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

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Abstract

Motilin, a peptide hormone produced in the upper intestinal mucosa, plays an important role in the regulation of gastrointestinal (GI) motility. In the present study, we first determined the cDNA and amino acid sequences of motilin in the Japanese quail and studied the distribution of motilin-producing cells in the gastrointestinal tract. We also examined the motilin-induced contractile properties of quail GI tracts using an in vitro organ bath, and then elucidated the mechanisms of motilin-induced contraction in the proventriculus and duodenum of the quail. Mature quail motilin was composed of 22 amino acid residues, which showed high homology with chicken (95.4%), human (72.7%), and dog (72.7%) motilin. Immunohistochemical analysis showed 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. In the organ bath study, chicken motilin induced dose-dependent contraction in the proventriculus and small intestine. On the other hand, chicken ghrelin had no effect on contraction in the GI tract. Motilin-induced contraction in the duodenum was not inhibited by atropine, hexamethonium, ritanserin, ondansetron, or tetrodotoxin. However, motilin-induced contractions in the proventriculus were significantly inhibited by atropine and tetrodotoxin. These results suggest that motilin is the major stimulant of GI contraction in quail, as it is in mammals and the site of action of motilin is different between small intestine and proventriculus.

Key words: Japanese quail, motilin, ghrelin, gastrointestinal motility

1.Introduction

Motilin, a 22-amino acid polypeptide that stimulates gastrointestinal (GI) motility (Poitras et al., 1994), was originally purified from pigs in the 1970s (Brown et al., 1972, 1973; Schubert and Brown, 1974). Since its discovery, mRNA and amino acid sequences of motilin have been identified mainly in mammals, including humans, cows, dogs, rabbits, and suncus (Banfield et al., 1992; Huang et al., 1999; Strausberg et al., 2002; Tsutsui et al., 2009). Structural analysis has revealed that the N-terminal amino acid of motilin is necessary for full motilin activity (Poitras et al., 1992). Motilin is produced in the upper small intestine and motilin-producing cells are localized in the crypts and villi of the mucosal layer, but are not present in the muscular layer (Tsutsui et al., 2009). Motilin-producing cells in mammals exist as two types, open-type cells in villi and closed-type cells in crypts (Satoh et al., 1995). Several reports have shown that the specific receptor for motilin (G-protein coupled receptor 38; GPR38) is expressed in the brain, pituitary gland, lung, stomach, and small intestine (Ohshiro et al., 2008; Suzuki et al., 2012; Takeshita et al., 2006; Ter Beek et al., 2008; Yamamoto et al., 2008), indicating that motilin has many physiological functions related to gastrointestinal motility. Moreover, stomach derived peptides like ghrelin belong to the motilin family of peptides, and GPR38 and the ghrelin receptor (growth hormone secretagogue receptor: GHSR) are both highly conserved (Ohno et al., 2010; Poitras and Peeters, 2008).

Patterns of gastrointestinal motility differ between the fasted and postprandial states and feature contractions referred to as the migrating motor complex (MMC), observed during the fasted

77 state, which consists of phase I (motor quiescent period), phase II (irregular and low amplitude
78 contraction period), and phase III (regular and high amplitude contraction period) in humans, dogs,
79 and suncus (Itoh et al., 1976; Janssens et al., 1983; Sakahara et al., 2010; Vantrappen et al., 1977).
80 Gastric phase III of the MMC is strongly associated with peak plasma motilin concentrations (Hall
81 et al., 1983; Itoh et al., 1976; Janssens et al., 1983; Vantrappen et al., 1979). Intravenous
82 administration of motilin causes gastric phase III-like contractions (Itoh et al., 1976; Janssens et al.,
83 1983; Kuroda et al., 2015; Wingate et al., 1976) and in vitro studies found that motilin induced
84 dose-dependent gastric contractions in monogastric animals including humans, rabbits, and suncus
85 (Broad et al., 2015; Kitazawa et al., 1994; Mondal et al., 2011; Strunz et al., 1975). Recent studies
86 in suncus have shown that ghrelin is involved in phase II via the vagal afferent nerve and
87 coordination of motilin and ghrelin stimulates phase III contractions (Miyano et al., 2013; Mondal
88 et al., 2012). In addition, motilin-induced gastric contractions are reporteded to be mediated via
89 cholinergic, adrenergic, serotonergic, opioidergic, and nitergic neurons (Mondal et al., 2011).
90 In avian species, it has been reported that the frequency of chicken MMCs is 77–122 min
91 and the duration of phase III is 5–8 min (Clench et al., 1989), indicating that the MMC in chickens
92 is similar to that in mammals. Moreover, in vitro studies using the chicken GI tract show that
93 motilin induces contractions in a dose-dependent manner through both neural and direct smooth
94 muscle pathways (De Clercq et al., 1996; Kitazawa et al., 1995, 1997). However, studies on motilin
95 and GI motility in avian species have been lacking, when compared to those in mammals.

In the present study, we determined the sequence of motilin in Japanese quail and localized motilin-producing cells. We also examined the effects of motilin on gastrointestinal contractions and its mechanisms of action in vitro.

2.Materials and Methods

2.1Drugs used

Chicken motilin (Peptide Institute Inc., Osaka, Japan); chicken ghrelin (provided by the Asubio Pharma Co., Ltd., 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); and ritanserin (Tocris Bioscience, Ellisville, USA) were all used in the present study. Ritanserin was dissolved in ethanol, and other drugs were dissolved in distilled water. The final concentration of ethanol was 0.01% v/v and there was no effect on GI contractions.

2.2Animals

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.

2.3Cloning of quail motilin cDNA

Total RNA was extracted from the duodenum by ISOGEN (Nippon Gene Co., Ltd., Tokyo, Japan) according to 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 by using 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 carried out using a Thermal Cycler (Bio-Rad, Hercules, California, USA). Initial template denaturation was programmed for 10 min at 95°C, and the cycle profile was then programmed as follows: 30 s at 94°C (denaturation), 1 min at 57°C (annealing and extension), running of 40 cycles of the profile, and 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).

2.4Tissue preparation

Animals were sacrificed by deep anesthesia with sodium pentobarbital (100 mg/kg, i.p.), after which approximately 1.5 cm of crop, proventriculus, duodenum, jejunum, ileum, and colon

were removed. Each part of the GI tract was opened along its longitudinal axis in Bouin's-Hollande fixative solution and incubated for 16 h. The tissue blocks were dehydrated with an ascending ethanol series and xylene, and embedded in Paraplast Plus (McCormick Scientific, St Louis, MO). Serial sections (10 µm thick) were made with a microtome and mounted on slides coated with silane (ShinEtsu Chemicals, Tokyo, Japan).

2.4.1Immunohistochemistry

Immunohistochemical detection of quail motilin cells using rabbit anti-pig motilin serum was carried out by the avidin–biotin–peroxidase complex (ABC) method. The sections were deparaffinized with xylene and rehydrated through descending 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 being washed 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-motilin serum (Tsutsui et al., 2009) diluted 1:8000 in TNBS. After being washed with PBS, the sections were then 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) prepared according to the manufacturer's instructions. After being washed with PBS for 10 min, the sections 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), then dehydrated with a graded ethanol series, cleared in xylene, mounted with Entellan (Merck, Darmstadt, Germany), and viewed under a light microscope (BX60, Olympus, Tokyo, Japan). All incubations were carried out in a humidity chamber at room temperature. For the antigen absorption test, the anti-pig 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.

2.4.2Morphometric analysis

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

2.5Organ bath study

Quails were anesthetized by 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 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). Initial load was set at 1.0 g 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. We confirmed that 10^{-4} M ACh showed the maximum contraction. 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}–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, based on the previously published report, we selected the dose of motilin and ghrelin concentrations to determine the coordinated effects of motilin and ghrelin in quail GI tract. Moreover, to determine the coordinative 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 contractions among all preparations was normalized by a standard contraction with ACh (10^{-4} M) and expressed as a relative contraction (%), and 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 and expressed as a percentage of the ACh (10^{-4} M) contractions.

2.6Statistical analysis

All values were expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Significance of differences between the values was determined at $p < 0.05$ by using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons and Student's *t*-test for control vs. antagonist treatment experiments.

3.Results

3.1Cloning of quail motilin cDNA

Quail motilin cDNA was cloned from mRNA of the duodenum and its sequence was determined (Fig. 1). The deduced amino acid sequence of quail mature motilin was 22 amino acids. Similar to motilin precursors in mammals, an endoproteinase cleavage site was present in quail motilin at Lys^{23}-Lys^{24} (Fig. 2). Mature quail motilin showed high sequence homology with other avian species, including chicken (95.4%) and turkey (90.9%), and moderate homology with mammalian species, including humans (72.7%), dogs (72.7%), and suncus (68.1%). Mature quail motilin differs in one amino acid (position 10) from chicken motilin, two amino acids (position 10 and 19) from turkey motilin, seven amino acids (position 2, 4, 7, 8, 9, 10, and 18) from *Suncus* motilin, six amino acids (position 4, 7, 9, 10, 13, and 14) from dog motilin, and six amino acids from human motilin (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 with that of the chicken and turkey (Fig. 2).

3.2Localization of motilin immunopositive (motilin-ip) cells in the GI tract of quail

Motilin-ip cells were observed in the mucosal layer of the duodenum (Fig. 3A), jejunum (Fig. 3B), and ileum (Fig. 3C), but were not observed in the crop, proventriculus, and colon. The motilin cell density was most abundant in the duodenum (23.4 ± 4.6 cells/mm^2) and gradually

237 decreased in the jejunum (15.2 ± 0.8 cells/mm^2) and ileum (2.5 ± 0.7 cells/mm^2) (Fig. 3D). Open-
238 and closed-type motilin-ip cells were also observed in these regions and the percentage of open-type
239 motilin cells among all immunopositive cells was 69.3% in the duodenum, 67.1% in the jejunum,
240 and 72.5% in the ileum. The immunoreactivities completely disappeared on using the antiserum that
241 absorbed with chicken motilin (Supplemental Fig. 2).
242
243 ***3.3Effects of chicken motilin in the GI tract of quail***
244 Motilin-induced contractions in the crop and colon were reflected by weak responses,
245 which were 27.3 ± 5.8% and 35.1 ± 6.0%, respectively (Fig 4 A, B). In the duodenum, motilin-
246 induced contraction from the 10^{-10} M treatment increased in a dose-dependent manner to reach
247 maximum contraction of 71.0 ± 9.7% (Fig. 4C). In the jejunum and ileum, motilin-induced
248 contraction was also observed from the 10^{-10} M treatment and reached a maximum of 81.7 ± 11.6%
249 and 74.1 ± 13.7%, respectively (Figs. 4 D, 4E). On the other hand, contractile activity in the
250 proventriculus reached a maximum of 46.2 ± 13.6% (Fig. 4F). Suncus (shrew) motilin induced no
251 contractions in the proventriculus or intestines (Supplemental Fig. 1).
252
253 ***3.4Effects of chicken ghrelin and co-administration of chicken motilin and ghrelin on the GI***
254 ***tract of quail***
255 Cumulative administration of chicken ghrelin (10^{-11} to 10^{-6} M) showed mild contractile

responses in the duodenum and proventriculus; however, this was not significant (Figs. 5A, 5B). In addition, administration of ghrelin induced no marked contractile responses in the crop, jejunum, ileum, or colon (data not shown). Pretreatment with chicken motilin (10^{-10} M) with cumulative administration of chicken ghrelin (10^{-11} to 10^{-6} M) showed no additive effects in the duodenum or proventriculus (Figs. 5C, 5D). Co-administration of motilin and ghrelin also showed no synergistic or additive effects in the crop, jejunum, ileum, or colon (data not shown).

3.5Mechanism 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), ritanserin (a 5-HT2A [5-hydroxytryptamine 2A] receptor antagonist), ondansetron (a 5-HT3 receptor antagonist), and TTX (a selective inhibitor of Na^+ channel conductance) were administered as pre-treatments, prior to motilin administration (Figs. 6A–C). None of the antagonists or inhibitors blocked motilin-induced duodenal contractions.

3.6Mechanism of chicken motilin-induced contraction in the proventriculus of quail

In the proventriculus, treatment with atropine reduced baseline contractions, but did not affect spontaneous contractions (data not shown). Pretreatment with atropine (10^{-6} M) significantly inhibited motilin-induced contractions at concentrations of 10^{-8} and 10^{-7} M motilin (Fig. 7A).

Pretreatment with TTX (10^{-6} M) significantly blocked motilin-induced contraction in the proventriculus at concentrations of 10^{-8} and 10^{-7} M motilin (Fig. 7B). On the other hand, pretreatment with ritanserin and ondansetron did not inhibit motilin-induced contractions in the proventriculus (data not shown).

4.Discussion

In the present study, we first determined the sequence of the mRNA coding region of quail motilin and localized motilin-producing cells in the GI tract of quail. The N-terminal amino acid of motilin is quite important and capable of effecting full activity, using cell lines that over-express the motilin receptor (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 with 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 induced no contractions in those tissues, suggesting that the N-terminal region of quail motilin is also important for specific binding of quail GPR38. 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 (Kishimoto et al., 1981; Polak et al., 1975; 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 luminal conditions in the small intestine are important for the release of motilin in quail.

The present study demonstrated that motilin stimulates contractions in the duodenum and

300 proventriculus of quail. Similar region-specific variations in motilin-induced contractions have also
301 been reported in chickens, with the strongest responses being observed in the small intestine (De
302 Clercq et al., 1996; Kitazawa et al., 1995). In addition to motilin, we examined the effects of ghrelin
303 on GI contraction and observed that ghrelin had no significant effects in quail. In the quail GI tract,
304 ghrelin did not induce any significant contraction (Kitazawa et al., 2009), which was consistent
305 with our results. On the other hand, ghrelin has been observed to induce gastric contractions in
306 rodents, which genetically, are motilin-knockout animals (Depoortere et al., 2005; Masuda et al.,
307 2000; Zheng et al., 2009). Ghrelin also stimulates gastric contractions in humans (Tack et al., 2006).
308 Recent studies in suncus (which are suitable motilin-producing animals for GI motility research)
309 revealed that ghrelin induces gastric contractions in vitro under the influence of low doses of
310 motilin, and motilin also induces strong gastric contractions under the influence of ghrelin in vivo
311 (Kuroda et al., 2015; Mondal et al., 2012). Moreover, motilin is not involved in contraction of the
312 small intestines (Janssens et al., 1983). Altogether, the present results suggest that the sites of action
313 of motilin and the mechanisms of motilin-induced gastric contractions are different between
314 mammals and birds.

315 The mechanisms of motilin-induced contractions in the stomach shows differences even
316 among mammalian species (Boivin et al., 1997; Mizumoto et al., 1993; Mondal et al., 2011; Van
317 Assche et al., 1997). In the dog and suncus, motilin induces gastric contractions through neural
318 pathways (Mizumoto et al., 1993; Mondal et al., 2011). In humans and rabbits, gastric contraction

319 induced by high doses of motilin is mediated through direct stimulation of smooth muscle; whereas
320 low doses of motilin exert its effects via a neural pathway (Broad et al., 2015; Coulie et al., 1998;
321 Dass et al., 2003; De Smet et al., 2009; Depoortere et al., 2003; Jarvie et al., 2007; Sanger, 2012;
322 Van Assche et al., 1997). On the other hand, motilin-induced contractile activity in the small
323 intestine of the rabbit, cat, dog, and human has been reported to interact directly with smooth
324 muscle (Adachi et al., 1981; Boivin et al., 1997; Depoortere et al., 1993; Depoortere et al., 1990;
325 Kitazawa et al., 1994; Poitras et al., 1987; Strunz et al., 1975). We found that atropine and TTX
326 pretreatments failed to suppress completely motilin-induced contractions in the duodenum but
327 decreased approximately 50% of the contractions in the proventriculus of quails. This finding
328 suggests that motilin induced proventricular contractions via the neural and smooth muscle direct
329 pathways. On the other hand, motilin induced duodenal contractions via direct stimulation of
330 smooth muscles. These results indicate that mechanisms of motilin-induced contractions differ
331 among various regions of the GI tract of quail. In chickens, it has shown that GPR38 mRNA
332 expression levels were different in different regions of the GI tract (Kitazawa et al., 2013).
333 Moreover, GPR38 expression is localized in the myenteric plexus and muscle layers of the human
334 stomach and small intestine (Broad et al., 2012; Takeshita et al., 2006; Ter Beek et al., 2008). Our
335 recent study in suncus showed that GPR38 mRNA expression is also higher in the upper corpus
336 than in other regions of the GI tract, and expression levels of GPR38 mRNA are consistent with
337 responses to motilin (manuscript in submission). Our findings suggest therefore, that response

differences in the GI tract may be linked to the expression levels and/or localization of GPR38 mRNA in the GI tract of quail.

The MMC occurs during the fasted state in mammals, and phase III of the MMC has been observed at approximately 90–120 min intervals (Takahashi, 2013). Biological and physiological relevance of the MMC is thought to be important for the mechanical clearance of remnants of indigestible food (Itoh, 1997; Vantrappen et al., 1977) and dysfunction of the MMC can lead to functional dyspepsia or other GI motility disorders (Gu et al., 1998; Kusano et al., 1997; Takahashi, 2013). Interestingly, the MMC cycle is highly conserved in humans, dogs, and suncus even though body weight and length of the GI tract vary widely (Itoh, 1997; Kuroda et al., 2015), suggesting 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 GI contractions associated with the MMC would be necessary to examine further the effects of motilin in quail.

In summary, the mRNA sequence of quail motilin and distribution of motilin-producing cells have been demonstrated in the present study. Motilin stimulated contractions in the proventriculus and duodenum in a dose-dependent manner in vitro. In addition, we demonstrated that motilin induced contractions in the proventriculus occur via both neural pathways and direct stimulation of smooth muscle, whereas motilin induced duodenal contractions occur only through

direct stimulation of smooth muscle. Further studies to determine expression levels and distribution of GPR38 mRNA within the GI tract is necessary for a better understanding of the mechanisms of motilin-induced GI contraction in quail.

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539 **Figure legends**

540 **Fig. 1.** Multiple alignment of nucleotide sequences encoding a motilin precursor from the human,
541 dog, suncus, turkey, chicken, and quail. Completely conserved regions between species are
542 indicated by an asterisk (*).The nucleotide sequence has been deposited in the
543 DDBJ/EMBL/GenBank databases with the Accession No. LC146647.1.

544

545 **Fig. 2.** Multiple alignment of the motilin precursor deduced from human, dog, suncus, turkey,
546 chicken, and quail nucleotide sequences. Completely conserved regions between species are
547 indicated by an asterisk (*). Conservation between groups with strongly similar properties is
548 indicated by two dots (:). Conservation between groups with weakly similar properties is indicated
549 by a single dot (.). Mature motilin peptide is indicated by a double-headed arrow. A dibasic cleavage
550 site (KK) was also found in the predicted quail motilin sequence (underlined). The amino acid
551 sequence of quail motilin (BAU80773.1) was aligned with those of human (AAI12315.1), dog
552 (NP_001300735.1), *Suncus* (BAI66099.1), turkey (XP_010722636.1), and chicken
553 (NP_001292058.1) motilins.

554

555 **Fig. 3.** Photomicrographs of motilin immunopositive (motilin-ip) cells in the intestine of quail.
556 Motilin-ip cells (arrowheads) stained by immunohistochemistry were present in the duodenum (A),
557 jejunum (B), and ileum (C). Insets show high magnification of motilin cells. Two types of cell, the
558 open- and closed-type, were present in the mucosa. Histogram showing the densities (cells/mm^2) of

559 motilin-ip cells in the GI tract (D). The density of motilin cells in the duodenum and jejunum was
560 significantly higher than that in the ileum. MU indicates mucosa. Scale Bars are 100 μm in (A), (B),
561 and (C), and 10 μm in insets. Densities of motilin cells in different tissues of the quail GI tract were
562 compared by one-way ANOVA followed by Tukey's post-hoc test. ** $p < 0.01$, *** $p < 0.001$; $n = 3$.
563
564 **Fig. 4**. Motilin-induced contractile activity in different isolated parts of the GI tract of quail. Left:
565 traces showing the representative contractile response to Acetylcholine (ACh) (10^{-4} M) in the
566 isolated crop (A), colon (B), and duodenum (C). Center: responses to cumulative administration of
567 chicken motilin (10^{-11}–10^{-6} M) in the isolated crop (A), colon (B), and duodenum (C). Right:
568 motilin-induced concentration-response curve of isolated crop (A), colon (B), and duodenum (C).
569 Concentration-response curve to cumulative doses of motilin (10^{-11}–10^{-6} M) in the jejunum (D),
570 ileum (E), and proventriculus (F) are also shown. Arrowheads indicate timing of administration of
571 motilin and numbers indicate concentration of chicken motilin (-Log M). Each value represents the
572 mean ± SEM ($n = 4$). Significance of differences was determined using one-way ANOVA followed
573 by Tukey's post-hoc test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. 10^{-11} M motilin treatment).
574
575 **Fig. 5**. Effects of chicken ghrelin and co-administration of chicken motilin and chicken ghrelin on
576 different parts of the GI tract of quail in vitro. Contractile responses to chicken ghrelin in isolated
577 duodenum (A) and proventriculus (B). Representative contractile responses of the coordinative
578 effects of motilin and ghrelin in the isolated duodenum (C) and proventriculus (D). Arrowheads

579 indicate timing of administration. Each value represents the mean ± SEM (n = 3). The statistical
580 analysis was performed using one-way ANOVA followed by Tukey's post-hoc test.
581
582 **Fig. 6.** Effects of cholinergic, serotonergic receptor antagonists, and tetrodotoxin on motilin-
583 induced contraction in the duodenum of Japanese quail. Pre-treatment with atropine (10^{-6} M) and
584 hexamethonium (200 μM) effected no changes in motilin-induced contraction in the duodenum (A).
585 Pre-treatment with ritanserin (10^{-7} M) and ondansetron (10^{-5} M) had no effect on motilin-induced
586 contractile activity (B). Tetrodotoxin (10^{-6} M) also had no effect on the motilin-induced contraction
587 in the duodenum (C). Each value represents the mean ± SEM (n = 4). Student's *t*-test was used to
588 compare differences on the same dose between control vs. antagonist treatment.
589
590 **Fig. 7.** Effects of atropine and tetrodotoxin on motilin-induced contraction in the proventriculus of
591 Japanese quail. Concentration-response curve showing significant inhibition of motilin-induced
592 contraction by pre-treatment with atropine (10^{-6} M) (A). Pre-treatment with tetrodotoxin (10^{-6} M)
593 also significantly reduced motilin-induced contractile activity in the proventriculus (B). Each value
594 represents the mean ± SEM (n = 4). Asterisk denotes statistical significance between control and
595 antagonist treatment. * $p < 0.05$, ** $p < 0.01$.
596
597 **Supplemental Fig. 1.** Effects of suncus motilin on contractions of the proventriculus, duodenum,

598 jejunum, and ileum in quail. Suncus motilin induced no contractions in the proventriculus (A),

599 duodenum (B), jejunum (C), and ileum (D). Each value represents the mean ± SEM (n = 4).

600

601 **Supplemental Fig. 2.** Specificity of anti-pig motilin antibody by antigen absorption test in quail

602 duodenum. Motilin-ip cells (arrowheads) stained by immunohistochemistry were observed when

603 the anti-pig motilin incubated without chicken motilin (A) but not observed with chicken motilin

604 5 μg/ml (B), 10 μg/ml (C), and 20 μg/ml (D). Negative control (E) and positive control (F) are also

605 shown. MU indicates mucosa. Scale bars are 100 μm.

606

Figure1

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Human       CGCCCTCCAAGATGGTATCCCGTAAGGCTGTGGCTGCTCTGCTGGTGGTGCATGCAGCTG
Dog         TGCTCCCTAGGATGGTGTCCCGAAAGGCCGTGGCTGCTCTGCTGGTGGTGCACGTGGCTG
Suncus      ----------------GTCCCGCAAAGCCATGGCAATGCTGCTGCTTGTGCACATGGCCA
Turkey      AGACTCTTGCGATGGTTTCGAAGAAGGCGGCGGCCGGTTTGCTCCTGGTGTACGTGATGG
Chicken     AGACTCTTGCAATGGCTTCGAAGAAGGCGGTGTCCGGTTTGCTCCTGCTGTACGTGATGT
Quail       --------------------------------CCGGTTTGCTCCTGGTGTACGTGATGT
                                             *     ****  *  ** *

Human       CCATGCTGGCCTCCCAGACGGAAGCCTTCGTCCCCATCTTCACCTATGGCGAACTCCAGA
Dog         CCATGCTGGCCTCCCAGACAGAAGCCTTCGTTCCCATCTTCACCCACAGTGAGCTCCAGA
Suncus      CCATGCTGGCCTCACAGATCGAAGCCTTCATGCCCATCTTCACCTATGGCGAACTTCAAA
Turkey      CAGTGCTGGCAGAACAGGCTGAAGGCTTTGTGCCCTTCTTCACCCAGAGCGACATCCAGA
Chicken     CAGTGCTGGCAGAACAGGCTGAAGGCTTTGTGCCCTTCTTCACCCAGAGCGACATCCAGA
Quail       CAGTGCTGGCAGAACAGGCTGAGGGCTTTGTGCCCTTCTTCACCCAGAGTGACTTCCAGA
            *  *******    ***   ** * ***  * *** ******** *  * **  * ** *

Human       GGATGCAGGAAAAGGAACGGAATAAAGGGCAAAAGAAATCCCTGAGTGTATGGCAGAGGT
Dog         AGATTCGGGAAAAGGAGCGCAACAAAGGGCAAAAGAAGTCCTTGATCTTACAGAAGAAGT
Suncus      AGATGCAGGAGAAGGAGCAAAACAAAGGCCAGAAGAAATCTCTGGGTGTGCAGCGCAGAG
Turkey      AAATGCAGGAAAAGGAGAGGATCAAAGGGCAGAAGAAATCCCTGACCTCTCTGCAGCAGC
Chicken     AAATGCAGGAAAAGGAGAGAAACAAAGGACAGAAGAAATCCCTGACACCTCTGCAGCAGC
Quail       AAATGCAGGAAAAGGAGAGGAACAAAGGGCAGAAGAAATCCCTGACCCCTCTGCAGCAGC
              ** * *** *****    *  ***** ** ***** **  **        *

Human       CTGGGGAGGAAGGTCCTGTAGACCCTGCGGAGCCCATCAGGGAAGAAGAAAACGAAATGA
Dog         CTGAGGAAGTGGGGCCTCTGGACTCTGTGGAGCCCACAGAGGAAGAAGAAAACCAAGTTA
Suncus      CCGAGGAATCAGGCCCCCTGGGCCTTGGGGACCCCACAGATGGAGAAGAAAGCCCCATGA
Turkey      TGGAAGAGGAAGGCTTCTCTGAGCAATCT---GGTGCAGATATCGAAGGAATGAAGACTA
Chicken     TGGAAGAAGACGACTTCTCTGAGCAACCT---GGGGCAGATGTTGACGGGATAAAGACTA
Quail       TGGAAGAGGAAGGCTTCTCTGAGAGGTCT---GATGCAGGTATCGACAGGATGAAGACTA
              *  **    *        *                        **    *        *

Human       TCAAGCTGACTGCTCCTCTGGAAATTGGAATGAGGATGAACTCCAGACAGCTGGAAAAGT
Dog         TCAAGTTGACTGCTCCTGTGGAAATTGGAATGAAGATGAACTCCAGGCAGCTGGAAAAGT
Suncus      TCAAGCTGACTGCTCCCGTGGAAATTGGGATATGGATGAACTCCAGGCAGCTGGAAAAGT
Turkey      TCCAGCTAGCTGTTCCTGTCAGAGCTGGGACGTGGCTCATACTGAGGCAGCTGGAAAAAT
Chicken     TCCAGCTAGCTGTTCCTGTCAGAGCTGGGATGTGGCTCATACTGAGGCAGCTGGAAAAAT
Quail       TCCAGCCAGCAGTTCCTGCCAGAGCTGGGATGTGGCTCATACTGAGACAGCTGGAAAAAT
            ** **    * * ***      *  *** *    * * *     ** *********** *

Human       ACCCGGCCACCCTGGAAGGGCTGCTGAGTGAGATGCTTCCCCAGCATGCAGCCAAGTGAT
Dog         ACTGGGCCGCCCTGGAAGAGCTGCTGAGTGAGGTGCTGCTGACCCCCCAGAACGACAAGT
Suncus      ACTGGGCCGCCCTGGAAGAGCTGCTGAGCCCAGCGCCACTGTCCACCCGGAACGAAACTG
Turkey      ACCAAGGTGTCCTGGAGAAACTGCTCACGGAGGTGTTACAGGACACCCCAAACGCTGACT
Chicken     ACCAAGGTGTCCTGGAGAAACTGCTCACGGAGGTGTTACAGGACACCCCAAACGCTGACT
Quail       ACCAAGGTGTCCTGGAGAAACTGCTCACGGAGGTGTTACAGGACACCCCAAACGCTGACT
            **   *    ******    ***** *       *   *            *
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Fig. 1

Figure2

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Human       MVSRKAVAALLVVHAAAMLASQTEAFVPIFTYGELQRMQEKERNKGQKKSLSVWQRSGEE
Dog         MVSRKAVAALLVVHVAAMLASQTEAFVPIFTHSELQKIREKERNKGQKKSLILQKKSEEV
Suncus      --------MLLLVHMATMLASQIEAFMPIFTYGELQKMQEKEQNKGQKKSLGVQRRAEES
Turkey      MVSKKAAAGLLLVYVMAVLAEQAEGFVPFFTQSDIQKMQEKERIKGQKKSLTSLQQLEEE
Chicken     MASKKAVSGLLLLYVMSVLAEQAEGFVPFFTQSDIQKMQEKERNKGQKKSLTPLQQLEED
Quail       ---------------MSVLAEQAEGFVPFFTQSDFQKMQEKERNKGQKKSLTPLQQLEEE
                           ::**.* *.*:*:** .::*:::***: *******   ::  *
                                  <-------------------->
                                  Mature motilin

Human       GPVDPAEPIREEENEMIKLTAPLEIGMRMNSRQLEKYPATLEGLLSEMLPQHAAK--
Dog         GPLDSVEPTEEEENQVIKLTAPVEIGMKMNSRQLEKYWAALEELLSEVLLTPQNDK-
Suncus      GPLGLGDPTDGEESPMIKLTAPVEIGIWMNSRQLEKYWAALEELLSPAPLSTRNETE
Turkey      GFSEQSG-ADIEGMKTIQLAVPVRAGTWLILRQLEKYQGVLEKLLTEVLQDTPNAD-
Chicken     DFSEQPG-ADVDGIKTIQLAVPVRAGMWLILRQLEKYQGVLEKLLTEVLQDTPNAD-
Quail       GFSERSD-AGIDRMKTIQPAVPARAGMWLILRQLEKYQGVLEKLLTEVLQDTPNAD-
                       :    *: :.* . *  :  ****** ..** **:
```

Fig. 2

Figure3

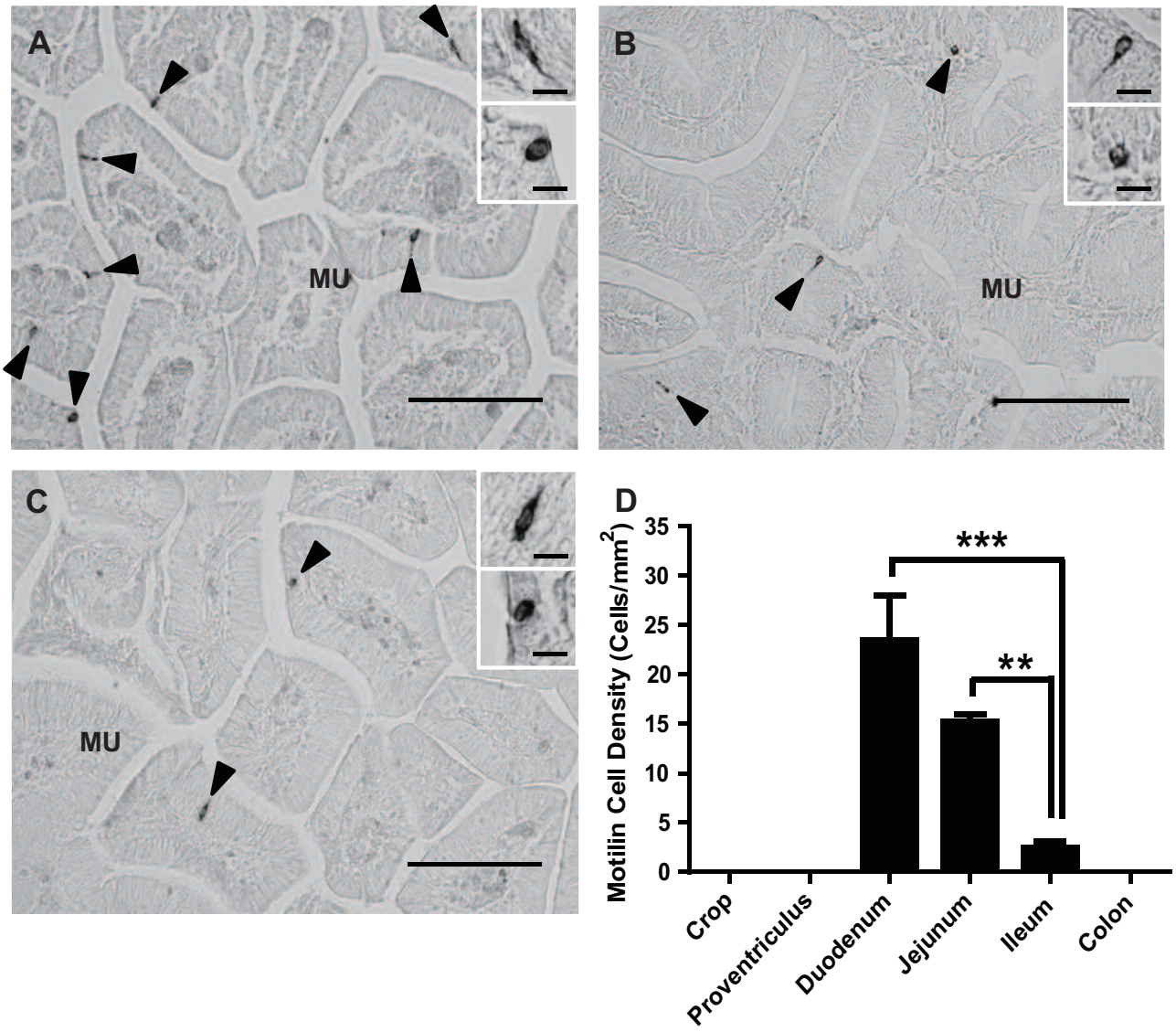


Fig. 3

Figure4

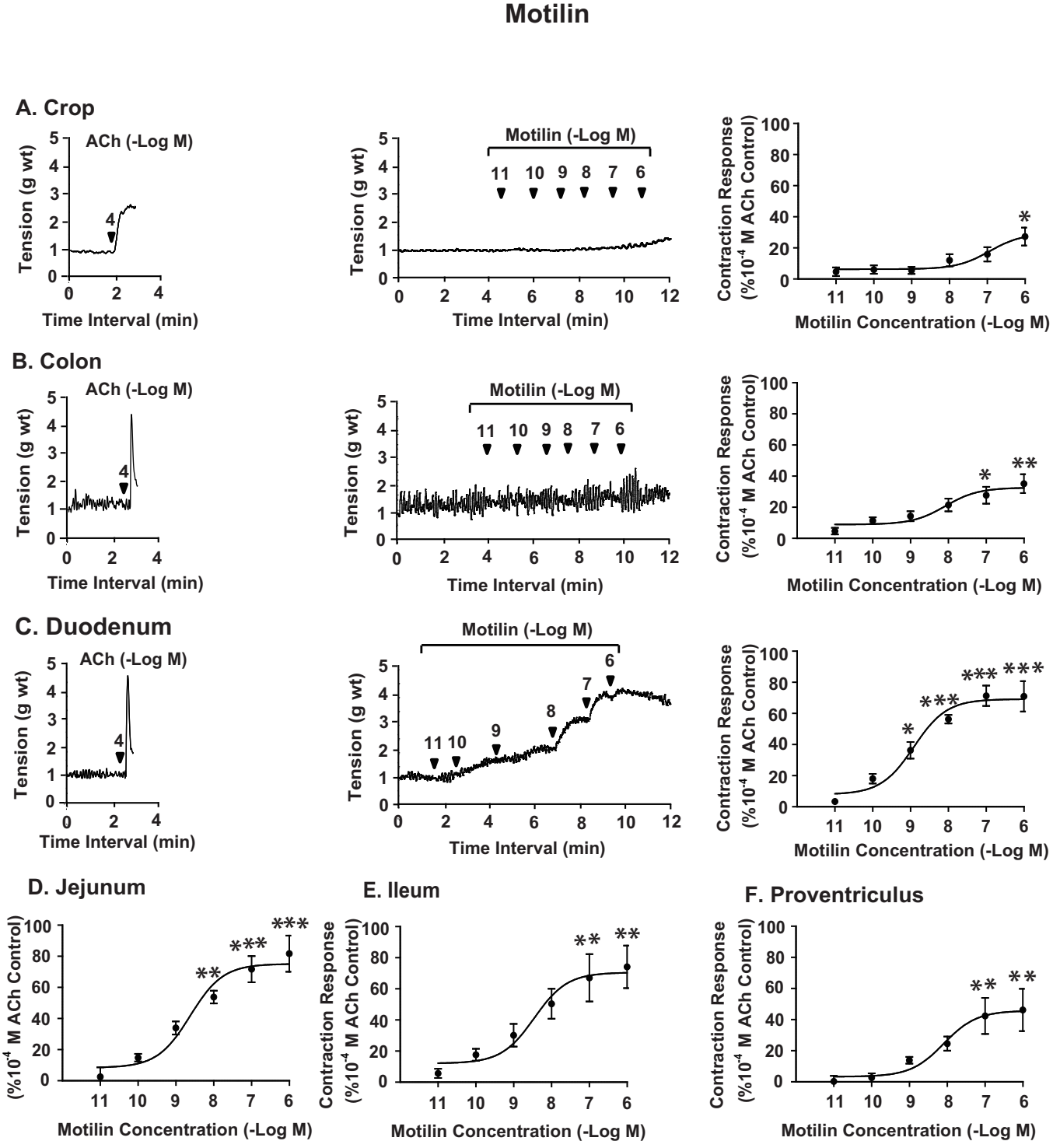


Fig. 4

Figure5

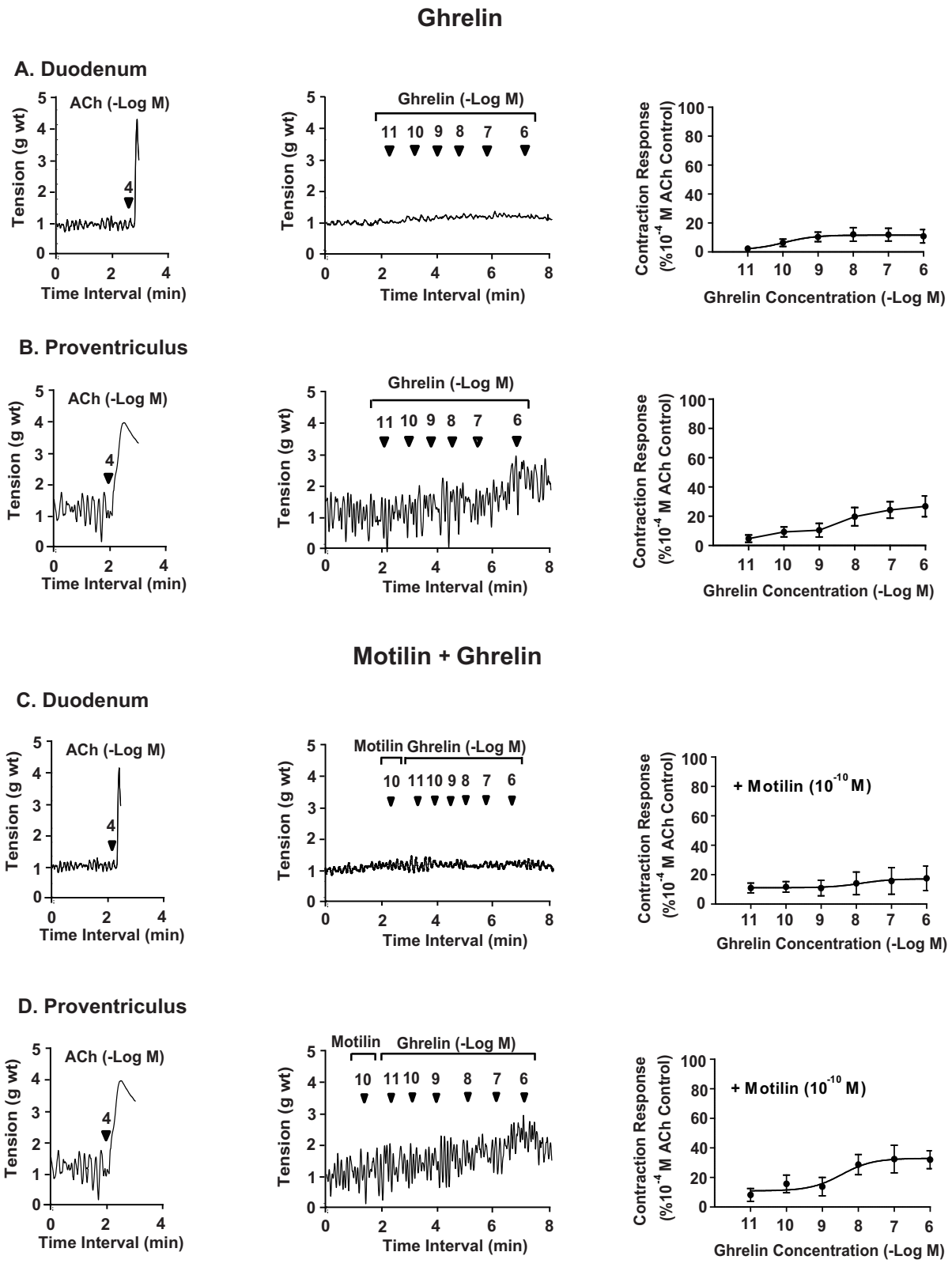
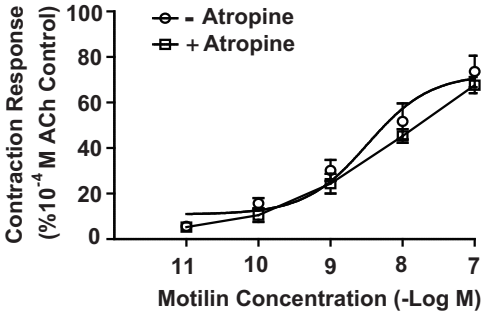


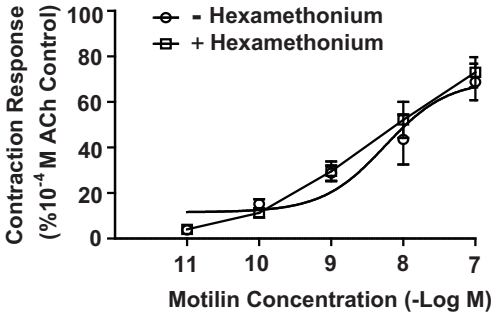
Fig. 5

Figure6

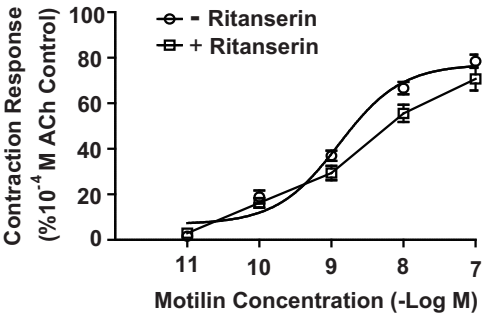
Duodenum

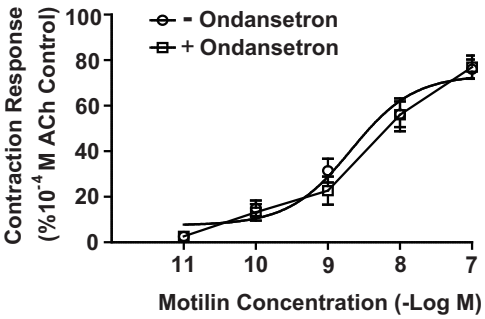
A. Cholinergic





B. Serotonergic





C. Tetrodotoxin (TTX)

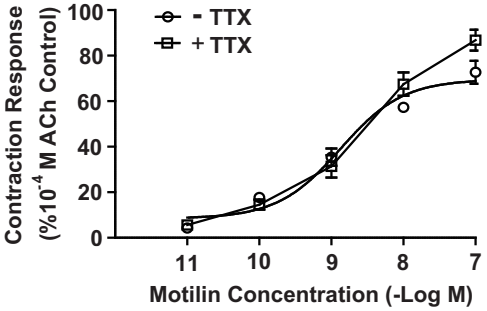
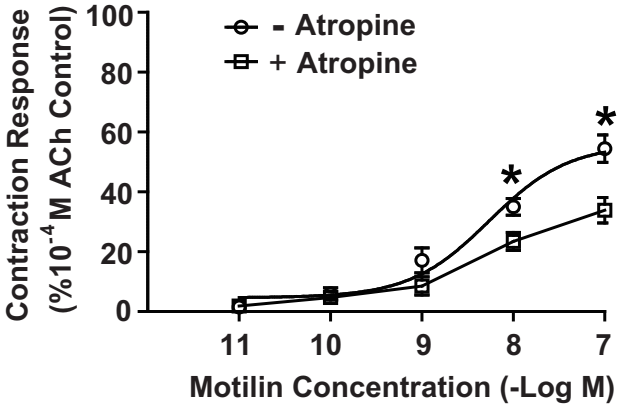


Fig. 6

Figure7

Proventriculus

A. Atropine



B. Tetrodotoxin (TTX)

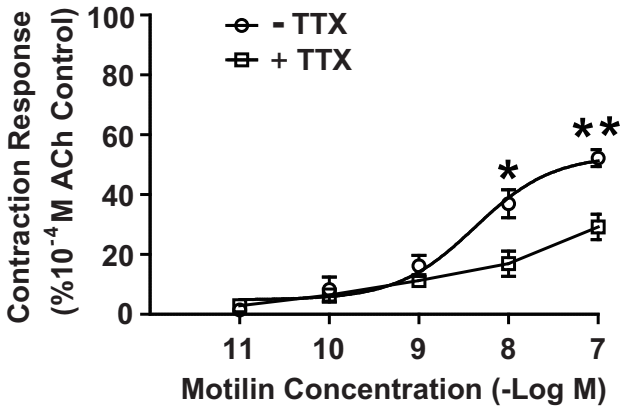


Fig. 7

Supplementary Figure1

Click here to download Supplementary Material: Supplimentary Figure 1.eps

Supplementary Material

Click here to download Supplementary Material: Supplemenatal Fig. 2.eps