that two distinct mechanisms exist in the quail proventriculus and duodenum. Similar to human and rabbit studies, motilin-induced quail proventricular contraction was mediated by both the neural pathway and by direct action on smooth muscle cells, and motilin-induced duodenal contraction was mediated only by direct action on smooth muscle cells.

We also found GPR38 expression in the liver, heart, and muscle of the Japanese quails. Previously, studies on chickens showed that in addition to the GI tract, GPR38 is expressed in the pituitary, brain, thymus, bursa of fabricus, liver, kidney, bone marrow, oviduct, ovary, and testis (Yamamoto *et al.*, 2008). Human and rabbit studies also showed GPR38 expression in the central nervous system, thyroid, and bone marrow, in addition to GI tissues (Depoortere *et al.*, 1997; Depoortere & Peeters, 1997; Feighner *et al.*, 1999). Although motilin was discovered decades ago, most studies regarding its function have been limited to its role in gastrointestinal motility. However, motilin was shown to stimulate gastric acid and pepsinogen secretion, based on dog and suncus studies (Konturek *et al.*, 1976; Goswami *et al.*, 2015a; Goswami *et al.*, 2015b). It also stimulates pancreatic bicarbonate and protein secretion in conscious dogs (Konturek *et al.*, 1976; Magee & Naruse, 1984). In humans, it stimulates hunger and food intake (Deloose *et al.*, 2016), and plays a role in gall bladder emptying

(Luiking *et al.*, 1998). The effect of motilin has also been observed on the reduction of nausea and vomiting in *Suncus murinus* (Javid *et al.*, 2013). Therefore, previous findings and those of this study support additional roles for motilin and GPR38 expression, other than GI motility.

3.5. Summary

We cloned quail GPR38 from cDNA derived from the medulla oblongata and obtained a partial sequence of 183 bp. Quail GPR38 showed high sequence homology to GPR38 of mammals including human, pig, dog, and rabbit. Partial GPR38 cDNA encoded a 52-amino acid polypeptide. The expression of GPR38 was observed throughout the GI tract of Japanese quails. An age-dependent reduction in motilin-induced contractions was observed in the proventriculus, but not in the duodenum, of quails. However, the identification and expression of GPR38 in different tissues would facilitate a more detailed study of the related physiological mechanism in Japanese quails.

4.0. CONCLUSION

The existence of motilin and GPR38 in Japanese quail was determined for the first time. The high responsiveness to motilin in the GI tract suggested a possible physiological role for motilin in the modulation of gastrointestinal motility in Japanese quails. Moreover, a distinct mechanism of motilin-induced contraction in the stomach and intestine indicates that the MMC in the stomach and intestine are regulated by different mechanisms in avian species. However, further studies are required to confirm the role of motilin in the regulation of MMC *in vivo*.

ABBREVIATIONS

ABC	:	Avidin-biotin-peroxidase complex
ACh	:	Acetylcholine chloride
ANOVA	:	Analysis of variance
BSA	:	Bovine serum albumin
bp	:	Base pair
cDNA	:	Complementary DNA
GI	:	Gastrointestinal
DAB	:	3,3′-diaminobenzidine tetrachloride
GHS-R	:	Growth hormone secretagogue receptor
GPR38	:	G protein-coupled receptor 38
H_2O_2	:	Hydrogen peroxide
ip	:	Immunopositive
MMC	:	Migrating motor complex
mRNA	:	Messenger RNA
NO	:	Nitric oxide
PBS	:	Phosphate-buffered saline

PCR	:	Polymerase chain reaction
Q-PCR	:	Quantitative polymerase chain reaction
RT	:	Reverse transcriptase
SEM	:	Standard error of the mean
TTX	:	Tetrodotoxin

REFERENCES

Adachi H, Toda N, Hayashi S, Noguchi M, Suzuki T, Torizuka K, Yajima H & Koyama K (1981). Mechanism of the excitatory action of motilin on isolated rabbit intestine. *Gastroenterology* **80,** 783-788.

Aerssens J, Depoortere, I., Thielemans, L., Mitselos, A., Coulie, B., Peeters, T. L. (2004). The rat lacks functional genes for motilin and for the motilin receptor. *Neurogastroenterol. Motil.* **16,** 841.

Ariga H, Tsukamoto K, Chen C, Mantyh C, Pappas TN & Takahashi T (2007). Endogenous acyl ghrelin is involved in mediating spontaneous phase III-like contractions of the rat stomach. *Neurogastroenterol Motil* **19,** 675-680.

Ariyasu H, Takaya K, Tagami T, Ogawa Y, Hosoda K, Akamizu T, Suda M, Koh T, Natsui K, Toyooka S, Shirakami G, Usui T, Shimatsu A, Doi K, Hosoda H, Kojima M, Kangawa K & Nakao K (2001). Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. *J Clin Endocrinol Metab* **86,** 4753-4758.

Banfield DK, MacGillivray RT, Brown JC & McIntosh CH (1992). The isolation and characterization of rabbit motilin precursor cDNA. *Biochim Biophys Acta* **1131,** 341-344.

Bang AS, Soule SG, Yandle TG, Richards AM & Pemberton CJ (2007). Characterisation of proghrelin peptides in mammalian tissue and plasma. *J Endocrinol* **192,** 313-323.

Barilani M, Deregnaucourt S, Gellego S, Galli L, Mucci N, Piomobo R, Puigcerver M, Rimondi S, Rodriguez-Teijeiro JD, Spano S & Randi E (2005). Detecting hybridization in wild (*Coturnix c. coturnix*) and domesticated (*Coturnix c. japonica*) quail populations. *Biol Conserv* **126**, 445–455.

Bloom SR, Mitznegg P & Bryant MG (1976). Measurement of human plasma motilin. *Scand J Gastroenterol Suppl* **39,** 47-52.

Boivin M, Pinelo LR, St-Pierre S & Poitras P (1997). Neural mediation of the motilin motor effect on the human antrum. *Am J Physiol* **272,** G71-76.

Bormans V, Peeters TL & Vantrappen G (1986). Motilin receptors in rabbit stomach and small intestine. *Regul Pept* **15,** 143-153.

Broad J, Mukherjee S, Samadi M, Martin JE, Dukes GE & Sanger GJ (2012). Regional- and agonist-dependent facilitation of human neurogastrointestinal functions by motilin receptor agonists. *Br J Pharmacol* **167,** 763-774.

Broad J, Takahashi N, Tajimi M, Sudo M, Goralczyk A, Parampalli U, Mannur K, Yamamoto T & Sanger GJ (2016). RQ-00201894: A motilin receptor agonist causing long-lasting facilitation of human gastric cholinergically-mediated contractions. *J Pharmacol Sci* **130,** 60-65.

Brown JC, Cook MA & Dryburgh JR (1972). Motilin, a gastric motor activity-stimulating polypeptide: final purification, amino acid composition, and C-terminal residues. *Gastroenterology* **62,** 401-404.

Brown JC, Cook MA & Dryburgh JR (1973). Motilin, a gastric motor activity stimulating polypeptide: the complete amino acid sequence. *Can J Biochem* **51,** 533-537.

Brown JC, Mutt V & Dryburgh JR (1971). The further purification of motilin, a gastric motor activity stimulating polypeptide from the mucosa of the small intestine of hogs. *Can J Physiol Pharmacol* **49,** 399-405.

Clench MH, Pineiro-Carrero VM & Mathias JR (1989). Migrating myoelectric complex demonstrated in four avian species. *Am J Physiol* **256,** G598-603.

Coulie B, Tack J, Peeters T & Janssens J (1998). Involvement of two different pathways in the motor effects of erythromycin on the gastric antrum in humans. *Gut* **43,** 395-400.

Daikh DI, Douglass JO & Adelman JP (1989). Structure and expression of the human motilin gene. *DNA* **8,** 615-621.

Dass NB, Hill J, Muir A, Testa T, Wise A & Sanger GJ (2003). The rabbit motilin receptor: molecular characterisation and pharmacology. *Br J Pharmacol* **140,** 948-954.

De Clercq P, Depoortere I, Macielag M, Vandermeers A, Vandermeers-Piret MC & Peeters TL (1996). Isolation, sequence, and bioactivity of chicken motilin. *Peptides* **17,** 203-208.

De Smet B, Mitselos A & Depoortere I (2009). Motilin and ghrelin as prokinetic drug targets. *Pharmacol Ther* **123,** 207-223.

Deloose E, Vos R, Corsetti M, Depoortere I & Tack J (2015). Endogenous motilin, but not ghrelin plasma levels fluctuate in accordance with gastric phase III activity of the migrating motor complex in man. *Neurogastroenterol Motil* **27,** 63-71.

Deloose E, Vos R, Janssen P, Van den Bergh O, Van Oudenhove L, Depoortere I & Tack J (2016). The motilin receptor agonist erythromycin stimulates hunger and food intake through a cholinergic pathway. *Am J Clin Nutr* **103,** 730-737.

Depoortere I, De Clercq P, Svoboda M, Bare L & Peeters TL (1997). Identification of motilin mRNA in the brain of man and rabbit. Conservation of polymorphism of the motilin gene across species. *Peptides* **18,** 1497-1503.

Depoortere I, De Winter B, Thijs T, De Man J, Pelckmans P & Peeters T (2005). Comparison of the gastroprokinetic effects of ghrelin, GHRP-6 and motilin in rats *in vivo* and *in vitro*. *Eur J Pharmacol* **515,** 160-168.

Depoortere I & Peeters TL (1997). Demonstration and characterization of motilin-binding sites in the rabbit cerebellum. *Am J Physiol* **272,** G994-999.

Depoortere I, Peeters TL, Vandermeers A, Vandermeers-Piret MC, Christophe J & Vantrappen G (1993). Purification and amino acid sequence of motilin from cat small intestine. *Regul Pept* **49,** 25-32.

Depoortere I, Peeters TL & Vantrappen G (1990). The erythromycin derivative EM-523 is a potent motilin agonist in man and in rabbit. *Peptides* **11,** 515-519.

Depoortere I, Peeters TL & Vantrappen G (1991). Motilin receptors of the rabbit colon. *Peptides* **12,** 89-94.

Depoortere I, Thijs T, Thielemans L, Robberecht P & Peeters TL (2003). Interaction of the growth hormone-releasing peptides ghrelin and growth hormone-releasing peptide-6 with the motilin receptor in the rabbit gastric antrum. *J Pharmacol Exp Ther* **305,** 660-667.

Dudani A, Aizawa S, Zhi G, Tanaka T, Jogahara T, Sakata I & Sakai T (2016). The proximal gastric corpus is the most responsive site of motilin-induced contractions in the stomach of the Asian house shrew. *J Comp Physiol B*.

Duxbury MS, Waseem T, Ito H, Robinson MK, Zinner MJ, Ashley SW & Whang EE (2003). Ghrelin promotes pancreatic adenocarcinoma cellular proliferation and invasiveness. *Biochem Biophys Res Commun* **309,** 464-468.

Feighner SD, Tan CP, McKee KK, Palyha OC, Hreniuk DL, Pong SS, Austin CP, Figueroa D, MacNeil D, Cascieri MA, Nargund R, Bakshi R, Abramovitz M, Stocco R, Kargman S, O'Neill G, Van Der Ploeg LH, Evans J, Patchett AA, Smith RG & Howard AD (1999). Receptor for motilin identified in the human gastrointestinal system. *Science* **284,** 2184-2188.

Follett BK, Kumar V & Juss TS (1992). Circadian nature of the photoperiodic clock in Japanese quail. *J Comp Physiol A* **171,** 533-540.

Folwaczny C, Chang JK & Tschop M (2001). Ghrelin and motilin: two sides of one coin? *Eur J Endocrinol* **144,** R1-3.

Fujino K, Inui A, Asakawa A, Kihara N, Fujimura M & Fujimiya M (2003). Ghrelin induces fasted motor activity of the gastrointestinal tract in conscious fed rats. *J Physiol* **550,** 227-240.

Fukumoto K, Nakahara K, Katayama T, Miyazatao M, Kangawa K & Murakami N (2008). Synergistic action of gastrin and ghrelin on gastric acid secretion in rats. *Biochem Biophys Res Commun* **374,** 60-63.

Goswami C, Shimada Y, Yoshimura M, Mondal A, Oda S, Tanaka T, Sakai T & Sakata I (2015a). Motilin Stimulates Gastric Acid Secretion in Coordination with Ghrelin in *Suncus murinus*. *PLoS One* **10,** e0131554.

Goswami C, Tanaka T, Jogahara T, Sakai T & Sakata I (2015b). Motilin stimulates pepsinogen secretion in *Suncus murinus*. *Biochem Biophys Res Commun* **462,** 263-268.

Gu C, Ke M & Wang Z (1998). Temporal and spatial relationship of pylorus to antroduodenal motility in functional dyspepsia. *Chin Med J (Engl)* **111,** 906-909.

Hall KE, Greenberg GR, El-Sharkawy TY & Diamant NE (1983). Vagal control of migrating motor complex-related peaks in canine plasma motilin, pancreatic polypeptide, and gastrin. *Can J Physiol Pharmacol* **61,** 1289-1298.

He J, Irwin DM, Chen R & Zhang YP (2010). Stepwise loss of motilin and its specific receptor genes in rodents. *J Mol Endocrinol* **44,** 37-44.

He Y, Wang H, Yang D, Wang C, Yang L & Jin C (2015). Differential expression of motilin receptor in various parts of gastrointestinal tract in dogs. *Gastroenterol Res Pract* **2015,** 970940.

Holmes DJ & Ottinger MA (2003). Birds as long-lived animal models for the study of aging. *Exp Gerontol* **38,** 1365-1375.

Houghton LA, Read NW, Heddle R, Horowitz M, Collins PJ, Chatterton B & Dent J (1988). Relationship of the motor activity of the antrum, pylorus, and duodenum to gastric emptying of a solid-liquid mixed meal. *Gastroenterology* **94,** 1285-1291.

Howard AD, Feighner SD, Cully DF, Arena JP, Liberator PA, Rosenblum CI, Hamelin M, Hreniuk DL, Palyha OC, Anderson J, Paress PS, Diaz C, Chou M, Liu KK, McKee KK, Pong SS, Chaung LY, Elbrecht A, Dashkevicz M, Heavens R, Rigby M, Sirinathsinghji DJ, Dean DC, Melillo DG, Patchett AA, Nargund R, Griffin PR, DeMartino JA, Gupta SK, Schaeffer JM, Smith RG & Van der Ploeg LH (1996). A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science* **273,** 974-977.

Huang Z, Depoortere I, De Clercq P & Peeters T (1999). Sequence and characterization of cDNA encoding the motilin precursor from chicken, dog, cow and horse. Evidence of mosaic evolution in prepromotilin. *Gene* **240,** 217-226.

Itoh Z (1997). Motilin and clinical application. *Peptides* **18,** 593-608.

Itoh Z, Honda R, Hiwatashi K, Takeuchi S, Aizawa I, Takayanagi R & Couch EF (1976). Motilin-induced mechanical activity in the canine alimentary tract. *Scand J Gastroenterol Suppl* **39,** 93-110.

Itoh Z, Takeuchi S, Aizawa I, Mori K, Taminato T, Seino Y, Imura H & Yanaihara N (1978). Changes in plasma motilin concentration and gastrointestinal contractile activity in conscious dogs. *Am J Dig Dis* **23,** 929-935.

Janssens J, Vantrappen G & Peeters TL (1983). The activity front of the migrating motor complex of the human stomach but not of the small intestine is motilin-dependent. *Regul Pept* **6,** 363-369.

Jarvie EM, North Laidler VJ, Corcoran S, Bassil A & Sanger GJ (2007). Differences between the abilities of tegaserod and motilin receptor agonists to stimulate gastric motility *in vitro*. *Br J Pharmacol* **150,** 455-462.

Javid FA, Bulmer DC, Broad J, Aziz Q, Dukes GE & Sanger GJ (2013). Anti-emetic and emetic effects of erythromycin in *Suncus murinus*: role of vagal nerve activation, gastric motility stimulation and motilin receptors. *Eur J Pharmacol* **699,** 48-54.

Kanamoto N, Akamizu T, Tagami T, Hataya Y, Moriyama K, Takaya K, Hosoda H, Kojima M, Kangawa K & Nakao K (2004). Genomic structure and characterization of the 5'-flanking region of the human ghrelin gene. *Endocrinology* **145,** 4144-4153.

Kayang BB, Vignal A, Inoue-Murayama M, Miwa M, Monvoisin JL, Ito S & Minvielle F (2004). A first-generation microsatellite linkage map of the Japanese quail. *Anim Genet* **35,** 195-200.

Kishimoto S, Polak JM, Buchan AM, Verhofstad AA, Steinbusch HW, Yanaihara N, Bloom SR & Pearse AG (1981). Motilin cells investigated by the use of region-specific antisera. *Virchows Arch B Cell Pathol Incl Mol Pathol* **36,** 207-218.

Kitamura N, Yamada J, Watanabe T & Yamashita T (1990). An immunohistochemical study on the distribution of endocrine cells in the gastrointestinal tract of the musk shrew, *Suncus murinus*. *Histol Histopathol* **5,** 83-88.

Kitazawa T, Ichikawa S, Yokoyama T, Ishii A & Shuto K (1994). Stimulating action of KW-5139 (Leu13-motilin) on gastrointestinal motility in the rabbit. *Br J Pharmacol* **111,** 288-294.

Kitazawa T, Kaiya H & Taneike T (2007). Contractile effects of ghrelin-related peptides on the chicken gastrointestinal tract *in vitro*. *Peptides* **28,** 617-624.

Kitazawa T, Maeda Y & Kaiya H (2009). Molecular cloning of growth hormone secretagogue-receptor and effect of quail ghrelin on gastrointestinal motility in Japanese quail. *Regul Pept* **158,** 132-142.

Kitazawa T, Taneike T & Ohga A (1995). Excitatory action of [Leu13]motilin on the gastrointestinal smooth muscle isolated from the chicken. *Peptides* **16,** 1243-1252.

Kitazawa T, Taneike T & Ohga A (1997). Functional characterization of neural and smooth muscle motilin receptors in the chicken proventriculus and ileum. *Regul Pept* **71,** 87-95.

Kitazawa T, Yoshida A, Tamano T, Teraoka H & Kaiya H (2013). Age-dependent reduction of ghrelin- and motilin-induced contractile activity in the chicken gastrointestinal tract. *Peptides* **43,** 88-95.

Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H & Kangawa K (1999). Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* **402,** 656-660.

Konturek SJ, Dembinski A, Krol R & Wunsch E (1976). Effects of motilin on gastric and pancreatic secretion in dogs. *Scand J Gastroenterol Suppl* **39,** 57-61.

Korbonits M, Goldstone AP, Gueorguiev M & Grossman AB (2004). Ghrelin--a hormone with multiple functions. *Front Neuroendocrinol* **25,** 27-68.

Kuroda K, Hequing H, Mondal A, Yoshimura M, Ito K, Mikami T, Takemi S, Jogahara T, Sakata I & Sakai T (2015). Ghrelin is an essential factor for motilin-induced gastric contraction in *Suncus murinus*. *Endocrinology* **156,** 4437-4447.

Kusano M, Sekiguchi T, Kawamura O, Kikuchi K, Miyazaki M, Tsunoda T, Horikoshi T & Mori M (1997). Further classification of dysmotility-like dyspepsia by interdigestive gastroduodenal manometry and plasma motilin level. *Am J Gastroenterol* **92,** 481-484.

Luiking YC, Peeters TL, Stolk MF, Nieuwenhuijs VB, Portincasa P, Depoortere I, van Berge Henegouwen GP & Akkermans LM (1998). Motilin induces gall bladder emptying and antral contractions in the fasted state in humans. *Gut* **42,** 830-835.

Maccarinelli G, Sibilia V, Torsello A, Raimondo F, Pitto M, Giustina A, Netti C & Cocchi D (2005). Ghrelin regulates proliferation and differentiation of osteoblastic cells. *J Endocrinol* **184,** 249-256.

Magee DF & Naruse S (1984). The role of motilin in periodic interdigestive pancreatic secretion in dogs. *J Physiol* **355,** 441-447.

Masuda Y, Tanaka T, Inomata N, Ohnuma N, Tanaka S, Itoh Z, Hosoda H, Kojima M & Kangawa K (2000). Ghrelin stimulates gastric acid secretion and motility in rats. *Biochem Biophys Res Commun* **276,** 905-908.

McKee KK, Tan CP, Palyha OC, Liu J, Feighner SD, Hreniuk DL, Smith RG, Howard AD & Van der Ploeg LH (1997). Cloning and characterization of two human G protein-coupled receptor genes (GPR38 and GPR39) related to the growth hormone secretagogue and neurotensin receptors. *Genomics* **46,** 426-434.

Miller P, Roy A, St-Pierre S, Dagenais M, Lapointe R & Poitras P (2000a). Motilin receptors in the human antrum. *Am J Physiol Gastrointest Liver Physiol* **278,** G18-23.

Miller P, Trudel L, St-Pierre S, Takanashi H & Poitras P (2000b). Neural and muscular receptors for motilin in the rabbit colon. *Peptides* **21,** 283-287.

Miyano Y, Sakata I, Kuroda K, Aizawa S, Tanaka T, Jogahara T, Kurotani R & Sakai T (2013). The role of the vagus nerve in the migrating motor complex and ghrelin- and motilin-induced gastric contraction in suncus. *PLoS One* **8,** e64777.

Mizumoto A, Sano I, Matsunaga Y, Yamamoto O, Itoh Z & Ohshima K (1993). Mechanism of motilin-induced contractions in isolated perfused canine stomach. *Gastroenterology* **105,** 425-432.

Mondal A, Kawamoto Y, Yanaka T, Tsutsui C, Sakata I, Oda SI, Tanaka T & Sakai T (2011). Myenteric neural network activated by motilin in the stomach of *Suncus murinus* (house musk shrew). *Neurogastroenterol Motil* **23,** 1123-1131.

Mondal A, Xie Z, Miyano Y, Tsutsui C, Sakata I, Kawamoto Y, Aizawa S, Tanaka T, Oda S & Sakai T (2012). Coordination of motilin and ghrelin regulates the migrating motor complex of gastrointestinal motility in *Suncus murinus*. *Am J Physiol Gastrointest Liver Physiol* **302,** G1207-1215.

Nakai N, Kaneko M, Nakao N, Fujikawa T, Nakashima K, Ogata M & Tanaka M (2004). Identification of promoter region of ghrelin gene in human medullary thyroid carcinoma cell line. *Life Sci* **75,** 2193-2201.

Nakamura T, Onaga T & Kitazawa T (2010). Ghrelin stimulates gastric motility of the guinea pig through activation of a capsaicin-sensitive neural pathway: *in vivo* and *in vitro* functional studies. *Neurogastroenterol Motil* **22,** 446-452, e107.

Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K & Matsukura S (2001). A role for ghrelin in the central regulation of feeding. *Nature* **409,** 194-198.

Ogawa A, Mochiki E, Yanai M, Morita H, Toyomasu Y, Ogata K, Ohno T, Asao T & Kuwano H (2012). Interdigestive migrating contractions are coregulated by ghrelin

and motilin in conscious dogs. *Am J Physiol Regul Integr Comp Physiol* **302,** R233-241.

Ohno T, Kamiyama Y, Aihara R, Nakabayashi T, Mochiki E, Asao T & Kuwano H (2006). Ghrelin does not stimulate gastrointestinal motility and gastric emptying: an experimental study of conscious dogs. *Neurogastroenterol Motil* **18,** 129-135.

Ohno T, Mochiki E & Kuwano H (2010). The roles of motilin and ghrelin in gastrointestinal motility. *Int J Pept* **2010**, 1-6.

Ohshiro H, Nonaka M & Ichikawa K (2008). Molecular identification and characterization of the dog motilin receptor. *Regul Pept* **146,** 80-87.

Okumura H, Nagaya N, Enomoto M, Nakagawa E, Oya H & Kangawa K (2002). Vasodilatory effect of ghrelin, an endogenous peptide from the stomach. *J Cardiovasc Pharmacol* **39,** 779-783.

Ottinger MA, Abdelnabi M, Li Q, Chen K, Thompson N, Harada N, Viglietti-Panzica C & Panzica GC (2004). The Japanese quail: a model for studying reproductive aging of hypothalamic systems. *Exp Gerontol* **39,** 1679-1693.

Pearse AG, Polak JM, Bloom SR, Adams C, Dryburgh JR & Brown JC (1974). Enterochromaffin cells of the mammalian small intestine as the source of motilin. *Virchows Arch B Cell Pathol* **16,** 111-120.

Peeters TL, Bormans V & Vantrappen G (1988). Comparison of motilin binding to crude homogenates of human and canine gastrointestinal smooth muscle tissue. *Regul Pept* **23,** 171-182.

Pemberton C, Wimalasena P, Yandle T, Soule S & Richards M (2003). C-terminal pro-ghrelin peptides are present in the human circulation. *Biochem Biophys Res Commun* **310,** 567-573.

Perboni S & Inui A (2010). Appetite and gastrointestinal motility: role of ghrelin-family peptides. *Clin Nutr* **29,** 227-234.

Perez-Tilve D, Heppner K, Kirchner H, Lockie SH, Woods SC, Smiley DL, Tschop M & Pfluger P (2011). Ghrelin-induced adiposity is independent of orexigenic effects. *FASEB J* **25,** 2814-2822.

Poitras P, Gagnon D & St-Pierre S (1992). N-terminal portion of motilin determines its biological activity. *Biochem Biophys Res Commun* **183,** 36-40.

Poitras P, Lahaie RG, St-Pierre S & Trudel L (1987). Comparative stimulation of motilin duodenal receptor by porcine or canine motilin. *Gastroenterology* **92,** 658-662.

Poitras P, Miller P, Gagnon D & St-Pierre S (1994). Motilin synthetic analogues and motilin receptor antagonists. *Biochem Biophys Res Commun* **205,** 449-454.

Poitras P & Peeters TL (2008). Motilin. *Curr Opin Endocrinol Diabetes Obes* **15,** 54-57.

Polak JM, Pearse AG & Heath CM (1975). Complete identification of endocrine cells in the gastrointestinal tract using semithin-thin sections to identify motilin cells in human and animal intestine. *Gut* **16,** 225-229.

Puigcerver M, Vinyoles D & Rodríguez-Teijeiro JD (2007). Does restocking with Japanese quail or hybrids affect native populations of common quail *Coturnix coturnix*? *Biol Conserv* **136**, 628–635.

Sakahara S, Xie Z, Koike K, Hoshino S, Sakata I, Oda S, Takahashi T & Sakai T (2010). Physiological characteristics of gastric contractions and circadian gastric motility in the free-moving conscious house musk shrew (*Suncus murinus*). *Am J Physiol Regul Integr Comp Physiol* **299,** R1106-1113.

Sakai T, Satoh M, Koyama H, Iesaki K, Umahara M, Fujikura K & Itoh Z (1994). Localization of motilin-immunopositive cells in the rat intestine by light microscopic immunocytochemistry. *Peptides* **15,** 987-991.

Sanger GJ (2012). Motilin receptor neuropharmacology: revised understanding. *Curr Opin Pharmacol* **12,** 641-646.

Satoh M, Sakai T, Koyama H, Shiba Y & Itoh Z (1995). Immunocytochemical localization of motilin-containing cells in the rabbit gastrointestinal tract. *Peptides* **16,** 883-887.

Schubert H & Brown JC (1974). Correction to the amino acid sequence of porcine motilin. *Can J Biochem* **52,** 7-8.

Seim I, Collet C, Herington AC & Chopin LK (2007). Revised genomic structure of the human ghrelin gene and identification of novel exons, alternative splice variants and natural antisense transcripts. *BMC Genomics* **8,** 298.

Seoane LM, Tovar S, Baldelli R, Arvat E, Ghigo E, Casanueva FF & Dieguez C (2000). Ghrelin elicits a marked stimulatory effect on GH secretion in freely-moving rats. *Eur J Endocrinol* **143,** R7-9.

Sjolund K, Sanden G, Hakanson R & Sundler F (1983). Endocrine cells in human intestine: an immunocytochemical study. *Gastroenterology* **85,** 1120-1130.

Strausberg RL, Feingold EA, Grouse LH, Derge JG, Klausner RD, Collins FS, Wagner L, Shenmen CM, Schuler GD, Altschul SF, Zeeberg B, Buetow KH, Schaefer CF, Bhat NK, Hopkins RF, Jordan H, Moore T, Max SI, Wang J, Hsieh F, Diatchenko L, Marusina K, Farmer AA, Rubin GM, Hong L, Stapleton M, Soares MB, Bonaldo MF, Casavant TL, Scheetz TE, Brownstein MJ, Usdin TB, Toshiyuki S, Carninci P, Prange C, Raha SS, Loquellano NA, Peters GJ, Abramson RD, Mullahy SJ, Bosak SA, McEwan PJ, McKernan KJ, Malek JA, Gunaratne PH, Richards S, Worley KC, Hale S, Garcia AM, Gay LJ, Hulyk SW, Villalon DK, Muzny DM, Sodergren EJ, Lu X, Gibbs RA, Fahey J, Helton E, Ketteman M, Madan A, Rodrigues S, Sanchez A, Whiting M, Young AC, Shevchenko Y, Bouffard GG, Blakesley RW, Touchman JW, Green ED, Dickson MC, Rodriguez AC, Grimwood J, Schmutz J, Myers RM, Butterfield YS, Krzywinski MI, Skalska U, Smailus DE, Schnerch A, Schein JE, Jones SJ & Marra MA (2002). Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. *Proc Natl Acad Sci USA* **99,** 16899-16903.

Strunz U, Domschke W, Mitznegg P, Domschke S, Schubert E, Wunsch E, Jaeger E & Demling L (1975). Analysis of the motor effects of 13-norleucine motilin on the rabbit, guinea pig, rat, and human alimentary tract *in vitro*. *Gastroenterology* **68,** 1485-1491.

Suzuki A, Ishida Y, Aizawa S, Sakata I, Tsutsui C, Mondal A, Kanako K & Sakai T (2012). Molecular identification of GHS-R and GPR38 in *Suncus murinus*. *Peptides* **36,** 29-38.

Tack J, Depoortere I, Bisschops R, Delporte C, Coulie B, Meulemans A, Janssens J & Peeters T (2006). Influence of ghrelin on interdigestive gastrointestinal motility in humans. *Gut* **55,** 327-333.

Takahashi T (2013). Interdigestive migrating motor complex-its mechanism and clinical importance. *J Smooth Muscle Res* **49,** 99-111.

Takeshita E, Matsuura B, Dong M, Miller LJ, Matsui H & Onji M (2006). Molecular characterization and distribution of motilin family receptors in the human gastrointestinal tract. *J Gastroenterol* **41,** 223-230.

Tatewaki M, Harris M, Uemura K, Ueno T, Hoshino E, Shiotani A, Pappas TN & Takahashi T (2003). Dual effects of acupuncture on gastric motility in conscious rats. *Am J Physiol Regul Integr Comp Physiol* **285,** R862-872.

Ter Beek WP, Muller ES, van den Berg M, Meijer MJ, Biemond I & Lamers CB (2008). Motilin receptor expression in smooth muscle, myenteric plexus, and mucosa of human inflamed and noninflamed intestine. *Inflamm Bowel Dis* **14,** 612-619.

Tolle V, Zizzari P, Tomasetto C, Rio MC, Epelbaum J & Bluet-Pajot MT (2001). *In vivo* and *in vitro* effects of ghrelin/motilin-related peptide on growth hormone secretion in the rat. *Neuroendocrinology* **73,** 54-61.

Tomasetto C, Karam SM, Ribieras S, Masson R, Lefebvre O, Staub A, Alexander G, Chenard MP & Rio MC (2000). Identification and characterization of a novel gastric peptide hormone: the motilin-related peptide. *Gastroenterology* **119,** 395-405.

Tschop M, Smiley DL & Heiman ML (2000). Ghrelin induces adiposity in rodents. *Nature* **407,** 908-913.

Tsutsui C, Kajihara K, Yanaka T, Sakata I, Itoh Z, Oda S & Sakai T (2009). House musk shrew (*Suncus murinus*, order: *Insectivora*) as a new model animal for motilin study. *Peptides* **30,** 318-329.

Van Assche G, Depoortere I, Thijs T, Janssens JJ & Peeters TL (1997). Concentration-dependent stimulation of cholinergic motor nerves or smooth muscle by [Nle13]motilin in the isolated rabbit gastric antrum. *Eur J Pharmacol* **337,** 267-274.

Vantrappen G, Janssens J, Hellemans J & Ghoos Y (1977). The interdigestive motor complex of normal subjects and patients with bacterial overgrowth of the small intestine. *J Clin Invest* **59,** 1158-1166.

Vantrappen G, Janssens J, Peeters TL, Bloom SR, Christofides ND & Hellemans J (1979). Motilin and the interdigestive migrating motor complex in man. *Dig Dis Sci* **24,** 497-500.

Wingate DL, Ruppin H, Green WE, Thompson HH, Domschke W, Wunsch E, Demling L & Ritchie HD (1976). Motilin-induced electrical activity in the canine gastrointestinal tract. *Scand J Gastroenterol Suppl* **39,** 111-118.

Wren AM, Small CJ, Ward HL, Murphy KG, Dakin CL, Taheri S, Kennedy AR, Roberts GH, Morgan DG, Ghatei MA & Bloom SR (2000). The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. *Endocrinology* **141,** 4325-4328.

Yakabi K, Kawashima J & Kato S (2008). Ghrelin and gastric acid secretion. *World J Gastroenterol* **14,** 6334-6338.

Yamamoto I, Kaiya H, Tsutsui C, Sakai T, Tsukada A, Miyazato M & Tanaka M (2008). Primary structure, tissue distribution, and biological activity of chicken motilin receptor. *Gen Comp Endocrinol* **156,** 509-514.

Yano H, Seino Y, Fujita J, Yamada Y, Inagaki N, Takeda J, Bell GI, Eddy RL, Fan YS, Byers MG & et al. (1989). Exon-intron organization, expression, and chromosomal localization of the human motilin gene. *FEBS Lett* **249,** 248-252.

Zhang JV, Ren PG, Avsian-Kretchmer O, Luo CW, Rauch R, Klein C & Hsueh AJ (2005). Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. *Science* **310,** 996-999.

Zheng J, Ariga H, Taniguchi H, Ludwig K & Takahashi T (2009). Ghrelin regulates gastric phase III-like contractions in freely moving conscious mice. *Neurogastroenterol Motil* **21,** 78-84.

Zietlow A, Nakajima H, Taniguchi H, Ludwig K & Takahashi T (2010). Association between plasma ghrelin and motilin levels during MMC cycle in conscious dogs. *Regul Pept* **164,** 78-82.

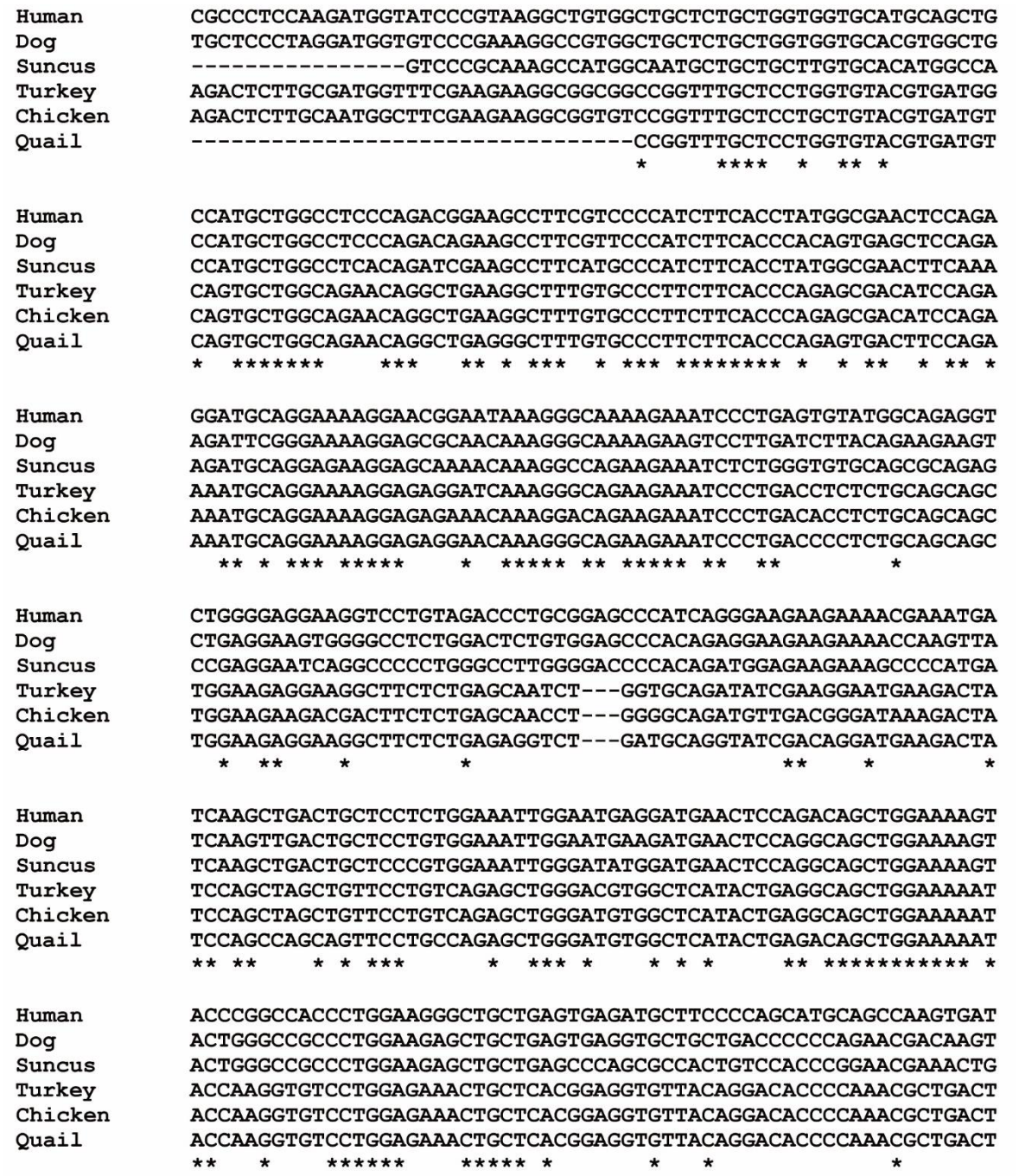

Figure 1: Multiple alignment of nucleotide sequences encoding a motilin precursor from human, dog, suncus, turkey, chicken, and quail. Asterisk (*) indicates entirely conserved regions among the species. The nucleotide sequence was deposited in the DDBJ/EMBL/GenBank databases with the Accession No. LC146647.1.

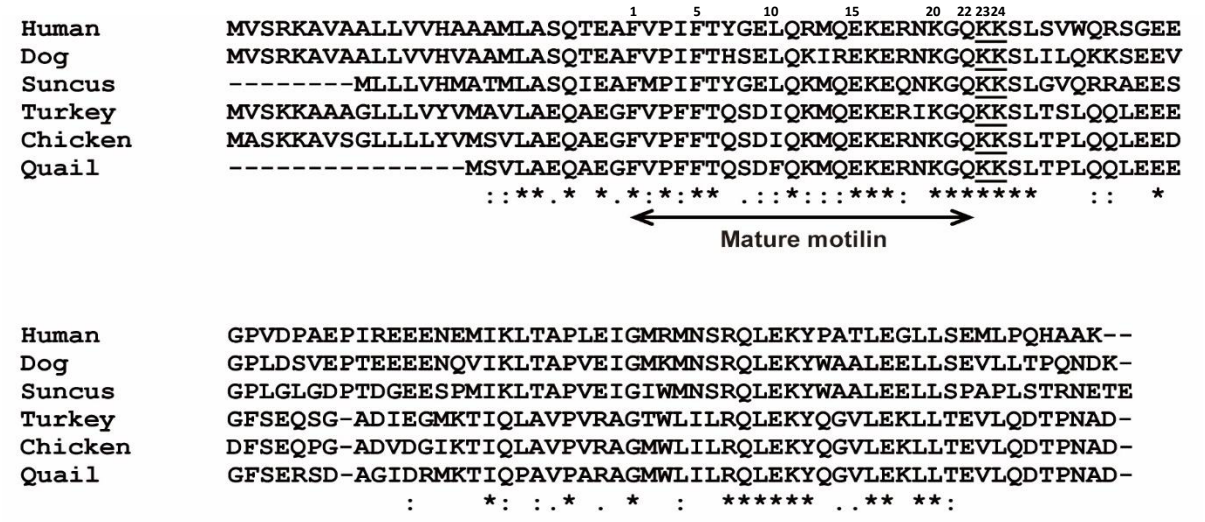

Figure 2: Multiple alignment of motilin precursor deduced from human, dog, suncus, turkey, chicken, and quail nucleotide sequences. Asterisk (*) indicates the entirely conserved regions among species. Dots (:) indicate conservation among groups (amino acids with strongly similar properties). A dot (.) indicates conservation among groups (amino acids with weakly similar properties). Double-headed arrow indicates the mature motilin peptide. A dibasic cleavage site (KK) (underlined) is also observed in the quail motilin sequence. The amino acid sequence of quail motilin (BAU80773.1) was aligned to that of human (AAI12315.1), dog (NP_001300735.1), suncus (BAI66099.1), turkey (XP_010722636.1), and chicken (NP_001292058.1) motilin.

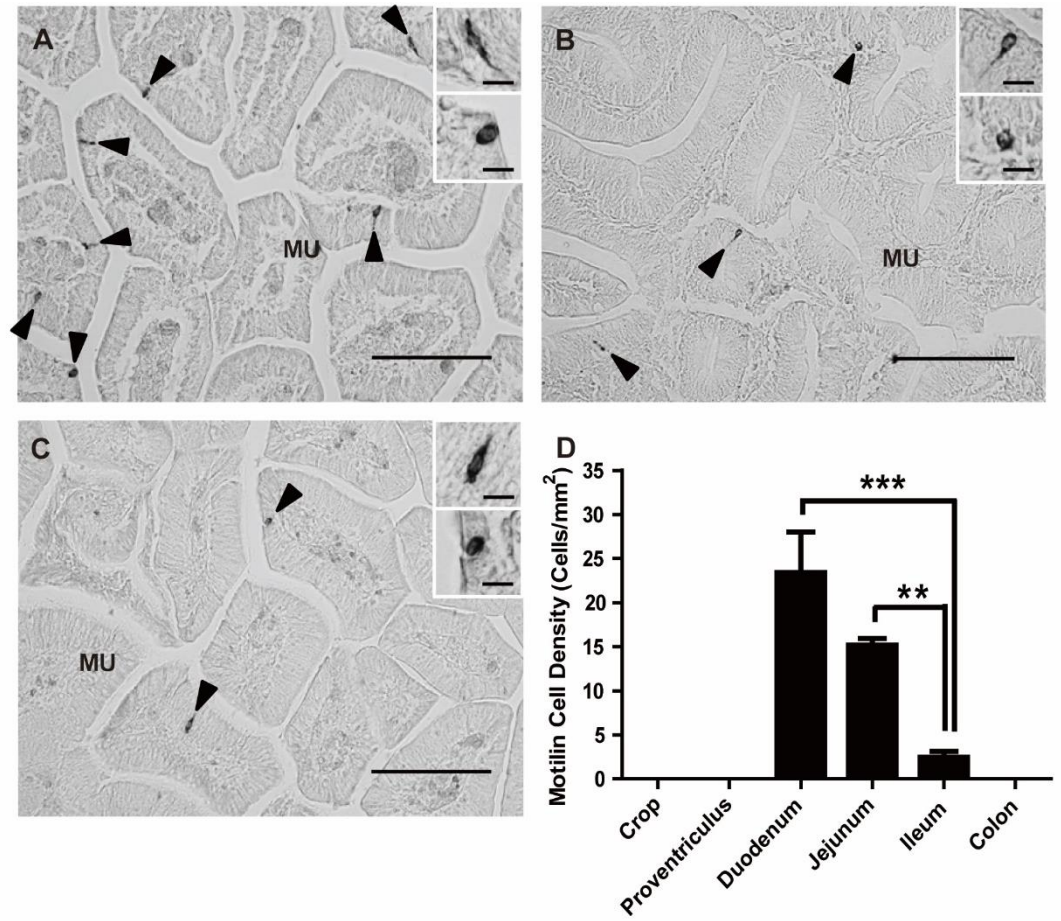

Figure 3: Microphotographs of motilin-ip cells in the quail intestine. Motilin-ip (motilin-immunopositive) cells (arrowheads) stained by immunohistochemistry were identified in the quail duodenum (A), jejunum (B), and ileum (C). Insets show high magnification of motilin cells. Two cell types, the open and closed types, were found in the mucosa. (D) Histogram showing the densities (cells/mm^2) of motilin-ip cells in the GI tract of quails. The densities of motilin-ip cells in the duodenum and jejunum were significantly higher than that in the ileum. Mu indicates mucosa. Scale Bars = 100 μm in (A), (B), and (C), and 10 μm in insets. Densities of motilin-ip cells in different tissues of the quail GI tract were compared by a one-way ANOVA followed by a Tukey's *post-hoc* test. ** $p < 0.01$, *** $p < 0.001$; $n = 3$.

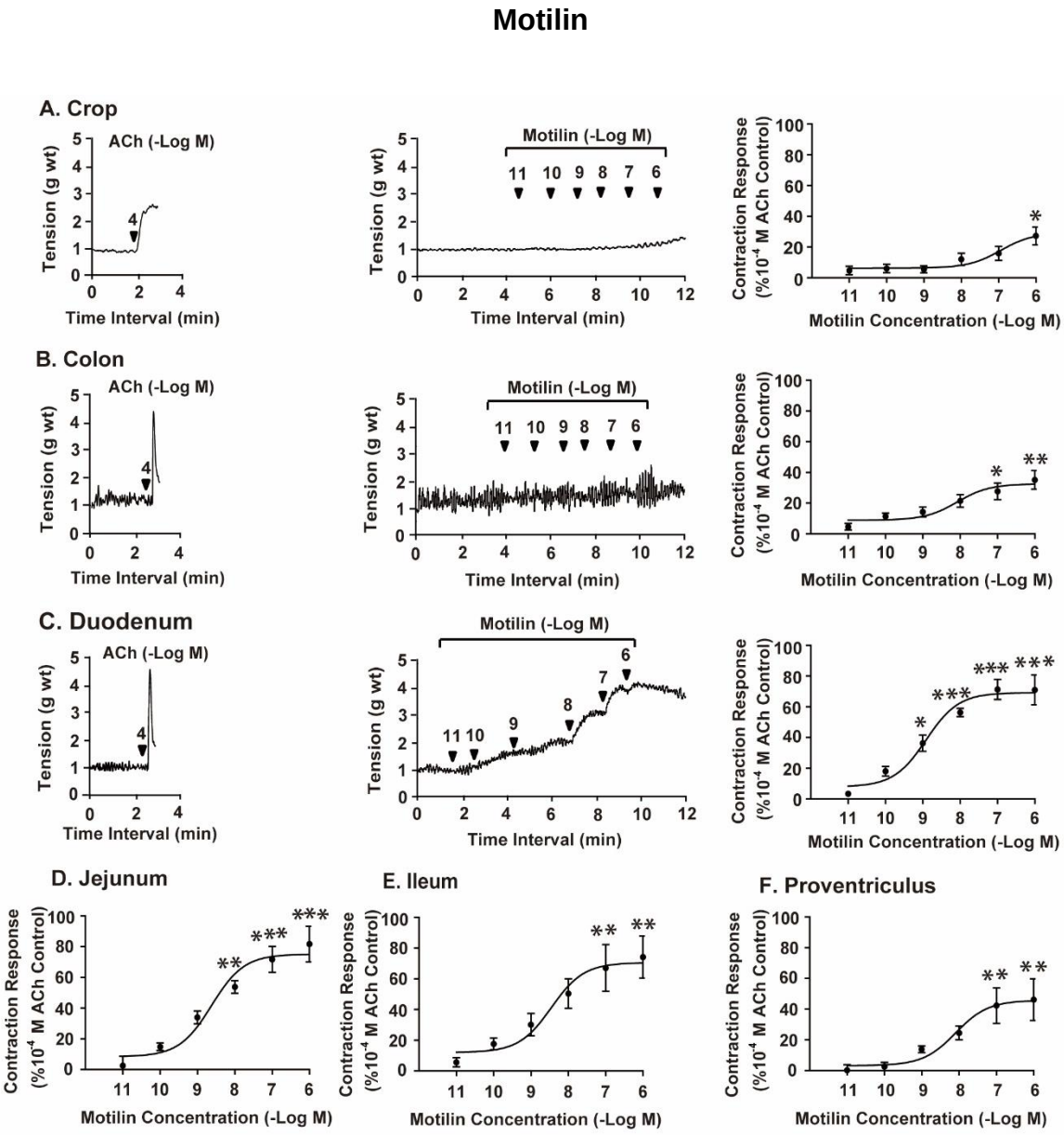

Figure 4: Chicken motilin-induced contractile activity in different regions of isolated quail GI tract. Left: traces showing the representative contractile response to ACh (10^{-4} M) in the isolated crop (A), colon (B), and duodenum (C). Center: responses to cumulative administration of chicken motilin (10^{-11}–10^{-6} M) in the isolated crop (A), colon (B), and duodenum (C). Right: motilin-induced concentration-response curve of isolated crop (A),

colon (B), and duodenum (C). The concentration-response curve to cumulative doses of motilin (10^{-11}–10^{-6} M) in the jejunum (D), ileum (E), and proventriculus (F) are also shown. Arrowheads indicate the timing of administration and the above number is the concentration of reagent (-Log M). Each value represents the mean ± SEM (n = 3). Significance was determined by a one-way ANOVA followed by a Tukey's *post-hoc* test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. 10^{-11} M motilin treatment).

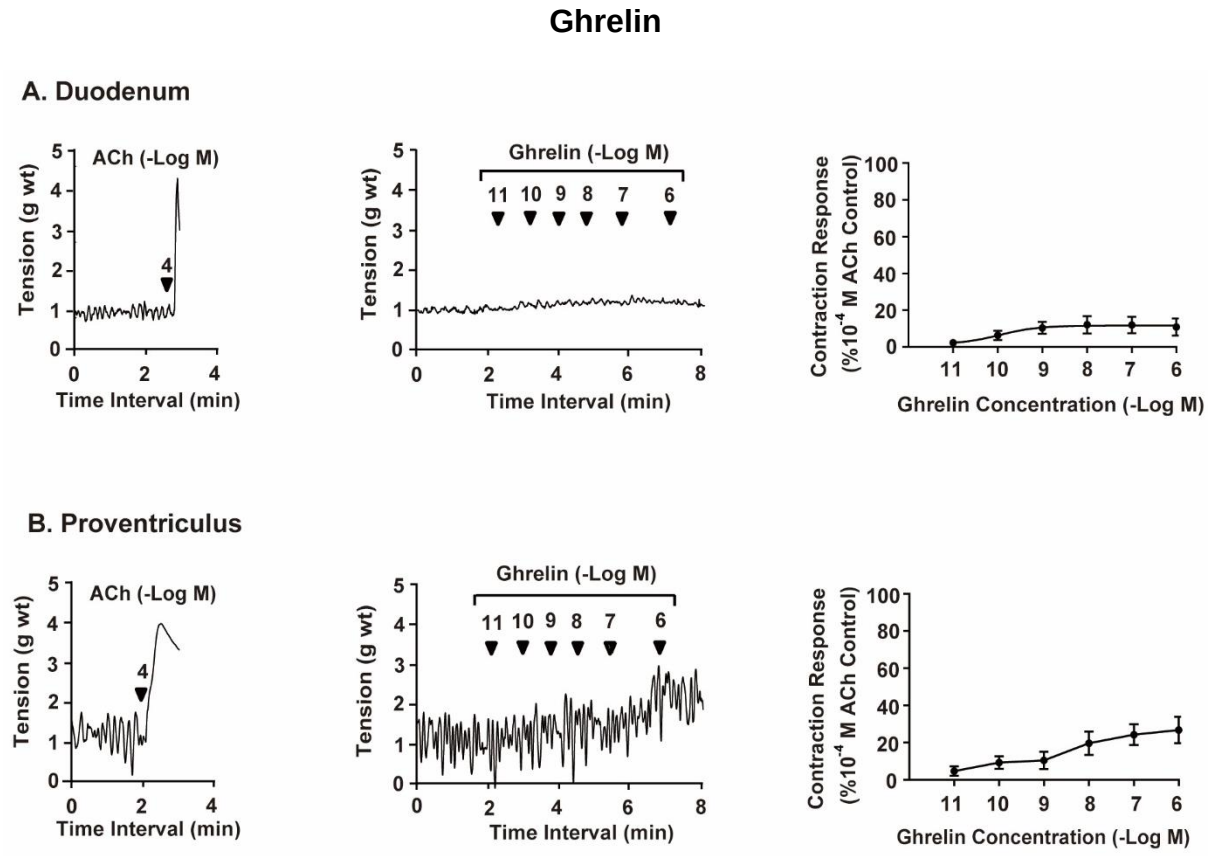

Figure 5: Effect of chicken ghrelin on quail duodenum and proventriculus *in vitro*. Left: traces showing the representative contractile response to ACh (10^{-4} M) in the isolated duodenum (A) and proventriculus (B). Center: responses to cumulative administration of chicken ghrelin (10^{-11}–10^{-6} M) in the isolated duodenum (A) and proventriculus (B). Right: chicken ghrelin-induced concentration-response curve in isolated duodenum (A) and proventriculus (B). Arrowheads indicate the timing of administration and above number is the concentration of reagent (-Log M). Each value represents the mean ± SEM (n = 4). Statistical analysis was performed by a one-way ANOVA followed by a Tukey's *post-hoc* test.

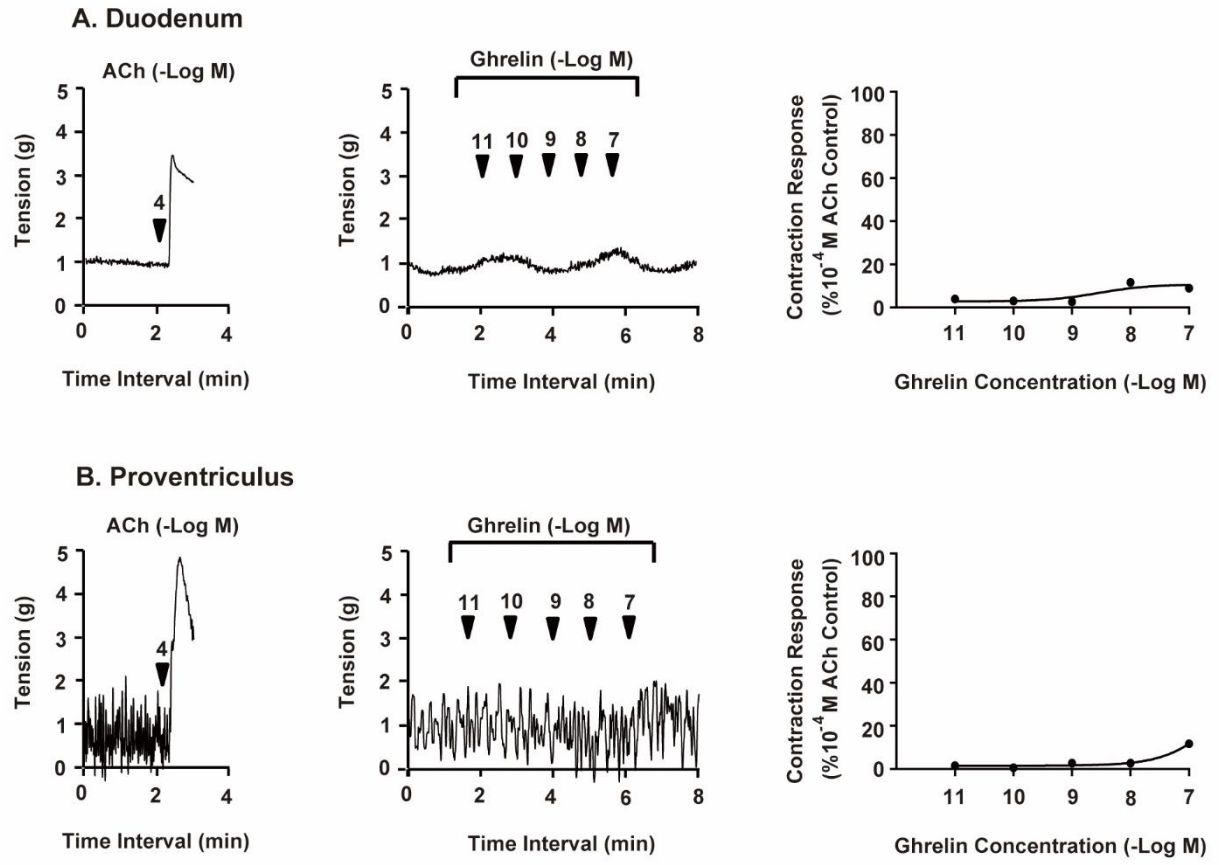

Figure 6: Effect of human ghrelin on contractions in the duodenum and proventriculus of Japanese quails. Left: traces showing the representative contractile response to ACh (10^{-4} M) in the isolated duodenum (A) and proventriculus (B). Center: responses to cumulative administration of human ghrelin (10^{-11}–10^{-7} M) in the isolated duodenum (A) and proventriculus (B). Right: human ghrelin-induced concentration-response curve in the isolated duodenum (A) and proventriculus (B). Arrowheads indicate the timing of administration and the above number is the concentration of the reagent (-Log M). Each value represents the mean ± SEM (n = 4). Statistical analysis was performed by a one-way ANOVA followed by a Tukey's *post-hoc* test.

Motilin + Ghrelin

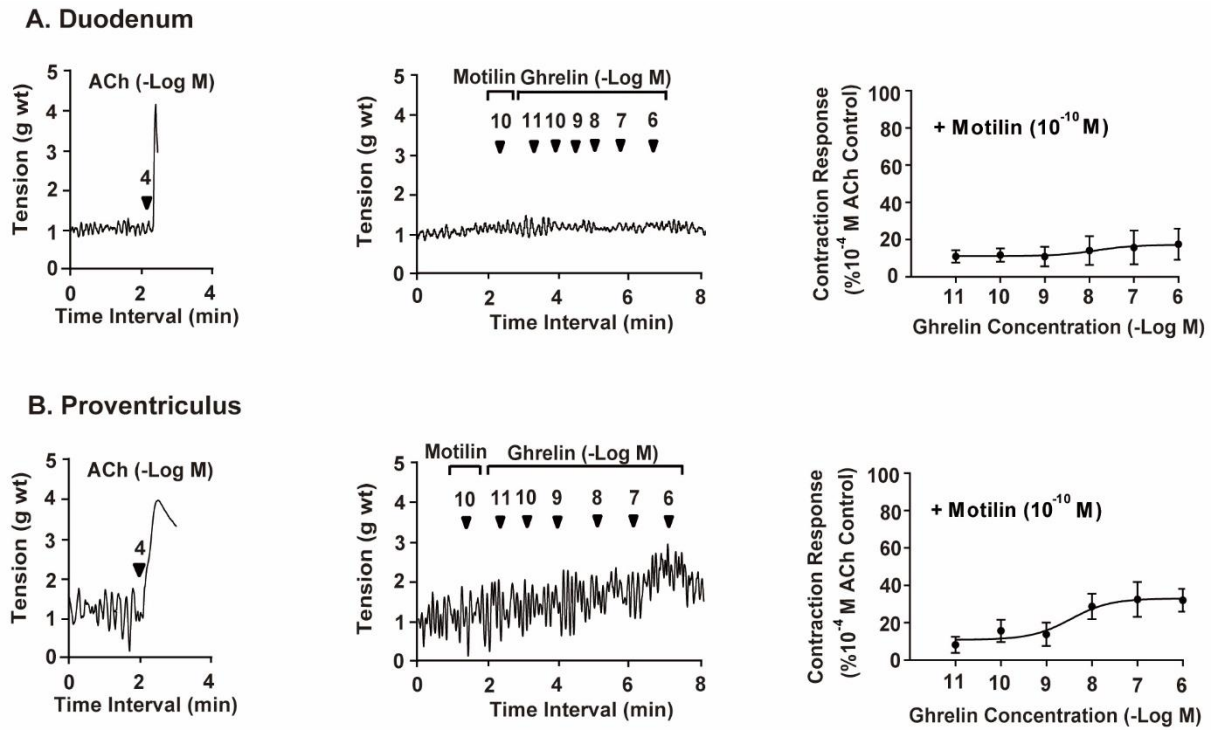

Figure 7: Effect of co-administration of chicken motilin and chicken ghrelin on the quail duodenum and proventriculus *in vitro*. Left: traces showing the representative contractile response to ACh (10^{-4} M) in the isolated duodenum (A) and proventriculus (B). Center: responses to cumulative administration of chicken ghrelin (10^{-11}–10^{-6} M) with pretreatment of 10^{-10} M chicken motilin in the isolated duodenum (A) and proventriculus (B). Right: co-administration of motilin and ghrelin-induced concentration-response curve in the isolated duodenum (A) and proventriculus (B). Arrowheads indicate the timing of administration and the above number is the concentration of the reagent (-Log M). Each value represents the mean ± SEM (n = 4). Statistical analysis was performed by a one-way ANOVA followed by a Tukey's *post-hoc* test.

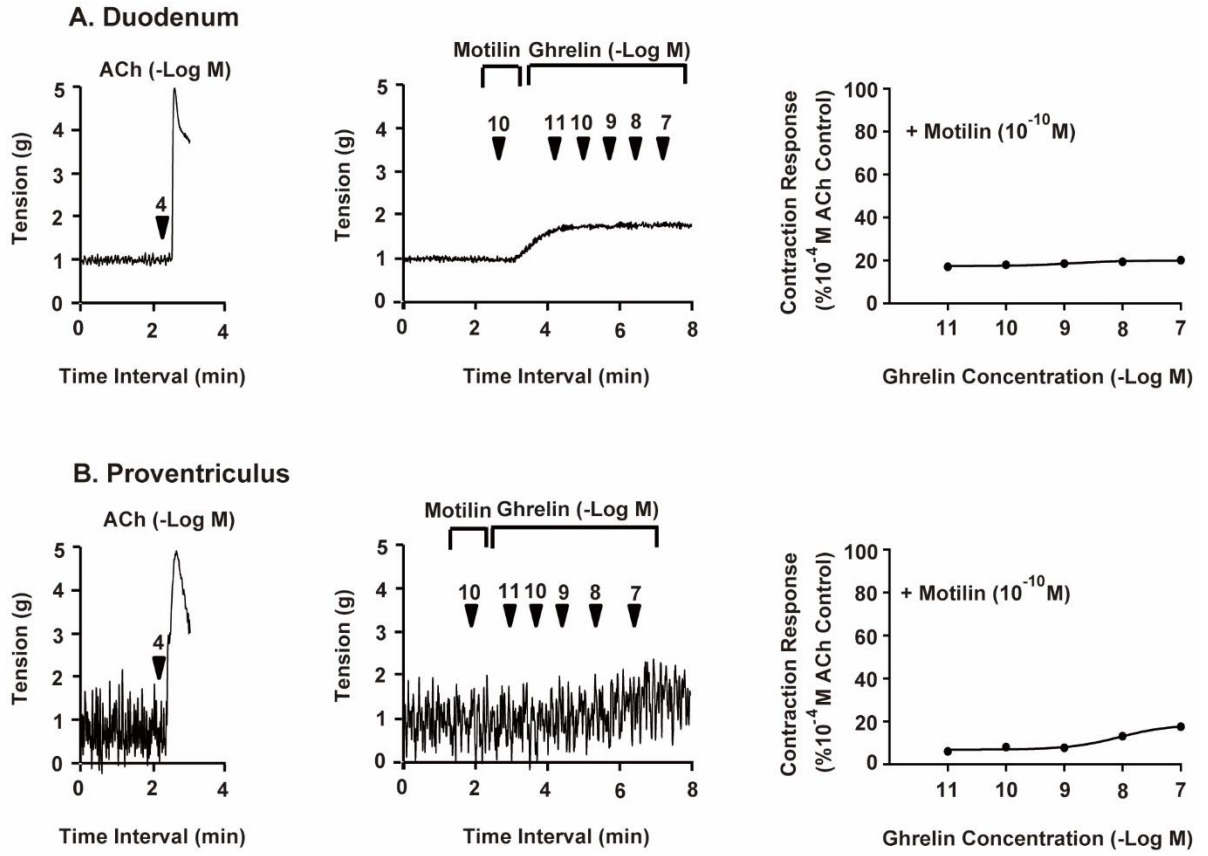

Figure 8: Effect of co-administration of chicken motilin and human ghrelin on contractions in the duodenum and proventriculus of Japanese quails. Left: traces showing the representative contractile response to ACh (10^{-4} M) in the isolated duodenum (A) and proventriculus (B). Center: responses to cumulative administration of human ghrelin (10^{-11}–10^{-7} M) with pretreatment of 10^{-10} M chicken motilin in isolated duodenum (A) and proventriculus (B). Right: co-administration of chicken motilin and human ghrelin-induced concentration-response curve in the isolated duodenum (A) and proventriculus (B). Arrowheads indicate the timing of administration and the above number is the concentration of the reagent (-Log M). Each value represents the mean ± SEM (n = 3). Statistical analysis was performed using a one-way ANOVA followed by a Tukey's *post-hoc* test.

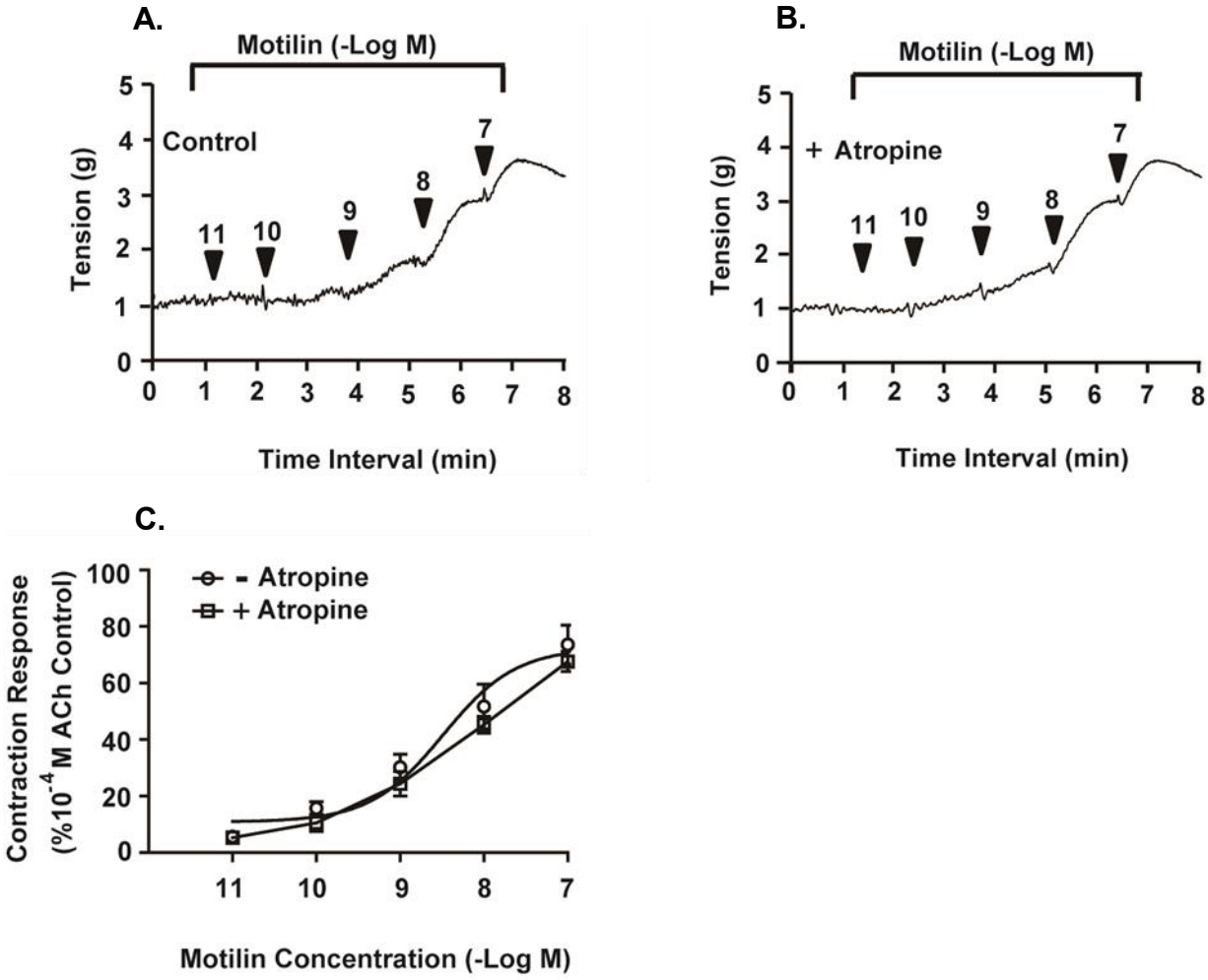

Figure 9: Effect of atropine on motilin-induced contraction in the duodenum of Japanese quails. (A) Motilin induced dose-dependent contraction in the duodenum. (B) Pretreatment of atropine (10^{-6} M) with chicken motilin (10^{-11}–10^{-7} M)-induced contraction. (C) The concentration-response curve showing that atropine did not inhibit motilin-induced duodenal contraction in Japanese quails. Arrowheads indicate the timing of administration and the above number is the concentration of motilin (-Log M). Each value represents the mean ± SEM (n = 4). A student's *t*-test was used to compare differences for the same dose between control and antagonist treatments. ○: Control; □: antagonist treatment.

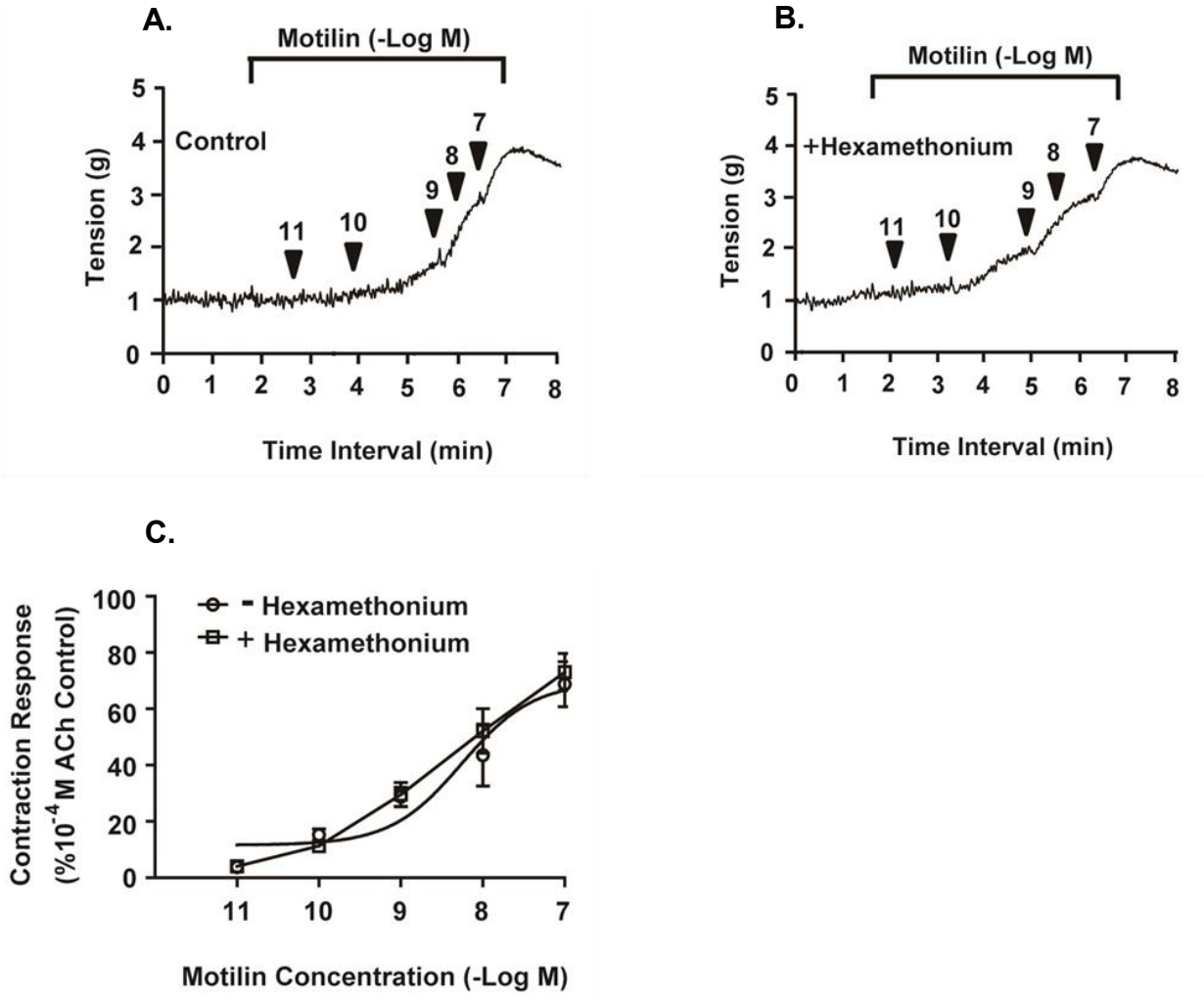

Figure 10: Effect of hexamethonium on motilin-induced contraction in the duodenum of Japanese quails. (A) Motilin induced dose-dependent contraction in the duodenum. (B) Pretreatment of hexamethonium (200 μM) with motilin-induced contraction. (C) Concentration-response curve showing that hexamethonium did not change motilin-induced contraction in the duodenum of Japanese quails. Arrowheads indicate the timing of administration and the above number is the concentration of motilin (-Log M). Each value represents the mean ± SEM (n = 4). A student's *t*-test was used to compare differences of the same dose between control and antagonist treatments. ○: Control; □: antagonist treatment.

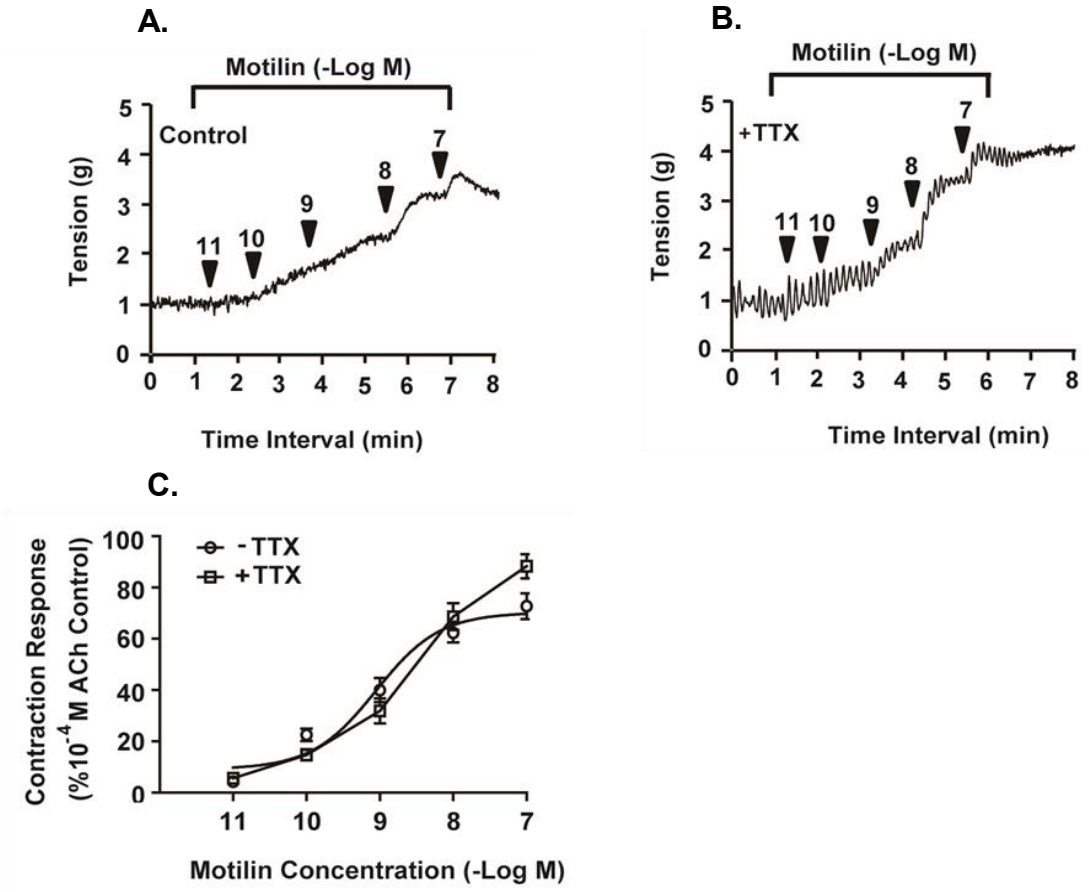

Figure 11: Effect of tetrodotoxin (TTX) on motilin-induced duodenal contraction in Japanese quails. (A) Motilin induced dose-dependent (10^{-11}–10^{-7} M) duodenal contraction in the Japanese quail, which started after 10^{-10} M motilin administration. (B) Pretreatment with tetrodotoxin (TTX; 10^{-6} M) did not inhibit motilin-induced quail duodenal contraction. (C) Concentration-response curve showing that motilin-mediated contraction in the duodenum was not affected by TTX pretreatment. Arrowheads indicate the timing of administration and the above number is the concentration of motilin (-Log M). Each value represents the mean ± SEM (n = 4). A student's *t*-test was used to compare differences of the same dose between control and antagonist treatments. ○: Control; □: TTX treatment.

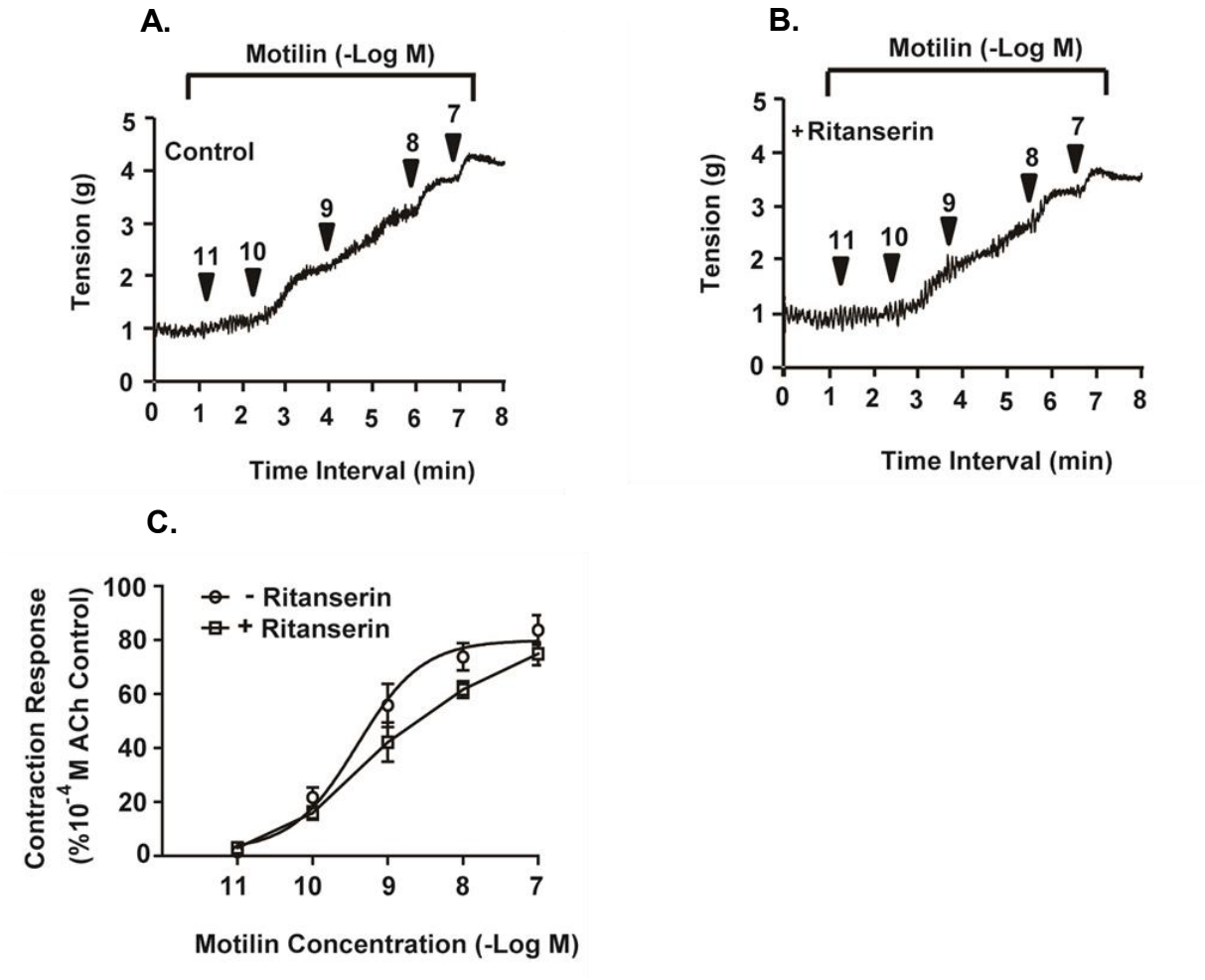

Figure 12: Effect of $5HT_2$ receptor antagonist (ritanserin) on motilin-induced contraction in the duodenum of Japanese quails. (A) Motilin induced dose-dependent contraction in the duodenum. (B) Ritanserin (10^{-7} M)-pretreated chicken motilin (10^{-11}–10^{-7} M)-induced contraction. (C) The concentration-response curve showing that ritanserin did not inhibit motilin-induced duodenal contraction. Arrowheads indicate the timing of administration and the above number is the concentration of motilin (-Log M). Each value is the mean ± SEM (n = 4). A student's *t*-test was used to compare differences of the same dose between control and antagonist treatments. ○: Control; □: antagonist treatment.

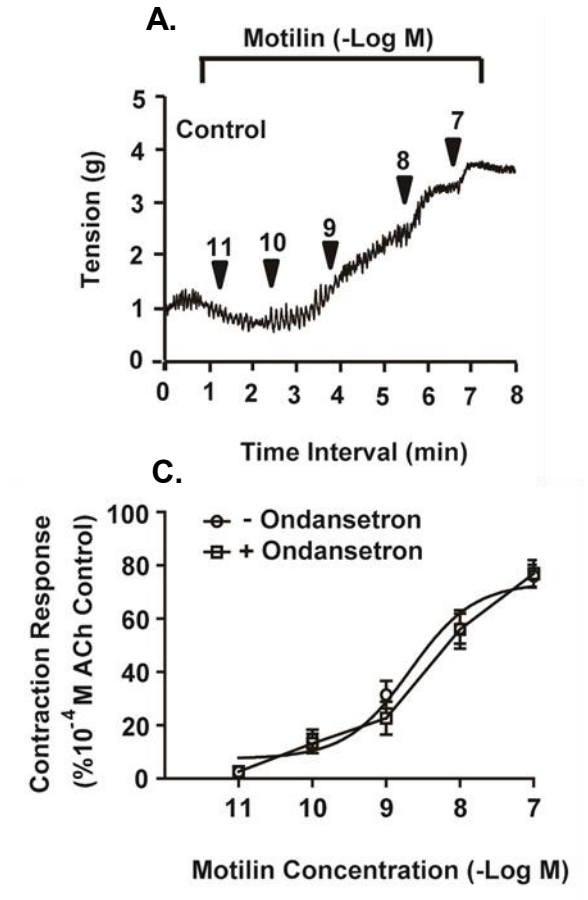

Figure 13: Effect of $5HT_3$ receptor antagonist (ondansetron) on motilin-induced duodenal contraction in Japanese quails. (A) Motilin induced dose-dependent contraction in the duodenum. (B) Motilin-induced contraction with ondansetron pretreatment (10^{-5} M). (C) Concentration-response curve showing that ondansetron had no effect on motilin-induced contraction. Arrowheads indicate the timing of administration and the above number is the concentration of motilin (-Log M). Each value represents the mean ± SEM (n = 4). A student's *t*-test was used to compare differences of the same dose between control and antagonist treatments. ○: Control; □: antagonist treatment.

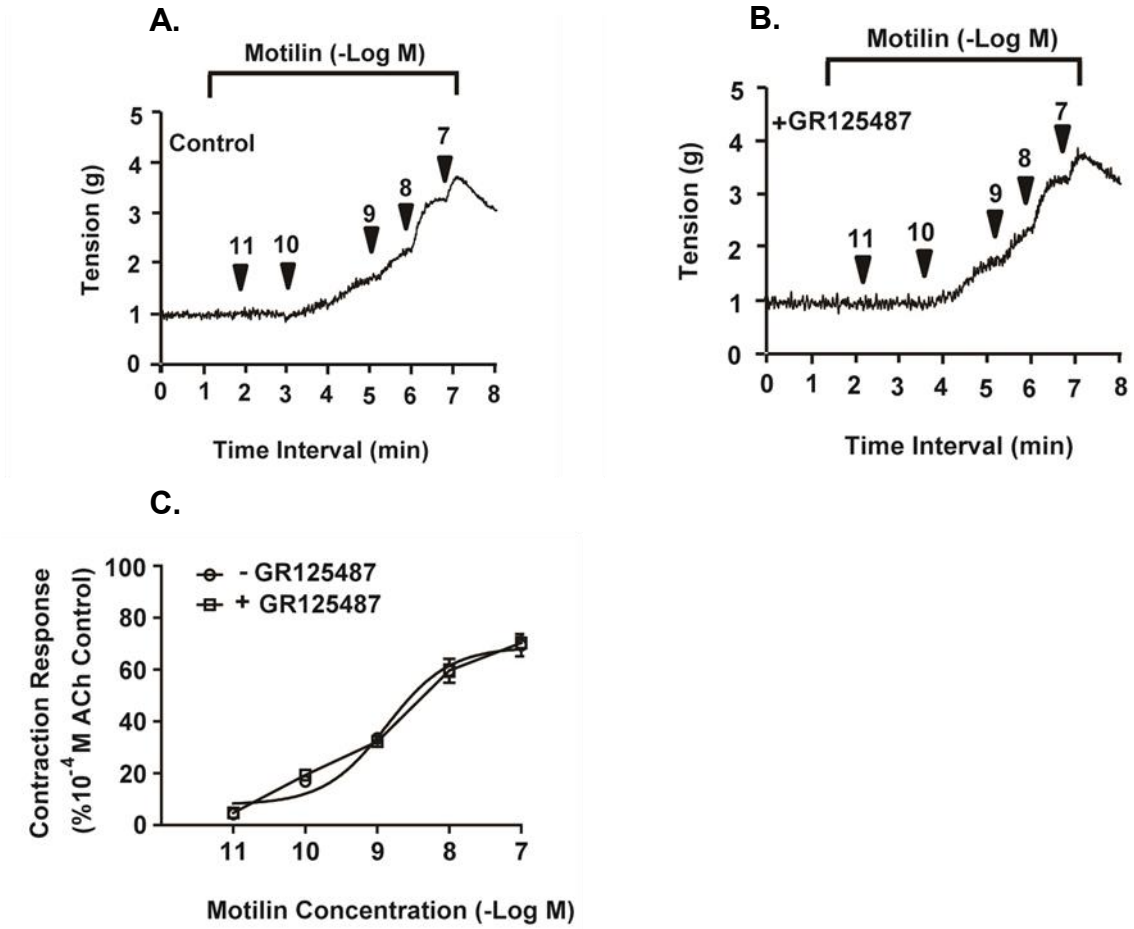

Figure 14: Effect of $5HT_4$ receptor antagonist (GR125487) on motilin-induced duodenal contraction in Japanese quails. (A) Motilin induced dose-dependent contraction in the duodenum. (B) Motilin-induced contraction with GR125487 pretreatment (10^{-8} M). (C) Concentration-response curve showing that GR125487 did not affect motilin-induced contraction in the duodenum of Japanese quails. Arrowheads indicate the timing of administration and the above number is the concentration of motilin (-Log M). Each value represents the mean ± SEM (n = 4). A student's *t*-test was used to compare differences of the same dose between control and antagonist treatments. ○: Control; □: antagonist treatment.

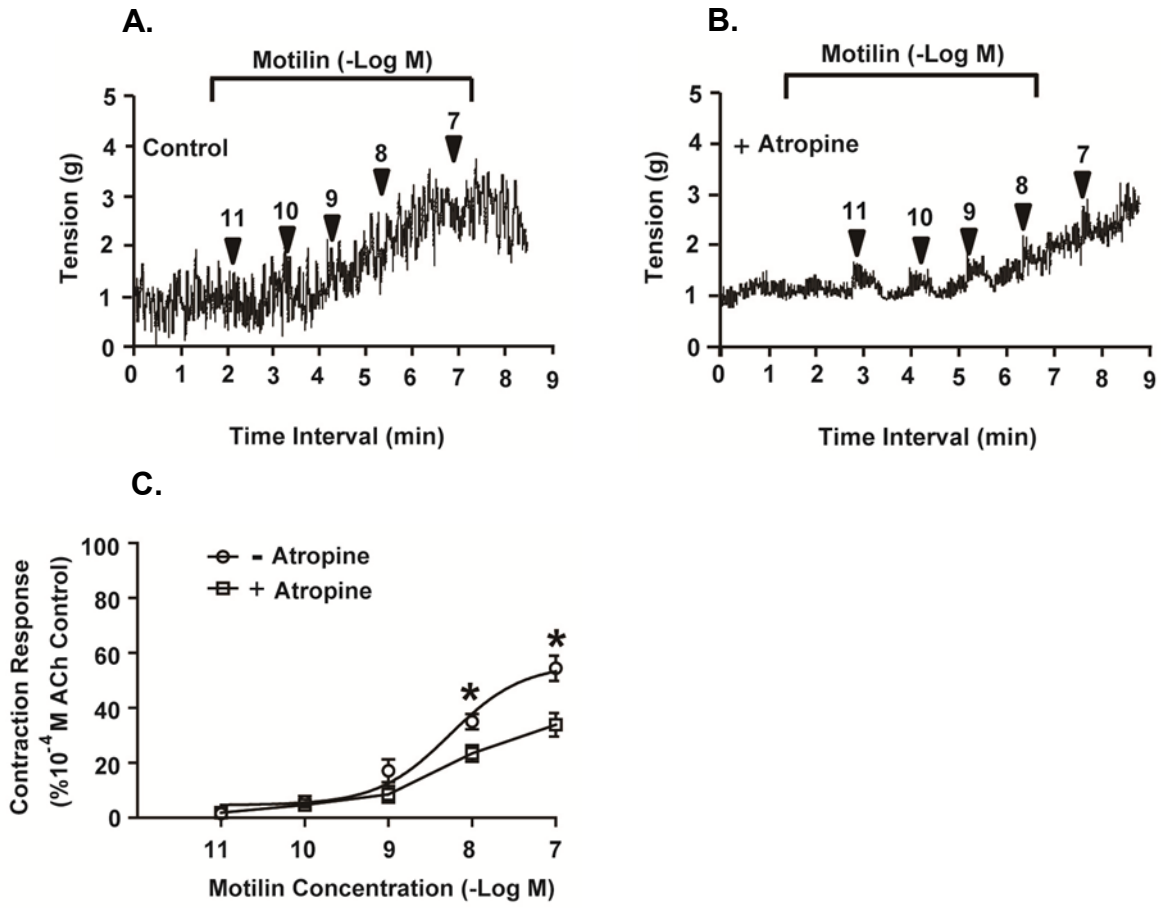

Figure 15: Effect of atropine on motilin-induced contraction in the proventriculus of Japanese quails. (A) Motilin evoked a dose-dependent contraction in the proventriculus. (B) Atropine (10^{-6} M) pretreatment in chicken motilin (10^{-11}-10^{-7} M)-induced contraction. (C) The concentration-response curve showing that motilin-induced proventricular contraction was significantly inhibited by atropine pretreatment. Arrowheads indicate the timing of administration and the above number is the concentration of motilin (-Log M). Each value represents the mean ± SEM (n = 4). ○: Control; □: antagonist treatment. Asterisk (*) denotes statistical significance between control and antagonist treatments. * $p < 0.05$.

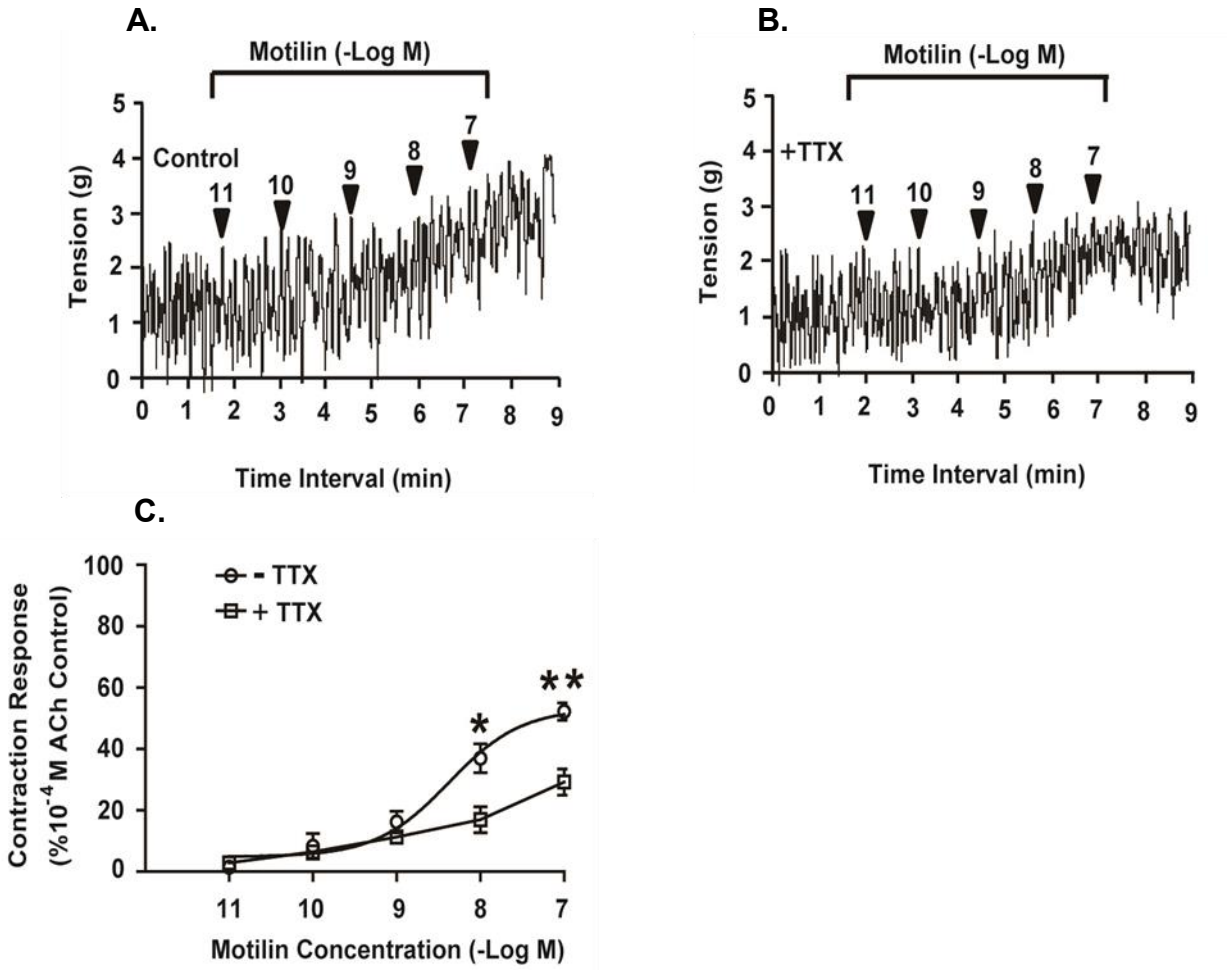

Figure 16: Effect of tetrodotoxin (TTX) on motilin-induced contraction in the proventriculus of Japanese quails. (A) Motilin evoked dose-dependent contraction in the proventriculus. (B) Motilin-evoked contraction with TTX pretreatment (10^{-6} M). (C) Concentration-response curve showing that TTX pretreatment significantly blocked motilin-evoked contraction in the proventriculus of Japanese quails. Arrowheads indicate the timing of administration and the above number is the concentration of motilin (-Log M). Each value represents the mean ± SEM (n = 4). ○: Control; □: antagonist treatment. Asterisk denotes statistical significance between control and antagonist treatments. * $p < 0.05$, ** $p < 0.01$.

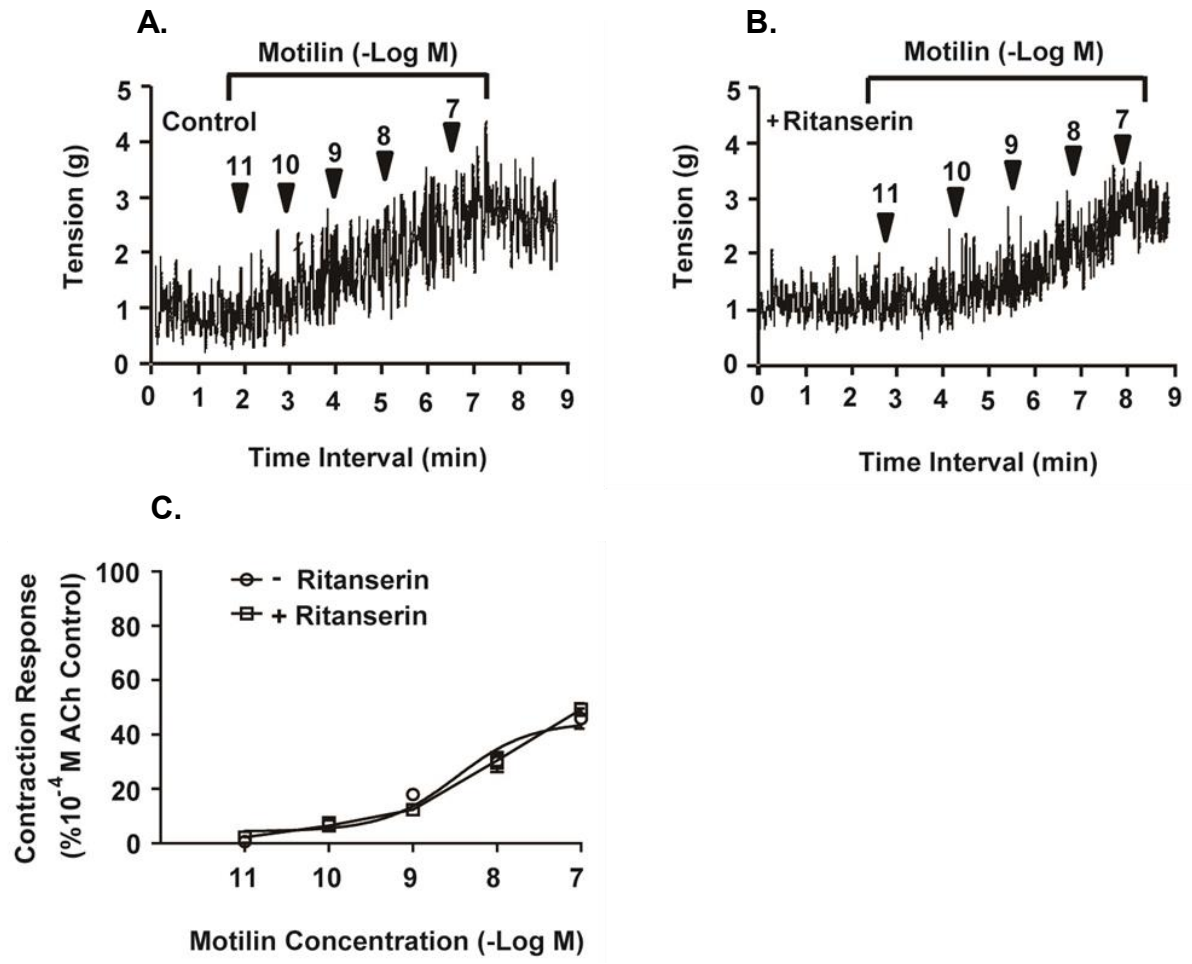

Figure 17: Effect of $5HT_2$ receptor antagonist (ritanserin) on motilin-evoked contraction in the proventriculus of Japanese quails. (A) Motilin evoked a dose-dependent contraction in the proventriculus. (B) Pretreatment of ritanserin (10^{-7} M) with chicken motilin (10^{-11}–10^{-7} M)-induced contraction. (C) The concentration-response curve showing that ritanserin did not inhibit motilin-induced contraction in the proventriculus of Japanese quails. Arrowheads indicate the timing of administration and the above number is the concentration of motilin (-Log M). Each value represents the mean ± SEM (n = 4). ○: Control; □: antagonist treatment. A student's *t*-test was used to compare differences of the same dose between the control and antagonist treatments.

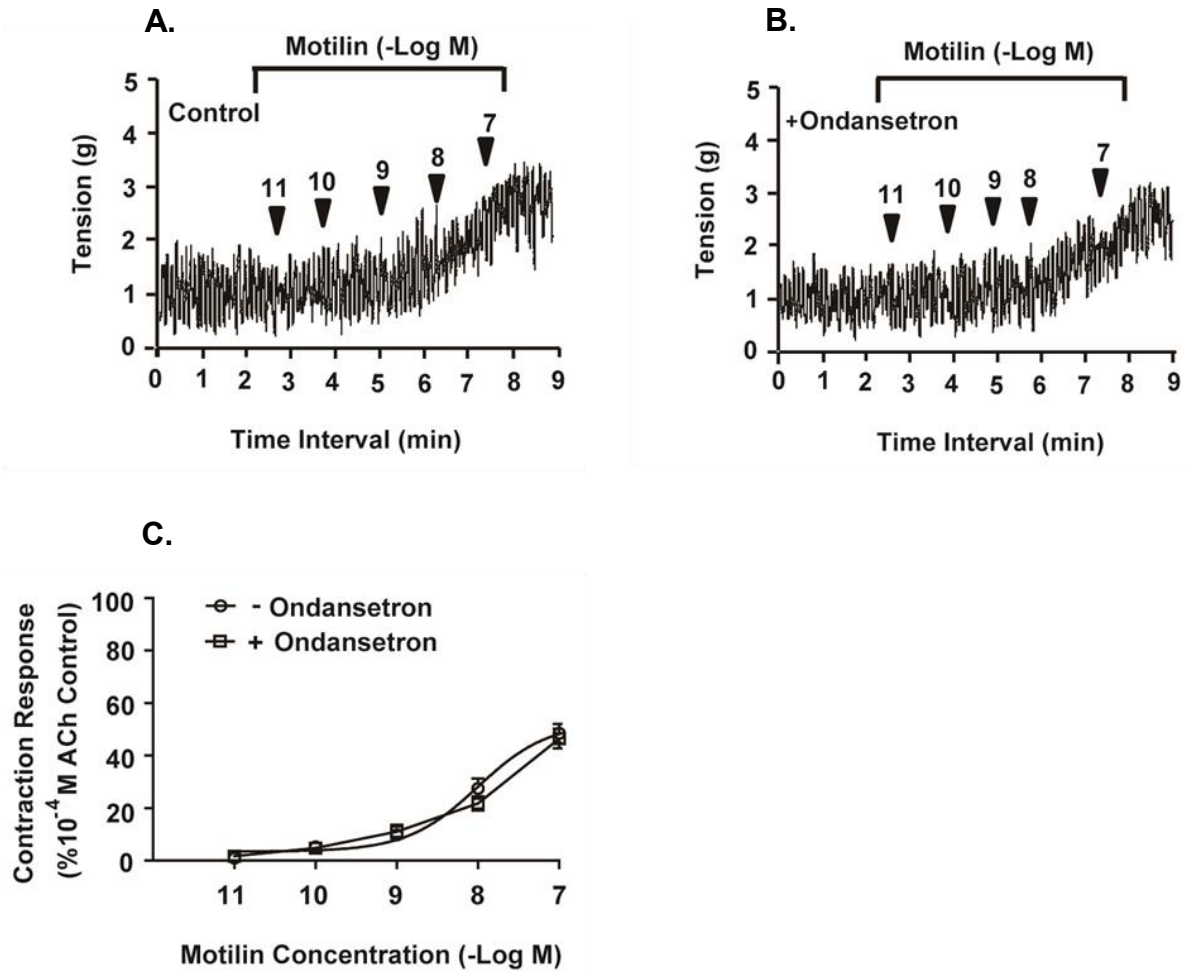

Figure 18: Effect of $5HT_3$ receptor antagonist (ondansetron) on motilin-evoked contraction in the proventriculus of Japanese quails. (A) Motilin induced dose-dependent contraction in the proventriculus. (B) Motilin-induced contraction with ondansetron pretreatment (10^{-5} M). (C) Concentration-response curve showing that ondansetron had no effect on motilin-induced contraction in the proventriculus of Japanese quails. Arrowheads indicate the timing of administration and the above number is the concentration of motilin (-Log M). Each value represents the mean ± SEM (n = 4). ○: Control; □: antagonist treatment. A student's *t*-test was used to compare differences of the same dose between control and antagonist treatments.

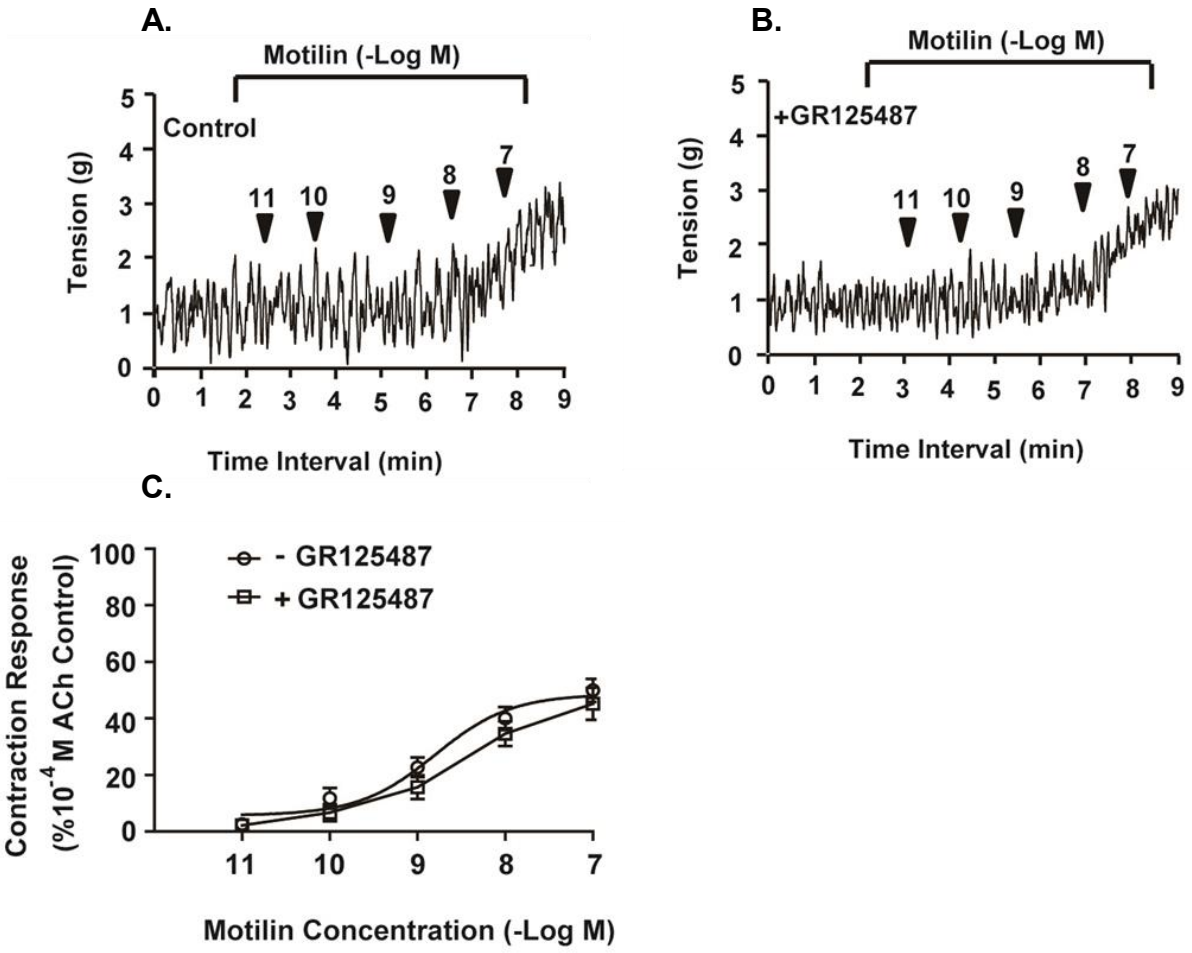

Figure 19: Effect of $5HT_4$ receptor antagonist (GR125487) on motilin-induced contraction in the proventriculus of Japanese quails. (A) Motilin evoked dose-dependent contraction in the proventriculus. (B) Motilin-induced contraction with GR125487 pretreatment (10^{-8} M). (C) Concentration-response curve showing that GR125487 did not affect motilin-induced contraction in the proventriculus of Japanese quails. Arrowheads indicate the timing of administration and the above number is the concentration of motilin (-Log M). Each value represents the mean ± SEM (n = 4). ○: Control; □: antagonist treatment. A student's *t*-test was used to compare differences in the same dose between control and antagonist treatments.

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Human           TCATCGGGCGGGAGCTGTGGAGCAGCCGGCGGCCGCTGCGAGGCCCGGCCGCCTCGGGGC
Pig             TCATCGGGCGGGAGCTGTGGCGGAGCCGCGGGCCGCTGCGAAGCCCGGCCATCCCCGGGC
Dog             GCATCGGCCGCGAGCTGCGGAGGCGCCGGGGGCCGCTGCGGGGCCGGGCCGCCTCGGGGC
Rabbit          TCATCGCGCGGCAGCTGTGGCGGGGTCGGGGCCCGCTGCGAGGCCCGGCGGCCACGGGTC
Chicken         TCATCGGGCGCGAGCTGTGGCGCAGCAGGGGCCGTCTGAGGGGTCCCGGCTCGGCCCTCC
Turkey          TCATCGGGCGCGAGCTGTGGCGCAGCAGGGGCCGCCTGCGGGGTCCCGGCGCTGCCCTCC
Quail           --------------------CGCAGCCGGGGCCGCCTCAGGGGTCCGGGCGCGGCCCTGC
                                     *  *  *  * *  **  *  * * *  *     *    *

Human           GGGAGAGAGGCCACCGGCAGACCGTCCGCGTCCTGCTGGTGGTGGTTCTGGCATTTATAA
Pig             GGGAGAAGGGCCACCGGCAGACCGTCCGCGTCCTGCTGGTGGTGGTTCTGGCATTTATAG
Dog             GCGAGCGGGGCCACCGCCAGGCCGTCCGCGTGCTGCTGGCCGTGGTGCTGGCGTTCCTGG
Rabbit          GGGAGAGGGGCCACCGGCAGACCGTCCGCGTCCTGCTGGTGGTGGTTCTGGCCTTTATAG
Chicken         GGGAGCGGGGCCACCGGCAGACCGTCAGGATCCTGGCTGTGGTGATCCTGGCCTTTGTAA
Turkey          GGGAGCGGGGCCACCGGCAGACCGTCAGGATCCTGGCTGTGGTGATTCTGGCCTTTGTAA
Quail           GGGAGCGGGGACACCGGCACACCGTCAGGATCCCGGCTGTGGTGATCCTGGCCTTTGTAA
                * ***   ** ***** **  ***** *  * * *   *  *** * ***** **  *

Human           TTTGCTGGTTGCCCTTCCACGTTGGCAGAATCATTTACATAAACACGGAAGATTCGCGGA
Pig             TTTGCTGGTTGCCTTTCCACGTTGGCAGAATCATTTACATAAATACTGAAGATTCCCGGA
Dog             TGTGCTGGCTGCCCTTCCACGTGGGCAGGATCATCTACATAAATACCGAAGACTCGCGCA
Rabbit          TGTGCTGGCTGCCTTTCCACGTTGGCAGGATCATTTACATAAACACCCAAGACTCGCGGA
Chicken         TTTGCTGGTTGCCTTTCCACATTGGCAGGATCATATTTATAAGCACCCGGGACACCAGAA
Turkey          TTTGCTGGTTGCCTTTCCACATTGGCAGGATCGTATTTATAAACACCCGAGACACCAGAA
Quail           CTTGCTGGTTGCCTTTCCACATTGGCAGGATCATATTTATAAGCACCCGGGACACCAGAA
                  ****** **** ****** * ***** *** * *  ****  **    **  *  * *

Human           TGATGTACTTCTCTCAGTACTTTAACATCGTCGCTCTGCA---
Pig             TGATGTACTTCTCTCAGTATTTTAACATCGTTGCTCTGCA---
Dog             TGATGCACTTCTCTCAGTACTTTAACATCGTGGCGCTGCA---
Rabbit          TGATGTACTTCTCCCAGTACTTTAACATTGTCGCGCTGCA---
Chicken         CGATGCTGTTCTCCCAATACTTCAACATCTTTGCCCTGCA---
Turkey          CGATGCTGTTCTCCCAGTACTTCAACATCTTTGCCCTGCAGCT
Quail           CGATGCTGTTCTCCCAATACTTC--------------------
                 ****   ***** ** ** **
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Figure 20: Comparison of partial sequence of quail GPR38 with known GPR38 sequences of human, pig, dog, rabbit, chicken, and turkey. Asterisk (*) indicates completely conserved regions among species.

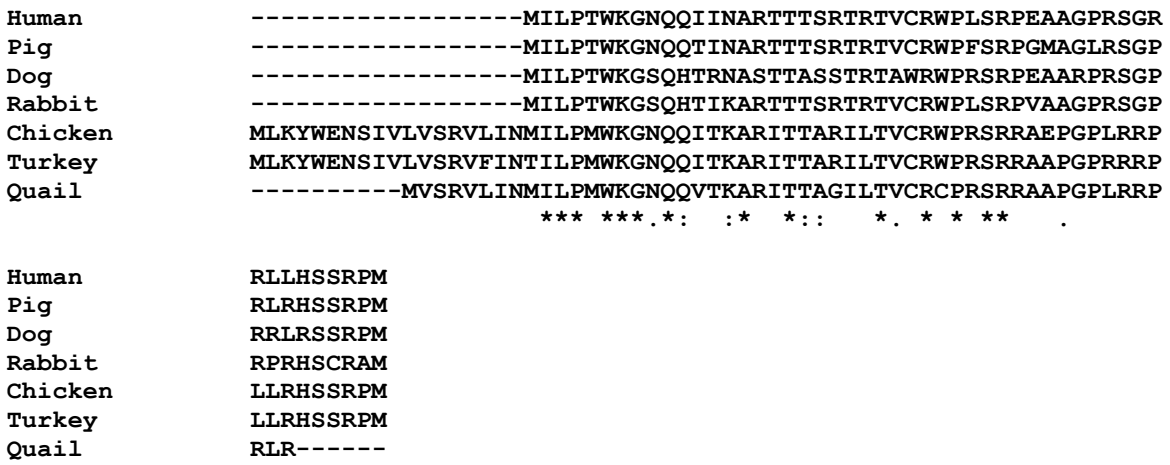


Figure 21: Comparison of partial GPR38 amino acid sequence deduced from human, pig, dog, rabbit, chicken, turkey, and quail nucleotide sequences. Asterisk (*) indicates completely conserved regions among species. Dots (:) indicate conservation among groups (amino acids with strongly similar properties). A dot (.) indicates conservation among groups (amino acids with weakly similar properties).

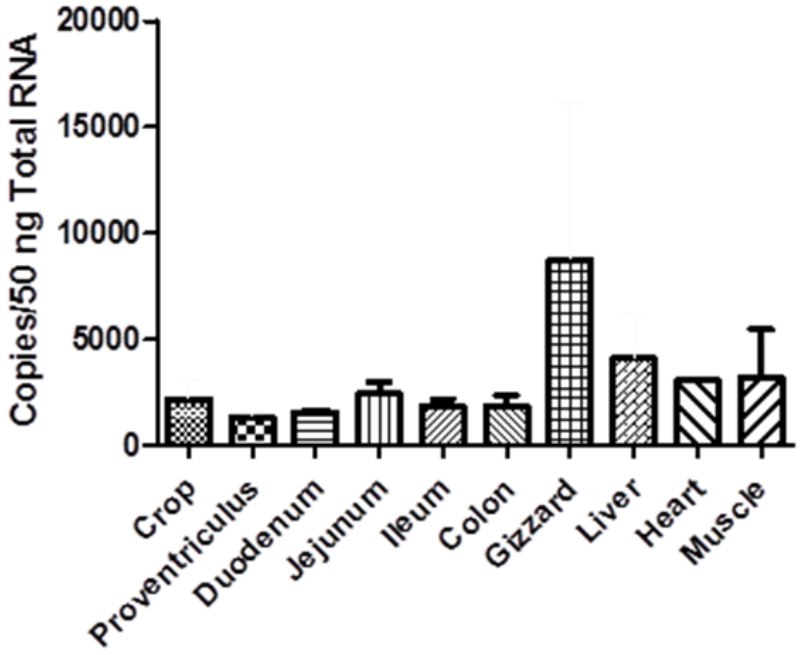


Figure 22: Expression of GPR38 mRNA in different quail tissues. Expression of GPR38 mRNA was determined by Q-PCR. Each value represents the mean ± SEM (n = 3).

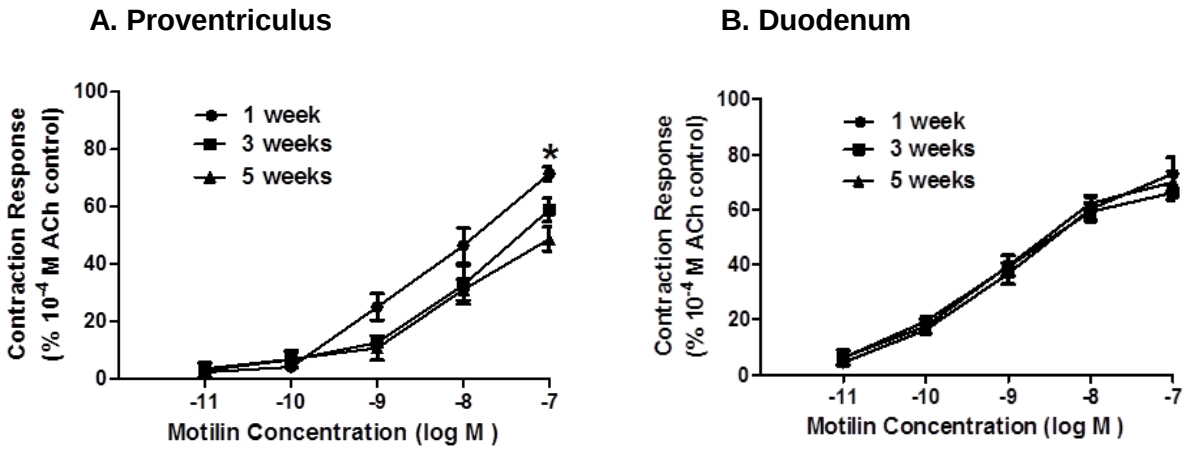
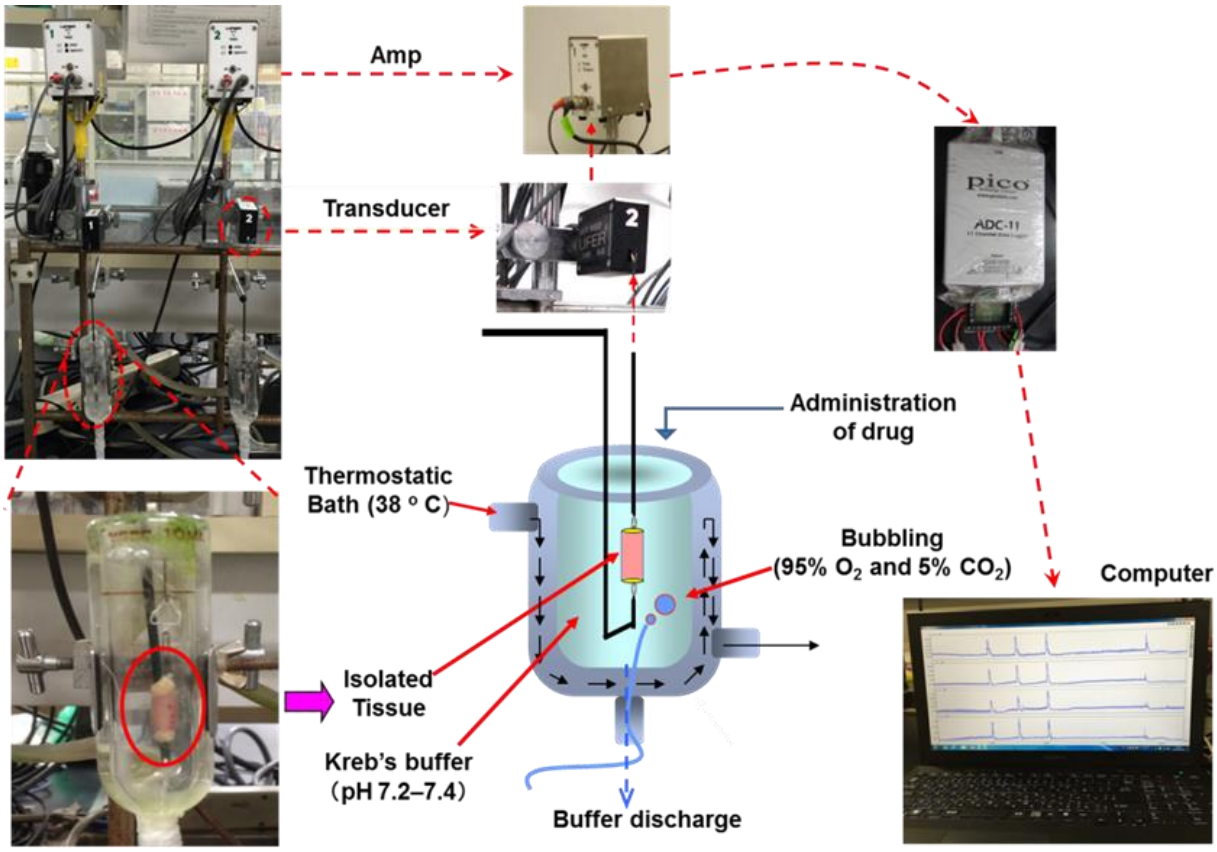
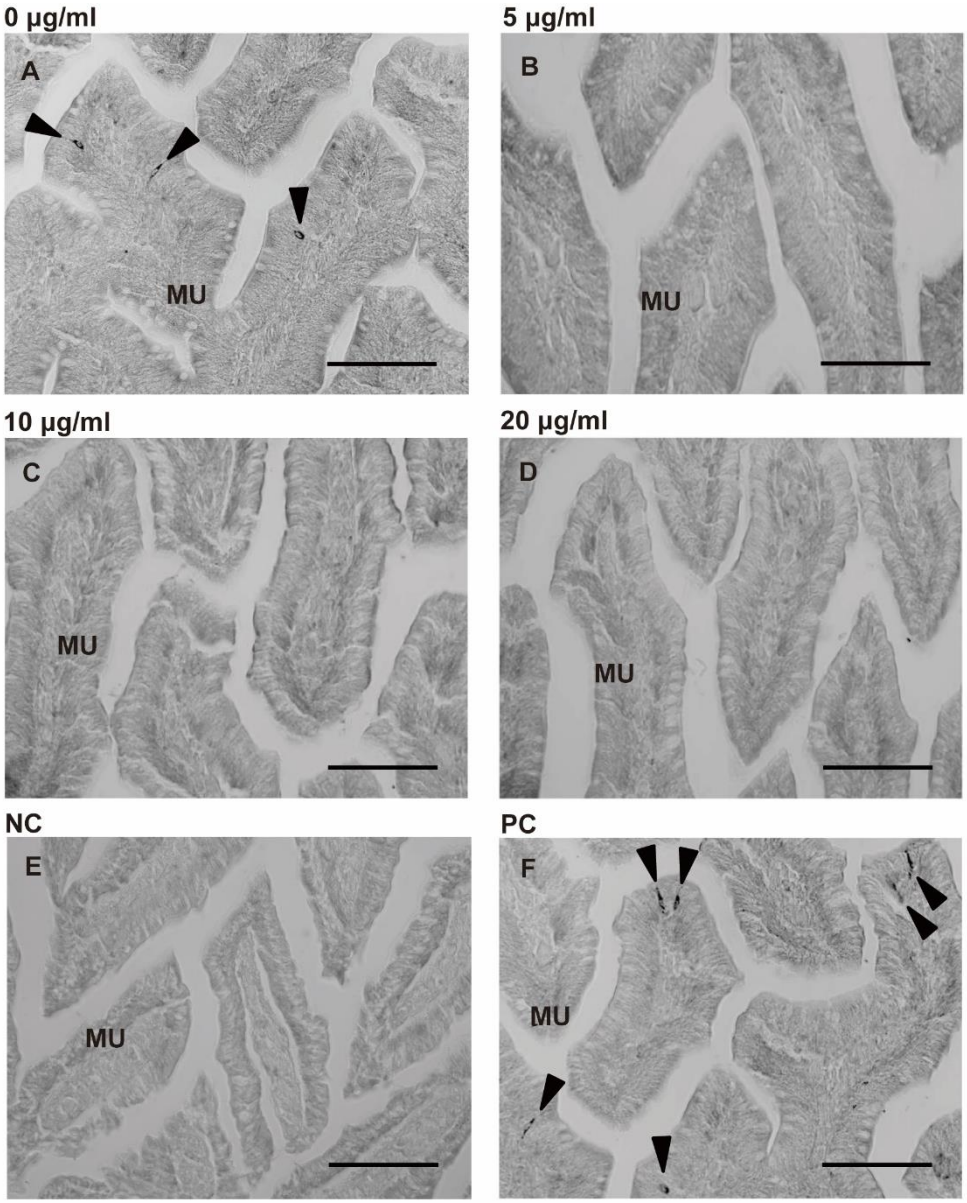


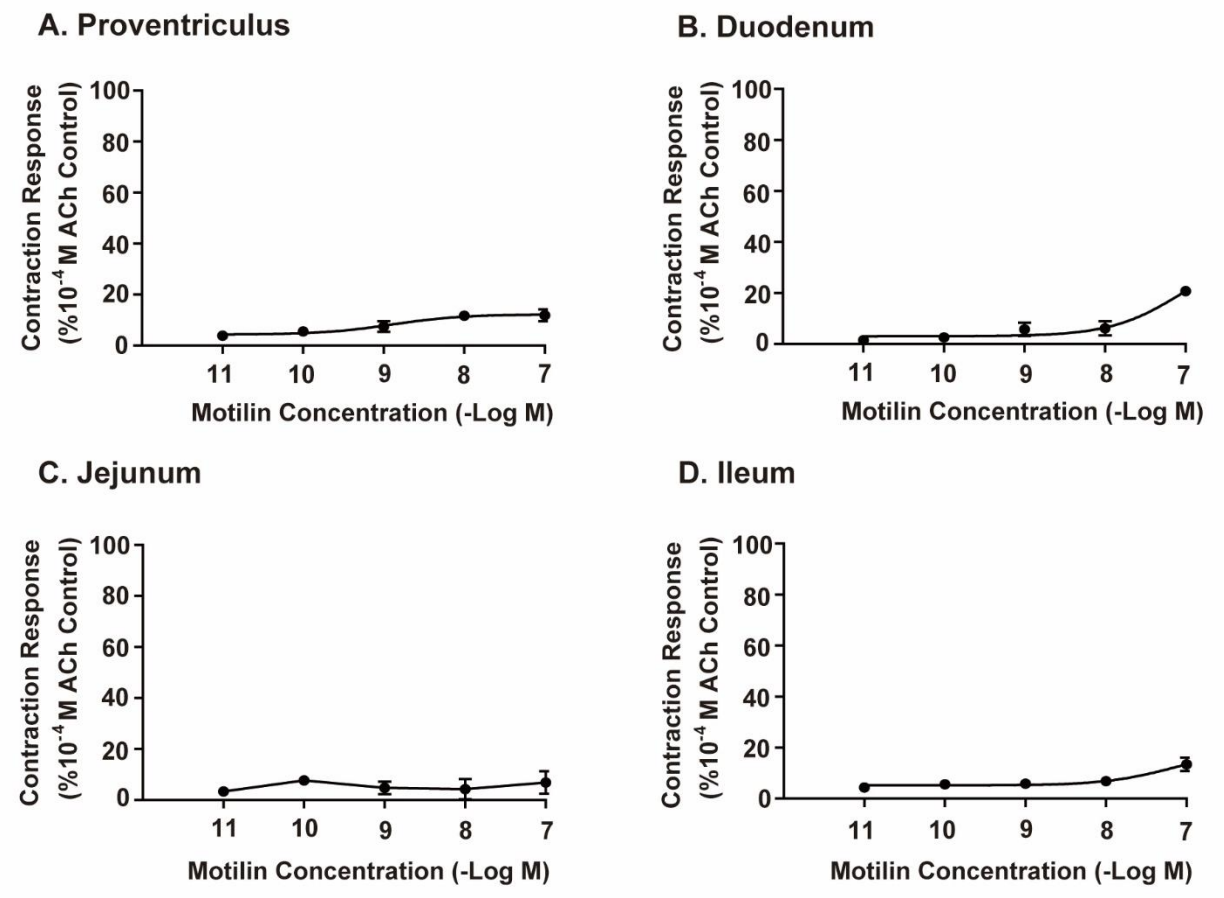
Figure 23: Age dependent changes in motilin-induced contractile activity in the proventriculus and duodenum of Japanese quails. Concentration response curves for motilin in the proventriculus (A) and duodenal strips (B) obtained from Japanese quails at the ages of 1, 3, and 5 weeks. Each value represents the mean ± SEM (n = 3). A one-way analysis of variance, followed by a Tukey's *post-hoc* test was performed to test for significant differences * $p < 0.05$.



Appendix I: Organ bath system for recording gastrointestinal contraction in Japanese quails.



Appendix II: Specificity of anti-porcine motilin antibody assessed by antigen adsorption test in quail duodenum. Motilin-ip cells (arrowheads) stained by immunohistochemistry were observed after staining with anti-porcine motilin incubated without chicken motilin (A), but were not observed with chicken motilin at 5 µg/ml (B), 10 µg/ml (C), and 20 µg/ml (D). Negative (E) and positive controls (F) are also shown. MU indicates mucosa. Scale bars = 100 µm.



Appendix III: Effect of suncus motilin on contractions of proventriculus, duodenum, jejunum, and ileum in quails. Suncus motilin did not evoke contractions of the proventriculus (A), duodenum (B), jejunum (C), and ileum (D) in quails. Each value represents the mean ± SEM (n = 3). Statistical analysis was performed by a one-way ANOVA followed by a Tukey's *post-hoc* test.

Appendix IV: Primers used for cloning quail GPR38

Primer name	**Sequence (5´ to 3´)**
qGPR38FWD#2669	TGCCTCWGCMTCCTSTACGG
qGPR38BWD#2670	RTTGATRGABGCRCTCAGRT
qGPR38nestFWD#2811	TCATCGGGCGCGAGCTGTGG
qGPR38nestBWD#2812	AGCTGCAGGGCAAAGATGTT

Appendix V: Primers used for Q-PCR

Primer name	**Sequence (5´ to 3´)**
qGPR38FWD#2825	GTCAGGATCCCGGCTGTG
qGPR38BWD#2826	GAAGTATTGGGAGAACAGCATC

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