Effects of ghrelin and motilin on smooth muscle contractility of the isolated gastrointestinal tract from the bullfrog and Japanese fire belly newt

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Abstract

Ghrelin has been identified in some amphibians and is known to stimulate growth hormone release and food intake as seen in mammals. Ghrelin regulates gastrointestinal motility in mammals and birds. The aim of this study was to determine whether ghrelin affects gastrointestinal smooth muscle contractility in bullfrogs (anuran) and Japanese fire belly newts (urodelian) *in vitro*. Neither bullfrog ghrelin nor rat ghrelin affected longitudinal smooth muscle contractility of gastrointestinal strips from the bullfrog. Expression of growth hormone secretagogue receptor 1a (GHS-R1a) mRNA was confirmed in the bullfrog gastrointestinal tract, and the expression level in the gastric mucosa was lower than that in the intestinal mucosa. In contrast, some gastrointestinal peptides, including substance P, neurotensin and motilin, and the muscarinic receptor agonist carbachol showed marked contraction, indicating normality of the smooth muscle preparations. Similar results were obtained in another amphibian, the Japanese fire belly newt. Newt ghrelin and rat ghrelin did not cause any contraction in gastrointestinal longitudinal muscle, whereas substance P and carbachol were effective causing contraction. In conclusion, ghrelin does not affect contractility of the gastrointestinal smooth muscle in anuran and urodelian amphibians, similar to results for rainbow trout and goldfish (fish) but different from results for rats and chickens. The results suggest diversity of ghrelin actions on the gastrointestinal tract across animals. This study also showed for the first time that motilin induces gastrointestinal contraction in amphibians.

Keywords: Growth hormone secretagogue receptor 1a, Ghrelin, Gastrointestinal motility, Motilin, Bullfrog, Japanese fire belly newt.

1. Introduction

Ghrelin was first identified in the rat stomach as an endogenous ligand for growth hormone secretagogue-receptor 1a (GHS-R1a). Two major ghrelin molecules (acylated ghrelin and unacylated ghrelin) are predominantly produced in the stomach, and only acylated ghrelin can bind and activate GHS-R1a, by which acylated ghrelin elicits its biological activities including growth hormone (GH)-releasing action, feeding-stimulating action and regulation of lipid metabolism, glucose metabolism, cardiovascular function and reproductive function (Kojima et al., 1999; Kojima and Kangawa, 2005; Hosoda et al., 2006).

From the similarity of the amino acid sequence of ghrelin to that of motilin, a gut peptide hormone produced in the duodenal mucosa, it has been thought that both peptides originated from the same ancestral gene (Peeters, 2005, Yamamoto et al., 2008). There is also a relation in receptors for the two peptides, and phylogenetic analysis showed that these two receptors are classified under the same umbrella (Mckee et al., 1997; Peeters, 2005). Since motilin stimulates gastrointestinal motility and is a mediator of interdigestive migrating motor complex (Itoh, 1997), a functional role of ghrelin in regulation of gastrointestinal motility has been examined first in mammals. Ghrelin was shown to stimulate contractility or to potentiate spontaneous phase III-like contractions in rats, mice and guinea pigs *in vivo* (Masuda et al., 2000; Fujino et al., 2003; Fukuda et al., 2004; Kitazawa et al., 2005; Depoortere et al., 2005; Nakamura et al., 2010). Ghrelin has been shown to be effective in isolated gastrointestinal smooth muscles of rats and mice *in vitro* through acting on the neural GHS-R1a (Edholm et al., 2004; Fukuda et al., 2004; Depoortere et al., 2005; Kitazawa et al., 2005). Recently, interaction of motilin and ghrelin for regulating gastrointestinal motility has been

reported in the Suncus and dogs (Ogawa et al., 2012; Mondal et al., 2013). These results indicate that ghrelin is one of the gut hormones regulating gastrointestinal motility in mammals.

Ghrelin has also been identified in non-mammalian vertebrates from elasmobranch fish to birds (Kaiya et al., 2008; 2011a). Ghrelin is predominantly produced in the mucosa of the stomach and intestine of all vertebrate species examined so far. The fundamental structure of ghrelin has been conserved during vertebrate evolution, and phylogenetic tree analysis demonstrated that ghrelin falls into five lineages: mammalian-type, avian/reptilian-type, amphibian-type, teleost fish-type and cartilaginous fish-type (Kaiya et al., 2011a). On the other hand, ghrelin receptors have been roughly divided into two groups, ghrelin receptor-like receptor (GHS-R1a-LR) and GHS-R1a (Kaiya et al., 2008; 2009; 2010; 2011a). GH release-stimulating action of ghrelin has been demonstrated in birds, frogs and fish (Baudet and Harvey, 2003; Kaiya et al., 2001, 2002; 2005). The endogenous ghrelin level is increased by fasting, and ghrelin stimulates food intake in rodents, goldfish and bullfrogs, whereas it inhibits food intake in chickens and rainbow trout (Kojima and Kangawa, 2005; Saito et al., 2005; Matsuda et al., 2006; Riley et al., 2005; Jönsson et al., 2010; Kaiya et al., 2011b; Shimizu et al., 2014), indicating that ghrelin regulates energy homeostasis both in mammalian and non-mammalian vertebrates. Regarding gastrointestinal motility, ghrelin was shown to cause contraction of gastrointestinal tracts isolated from chickens and Japanese quails *in vitro* (Kitazawa et al., 2007; 2009) as was demonstrated for rodents. However, ghrelin did not affect gastrointestinal motility in fish such as rainbow trout and goldfish (Kitazawa et al., 2012) and caused only very small contraction of the isolated zebrafish intestine (Olsson et al., 2008). These results suggest that the

gastrointestinal-stimulating action of ghrelin is not common in all vertebrates. Bullfrogs and Japanese fire belly newts are good models for ghrelin study because ghrelin and GHS-R1a have been identified and because functional roles of ghrelin such as GH release and regulation of food intake have been reported (Kaiya et al., 2001, 2011b, 2015; Shimizu et al., 2014). Since vertebrate ghrelin has been divided into five families including amphibian-type and since bullfrog ghrelin has a unique *n*-octanoic acid modification of the third threonine residue (In general, the third position of ghrelin is serine.) (Kaiya et al., 2001; 2011b), clarification of the gastrointestinal tract motility-regulating roles of ghrelin in amphibians might be important for estimating ontogenic change in the physiological function of ghrelin from fish to mammals. However, effects of ghrelin on gastrointestinal contractility have not been examined in amphibians.

Motilin, a peptide related to ghrelin, caused contraction of isolated gastrointestinal strips of mammals (rabbit, human, cat, suncus) and avians (chicken and quail) in *in vitro* studies (Ludtke et al., 1989; Depoortere et al., 1993; Kitazawa et al., 1994, 2009; Mondal et al., 2011). Although motilin-related peptides have been identified in lower vertebrates such as fish (Liu et al., 2013), motilin has not been identified in amphibians, and the action of motilin on the gastrointestinal tract has never been examined.

The aim of the present study was to determine whether bullfrog ghrelin/newt ghrelin affects contractility of gastrointestinal strips isolated from the bullfrog and Japanese fire belly newts. The bullfrog and Japanese fire belly newt are good amphibian models in which structures of ghrelin and GHS-R1a have already been identified (Kaiya et al., 2001, 2011a, 2011b, 2015). Expression of GHS-R1a mRNA in the bullfrog and

newt gastrointestinal tracts was examined by quantitative RT-PCR to understand the localization of ghrelin action sites. Effects of motilin on the gastrointestinal contractility have been investigated and compared with those of ghrelin in some animals (suncus, rats and chickens) (Depoortere et al., 2005; Kitazawa et al., 2007; Mondal et al., 2013). Therefore effect of human motilin was also examined to determine the functional role of motilin in the regulation of gastrointestinal motility in amphibians.

2. Materials and methods

All experiments were performed in accordance with Institutional Guidelines for Animal Care at Rakuno Gakuen University, Hokkaido, Japan..

2.1. Animals and tissue preparations

Bullfrogs (*Rana catesbeiana,* 200-250 g) of both sexes were commercially obtained from an animal supplier (Hokudo, Sapporo, Japan) and kept in a humid plastic case under natural photoperiod and room temperature and used within 2-3 days. Japanese fire belly newts (*Cynops pyrrhogaster,* 4-6 g) of both sexes were obtained another animal supplier (Sankyo Laboratory, Sapporo, Japan) and kept in a tank containing dechlorined tap water for 1 week under natural light/dark conditions at room temperature (20-24℃) before use. The newts were fed once in a day by commercially available granular feed. Bullfrogs and newts were sacrificed by decapitation and pithing the spinal cord by fine needles. The whole gastrointestinal tract from the stomach to anus was carefully isolated and placed in an ice-cold physiological salt solution of the following composition described in a previous study (Yano et al., 1994): NaCl, 80 mM; KCl, 2.5

mM; $CaCl_2$, 1.8 mM; NaH_2PO_4, 0.12 mM; $NaHCO_3$, 24 mM and glucose, 1.1 mM. The bullfrog gastrointestinal tract was divided in four parts: stomach, upper small intestine, middle small intestine and lower small intestine (length of each region being about 60-70 mm), and they were used for both molecular and contraction studies. After removing the mucosal layer, smooth muscle strips of the bullfrog stomach in the longitudinal and circular muscle directions were prepared. Only longitudinal muscle strips were prepared from the intestinal tract because the intestine was a small tubular organ with a diameter of 3-4 mm, and it was difficult to make circular muscle strips. For molecular study, the isolated bullfrog gastrointestinal preparations were divided into three parts, smooth muscle layer, mucosal layer and the whole preparation including both muscle and mucosal layers, and cut into small pieces. These bullfrog gastrointestinal preparations were soaked in RNAlater (Ambion Inc., Texas, USA) for 16 h and frozen until used. Expression of GHS-R1a mRNA among gastrointestinal regions or between muscle layer and mucosal layer was compared.

In the case of fire belly newt gastrointestinal tracts, only longitudinal muscle strips were prepared from the stomach and upper small intestine for contraction study because of small diameter of the tract. For molecular study, the newt gastrointestinal tract was divided into the stomach, upper, middle and lower small intestine, and large intestine. Due to the small size and tight bond between muscle layer and mucosa, separation of mucosal layer and muscle layer was not carried out in the newts. Expression levels of GHS-R1a mRNA in the five regions of gastrointestinal tract of the newts were measured and compared.

2.2. Quantitative real-time PCR (qPCR)

First-strand cDNAs were synthesized from 1 μg total RNA using the QuantiTect RT Kit (QIAGEN) with oligo-dT_{12-18} primers. Quantitative real-time PCR (qPCR) was performed using a LightCycler 480 (Roche Applied Sience, Mannheim, Germany) with the QuantiFast SYBR Green PCR Kit (QIAGEN GmbH) in combination with a primer set for bullfrog GHS-R1a (bfGHSR-Q-s: AGA ATG GTA CCA ATC CTT TTG AGA, bfGHSR-Q-AS: CAG CTA GCA TTT TTA CAGTCT GTC [240-bp amplicon]) or for newt GHS-R1a (ntGHSR-Q-s: TTG GTC GGG GTA GAA CAC GAG AAT, ntGHSR-Q-AS: CAC AAC AAG CAT TTT TAC AGT CTG [261-bp amplicon]). The reaction mixtures consisted of 250 nM of primer and template (100 ng total RNA equivalent) in 1 × master mix. The amplification reactions were 95˚C for 5 min and subsequent 35 cycles of 95˚C for 10 sec and 60˚C for 30 sec. To estimate mRNA copy numbers, qPCR samples were run with a serially diluted (10^3 to 10^6 copies) pCRII plasmid vector that consisted of an Xba-I linearized full-length target cDNA.

2.3. In vitro contraction study of gastrointestinal strips

Longitudinal muscle (LM) and circular muscle (CM; 20 mm in length and 2 mm in width) preparations from the stomach and LM strips from upper, middle or lower intestine of the bullfrogs were suspended vertically in an organ bath (5 ml) to measure contraction of muscle preparations. For the newts, LM strips from the stomach and upper intestine were used in the contraction study. The organ bath contained warmed physiological salt solution (23^{o}C) bubbled with 95% O_2 + 5% CO_2. (pH =7.4-7.6). Mechanical activity of the preparation was measured with an isometric force transducer (SB-11T, Nihon Kohden, Tokyo, Japan) and then analyzed using a computer-aided analysis system (Power Lab 2/25, Lab Chart Ver.6.1.1., Japan Bioresearch Center,

Gifu-hashima, Japan). The initial load was set at 0.5 g for each preparation. The preparations were rinsed with the physiological salt solution every 15 min and allowed to equilibrate for 1 h. Prior to the addition of test substances, it was confirmed that spontaneous contraction appeared and that depolarization by 50 mM KCl caused contraction of each muscle strip.

Synthetic octanoylated bullfrog ghrelin (GLT[O-n-octanoyl]FLSPADMQKIA ERQSQNKLRHGNM) (Kaiya et al., 2001, custom-ordered from Peptide Institute Inc. Osaka, Japan), Japanese fire belly newt ghrelin (GSS[O-n-octanoyl]FLSPADLHKPQPRKPARKIIPNNPQ) (Kaiya et al., 2011a, custom-ordered from Peptide Institute Inc. Osaka, Japan) or rat ghrelin at was applied to the organ bath at 1h intervals, and evoked mechanical changes in muscle strips were observed. The effect of human motilin (Peptide Institute Inc.) on the isolated amphibian gastrointestinal strips was also examined in the present experiments. The mechanical responses of carbamylcholine chloride (Carbachol, Sigma), human neurotensin (Peptide Institute, Inc), substance P (Peptide Institute, Inc) were also examined as possible effective substances. Mechanical changes in muscle tonus (amplitude) caused by these substances were normalized by a standard contraction caused by 50 mM KCl and expressed as a relative change in muscle tonus (%) for comparison. All drugs were dissolved and diluted in distilled water and applied to the organ bath at indicated concentrations. The administration volume of each drug was less than 1% of the bath volume except for KCl (2.5 % of bath volume).

2.4. Statistical analysis

All data are expressed as means ± S.E.M. of at least three experiments. The

significance of differences between values was determined at $P < 0.05$ using Student's *t*-test (paired and unpaired) for single comparisons or one-way ANOVA followed by Dunnett's test for multiple comparisons.

3. Results

3.1. Bullfrog stomach

After equilibration, LM and CM preparations from the bullfrog stomach showed a spontaneous contractility, and application of high K^+ solution (50 mM) caused transient contraction. The frequencies and amplitudes of spontaneous contraction were 20.5 ± 1.4 contractions / 10 min and 0.1 ± 0.02 g in the LM strips (n = 7) and 18.9 ± 1.5 contractions / 10 min and 1.54 ± 0.48 g in the CM strips (n = 9). The amplitude of high-K^+-induced contractions were 0.25 ± 0.15 g in the LM strips (n = 7) and 2.88 ± 0.66 g in the CM strips (n = 9). Contractile force observed in the CM strips was significantly stronger than that in the LM strips (Student's *t*-test). Figs. 1 and 2 show typical mechanical responses to rat ghrelin at 1 μM and bullfrog ghrelin at 1 μM in LM and CM strips from the bullfrog stomach. Neither rat ghrelin nor bullfrog ghrelin caused any mechanical changes in the muscle strips. Neurotensin at 1 μM also did not cause any contraction of the gastric strips. In contrast, carbachol at 10 μM and substance P at 1 μM caused contractions. Carbachol was effective to cause contraction at a lower concentration (100 nM – 1 μM). Relative changes in smooth muscle tonus induced by carbachol, rat ghrelin, bullfrog ghrelin, neurotensin and substance P are summarized in Fig. 4.

3.2. Bullfrog small intestine

241 Rat ghrelin and bullfrog ghrelin also did not cause any contraction of LM strips from
242 the upper intestine and lower intestine (Figs. 3 and 4). However, carbachol and
243 substance P caused contraction of intestinal LM strips. The responsiveness of upper
244 small intestinal strips to substance P was examined because it was a potent stimulant.
245 Substance P (0.1 nM -1 μM) caused concentration-dependent contractions, and the EC_{50}
246 value was 3.9 ± 1.1 nM (n = 5). In contrast to gastric muscle strips, neurotensin (1 μM)
247 caused contraction of intestinal strips. Relative contractions were 74.4 ± 18.4% (n = 6)
248 in the upper small intestinal LM strips and 136.8 ± 45.4 % (n = 3) in the lower small
249 intestinal LM strips.
250
251 ***3.3. Expression of GHS-R1a mRNA in the bullfrog gastrointestinal tract***
252 In the whole preparations, GHS-R1a mRNA expression levels were comparable in all
253 gastrointestinal regions: stomach (1681 ± 1274 copies / 100 ng total RNA, n = 4), upper
254 small intestine (1641 ± 490 copies / 100 ng total RNA, n=4) and lower small intestine
255 (1402 ± 314 copies / 100 ng total RNA, n = 4). Whole preparations were divided
256 mechanically into smooth muscle layer and mucosal layer **at each region**. GHS-R1a
257 mRNA expression in the muscle layer tended to decrease from the stomach to lower
258 intestine, but the difference was not significant (Fig. 5A). On the other hand, GHS-R1a
259 expression in the intestinal mucosa increased significantly compared with the
260 expression in the gastric mucosa (Fig. 5A).
261
262 3.4. Effects of human motilin in the bullfrog gastrointestinal tract
263 Motilin at 1 μM did not cause any mechanical changes in stomach LM (0.13 ± 0.1%,
264 n = 4) and CM strips (0.24 ± 0.2%, n = 5) (Fig. 6A) but caused contraction in the upper

intestinal LM strips. Contractile responses in eight different strips were 2.6%, 10.6%, 14.4%, 19.2%, 20.0%, 45.5%, 50.5% and 57.1% (mean=27.5 ± 7.7%, n = 8). In the middle (0.04 ± 1.5%, n = 5) and lower small intestinal LM strips (1.4 ± 1.8%, n = 9), motilin did not cause obvious changes in smooth muscle tonus (Fig. 6B).

3.5. Fire belly newt gastrointestinal tract

Similar to the bullfrog gastric LM strips, isolated gastric LM strips of Japanese fire belly newt contracted spontaneously in the present experimental conditions. High-K^+ solution (50 mM) caused transient contraction. Carbachol (10 μM) and substance P (1 μM) caused contractile activity with similar amplitudes (High-K^+ = 100%, carbachol: 110.6 ± 17.9%, n = 7, substance P: 119.8 ± 55.0%, n = 6). However, rat ghrelin (1 μM, 3.3 ± 1.9%, n = 7) and newt ghrelin (100 nM, -0.6 ± 3.6%, n = 5; 1 μM, 0 ± 3.1%, n = 8) did not cause any mechanical changes in the LM strips. Motilin (1 μM) also did not cause any mechanical changes in the tonus of gastric LM strips (Fig. 7).

In upper small intestinal LM strips, carbachol (10 μM) and substance P (1 μM) caused contractions of intestinal strips (High-K^+ = 100%, carbachol: 128 ± 35.4%, n = 4, substance P: 193 ± 55.0%, n = 4). However, rat ghrelin (1 μM, 7.6 ± 3.5%, n = 4) and newt ghrelin (100 nM, 6.7 ± 2.5%, n = 4; 1 μM, 2.7 ± 2.9%, n = 6) did not cause any mechanical changes. Motilin also did not affect tonus of the upper small intestinal LM strips (Fig. 8). Amplitudes of the responses to motilin ranged from 7.1% to 15.4% (10.5 ± 2.4%, n = 4), but these values were smaller than those for carbachol or substance P.

3.6. GHS-R1a mRNA expression in the newt gastrointestinal tract

GHS-R1a mRNA expression in the stomach of the newt was 12 ± 3 copies / 100 ng

289 total RNA (n = 5) (Fig. 5). **Although the mean value was low compared with that of**
290 **bullfrog whole stomach, the expression level** was not significantly different from that
291 in the bullfrog stomach (Student's *t* test, p=0.28). The expression levels in the upper,
292 middle and lower small intestine were 59.8 ± 3.9 copies / 100 ng total RNA (n = 5),
293 68.7± 14.9 copies / 100 ng total RNA (n = 5) and 73.6 ± 10.5 copies / 100 ng total RNA
294 (n = 5), respectively. The expression level in the intestine was significantly higher than
295 that in the stomach. In the large intestine, the expression level was intermediate between
296 the levels in the stomach and small intestine (Fig. 5B).
297
298 **4. Discussion**
299 The ghrelin/GHS-R1a system is conserved in many vertebrate species from fish to
300 mammals and is involved in various physiological functions, which are common or
301 differ among species (Kaiya et al., 2008; 2011a). GH-releasing ability and/or glucose
302 homeostasis are common actions among vertebrates examined so far, although effects of
303 the ghrelin/GHS-R1a system on feeding regulation and gastrointestinal motility vary in
304 animal species (see review by Kaiya et al., 2013). For example, ghrelin stimulates food
305 intake in dogs, rodents, tilapia and goldfish but inhibits food intake in chickens and
306 rainbow trout. The different actions may reflect their variable living habitats or
307 metabolic activity. Since ghrelin and GHS-R1a have some similarities to a gut
308 motility-stimulating hormone, motilin, and its receptor, effects of ghrelin on
309 gastrointestinal motility have been studied and gastrointestinal motility-stimulating
310 action of ghrelin in birds and mammals has been already demonstrated (Masuda et al.,
311 2000; Fujino et al., 2003; Fukuda et al., 2004; Depoortere et al., 2005; Kitazawa et al.,
312 2005, 2007, 2009). However, this action also has a species-related variation; ghrelin

313 does not affect gut motility in fish (Kitazawa et al., 2012). In this regard, investigation
314 of the effect of ghrelin on gut motility in amphibians might be interesting from the view
315 point of comparative endocrinology. The present study is the first study in which
316 ghrelin-induced responses in isolated gastrointestinal strips of the bullfrog and Japanese
317 fire belly newt were examined. The results demonstrated that ghrelin, even though its
318 structure was homologous, did not affect the gastrointestinal motility (especially
319 contractility of LM) in either of the amphibians *in vitro*.

320 We used two species of amphibians with different characteristics: the bullfrog is an
321 anuran amphibian and the Japanese fire belly newt is urodelian amphibian. It might be
322 interesting to compare the responses to ghrelin and related peptides in the two different
323 species. The structure of GHS-R1a was recently clarified in the bullfrog and Japanese
324 fire belly (Kaiya et al., 2011a, 2011b; 2015). Ghrelin was identified in both amphibians,
325 and bullfrog ghrelin is unique in that it has threonine instead of serine at the acylated
326 amino acid residue and mainly synthesized in the gastric mucosa (Kaiya et al., 2001;
327 2006; 2011a). Isolated gastric LM and CM strips showed spontaneous contractile
328 activity, and a strong contraction was caused by high-K^+ stimulation, the muscarinic
329 receptor agonist carbachol and substance P, indicating that the smooth muscle strips can
330 respond to depolarization and hormonal receptor activation. However, neither bullfrog
331 ghrelin nor rat ghrelin caused mechanical changes in gastric LM and CM strips or the
332 upper and lower small intestinal LM strips of the bullfrog. Kaiya et al. (2011) have
333 already confirmed that **synthesized** bullfrog ghrelin used in this experiment increased
334 intracellular Ca^{2+} concentration in the bullfrog GHS-R1a-transfected-HEK293 cells.
335 GHS-R1a mRNA showed homogenous expression in the bullfrog stomach and small
336 intestine. Relationships between GHS-R1a mRNA expression and ghrelin-induced

mechanical responses in the gastrointestinal tract have been classified into two patterns. One is observed in chickens, rats and guinea-pigs, and the ghrelin-induced response was shown to be dependent on the expression level of GHS-R1a mRNA (Kitazawa et al., 2009, 2011, 2013; Nunoi et al., 2012). The other is observed in goldfish and rainbow trout, and although GHS-R1a mRNA was expressed at a moderate level, ghrelin failed to cause any mechanical changes (Kitazawa et al., 2012). One possible explanation for the discrepancy is that GHS-R1a mRNA is not translated in the GHS-R1a protein. Expression of GHS-R 1a mRNA is dissociated with its protein distribution as previously demonstrated in the case of ghrelin and ghrelin mRNA (Ghelardoni et al., 2006). The second explanation is that GHS-R1a protein is expressed in the gastrointestinal tract, but the region of expression is not on enteric neurons and smooth muscle cells linked to muscle contraction but on mucosal cells for endocrine and exocrine systems. It has been demonstrated that GHS-R1a mRNA is expressed in the mucosa of the human intestine (Takeshita et al., 2006). The third is that, recent study suggested the importance of intrinsic primary afferent neurons (IPAN) in the mucosa for the ghrelin-induced mechanical action of the suncus gastrointestinal tract (Mondal et al., 2013). Removal of the mucosal layer for making smooth muscle preparations in the present study might have destroyed neural networks included in the actions of ghrelin. In fact, GHS-R1a mRNA was expressed in the bullfrog mucosa in the present study. Anyway, distribution of GHS-R1a protein in the gastrointestinal tract of two amphibian species should be investigated in the future studies to clarify the discrepancy between functional results and level of GHS-R1a mRNA expression.

We compared GHS-R1a mRNA expression levels in the muscle layer and mucosa of the bullfrog. Although average GHS-R1a mRNA expression levels in the muscle layer

tended to decrease from the stomach to lower small intestine, expression of GHS-R1a mRNA in three different regions were not significantly different whereas that in the mucosal layer increased significantly in the lower small intestine. In chickens, gastrointestinal region-related expression of GHS-R1a was more emphasized in muscle preparations than that in whole preparations containing the mucosa and muscle, suggesting that GHS-R1a mainly exists in smooth muscle layers including the myenteric plexus, and ghrelin affected the smooth muscle contractility (Kitazawa et al., 2009) as shown in a previous immunohistochemical study (Dass et al., 2003). However, the results of present study indicated that GHS-R1a is mainly distributed in the mucosa in the lower small intestine, suggesting that ghrelin regulates mucosal functions such as absorption of nutrients, electrolytes and water and secretion of hormone, electrolyte and water rather than gut motility. In support of this notion, GHS-R1a expression increased in 10 days after dehydration in the bullfrog stomach (Kaiya et al., 2011b).

Isolated gastrointestinal preparations of Japanese fire belly newt also contracted spontaneously, but both newt ghrelin and rat ghrelin did not cause any mechanical changes of gastrointestinal LM strips. However, high-K^+ (depolarization), carbachol and substance P caused marked contractions, indicating that there was no problem in the LM preparations. In addition, bioactivity of the present newt ghrelin has been already confirmed in the newt GHS-R1a-transfected-HEK293 cells (Kaiya et al., 2015). Therefore in addition to the results for the bullfrog, our results showed that ghrelin does not change contractility of the newt gastrointestinal LM in *in vitro* experimental condition.

Motilin, produced in the mucosa of the duodenum, stimulates gastrointestinal motility and its structure has been determined in the chicken and several species of

mammals including humans, dogs, cats, rabbits and suncus (Depoortere et al., 1993; Kitazawa et al., 1994, 2009; De Clercq et al., 1996; Itoh, 1997; Yamamoto et al., 2008; Tsutsui et al., 2009; Mondal et al., 2011). Recently, the motilin/motilin receptor (GPR38) system has been demonstrated in a teleost, zebrafish, through molecular cloning and functional studies (Olsson et al., 2008; Liu et al., 2013). Presence of motilin/motilin receptor system in fish, birds and mammals suggests that the motilin system is conserved in vertebrates and also exerts function in the amphibian gastrointestinal tract (bullfrog and newt). In the present study, human motilin caused contraction of bullfrog intestinal strips, and the contractile response was restricted to the upper small intestine corresponding to the duodenum in mammals. This is the first report to demonstrate the involvement of motilin in bullfrog gastrointestinal motility. Region-related contraction of isolated gastrointestinal strips by motilin has been already demonstrated in rabbits, chickens and Japanese quails (Kitazawa et al., 1994; 2009), and high expression of the motilin receptor has been demonstrated in the cat duodenum (Depoortere et al., 1993) and in the chicken small intestine (Kitazawa et al., 2013). The presence of the motilin system in amphibians has not yet been demonstrated, but the results of the present study showing motilin had contractile activity in upper intestinal strips of bullfrogs suggest that the motilin system is present in the bullfrog. However, intestinal smooth muscle strips of newts did not respond to human motilin, indicating a species difference in the responsiveness to motilin in the amphibian gastrointestinal tract as observed in the mammals. Bullfrogs and newts possess different characteristics as amphibians (an anuran amphibian vs. aurodelian amphibian). Since human motilin was used in the present study, the difference in motilin structure and motilin receptor structure between bullfrogs and Japanese fire belly newts might explain the different

actions of human motilin. Anyway the bullfrog would be a good model for further investigation of motilin function in amphibians.

In summary, we examined the action of ghrelin on isolated gastrointestinal tracts of the bullfrog and Japanese fire belly newt using homologous ghrelin. Despite the fact that GHS-R1a mRNA was expressed throughout the gastrointestinal tract, ghrelin did not cause mechanical change of the gastrointestinal LM in the two amphibian species, although neurotensin and substance P were capable of contracting the preparations. These results indicate that ghrelin does not play a crucial role in gastrointestinal motor function in these amphibians, being different from results obtained for avian and mammals, and indicate diversity of biological actions of ghrelin in regulation of gastrointestinal motility among vertebrates. However, except for bullfrog stomach, the effects of ghrelin were examined only in LM strips and it has been shown that ghrelin can act on the terminals of vagus nerves or intrinsic primarily afferent neurons in the intestinal wall (Fukuda et al., 2004; Nakamura et al., 2009; Mondal et al., 2013). Therefore, further studies to investigate gastrointestinal motility using isolated CM strips (*in vitro*) and whole animals (*in vivo* study) are needed to determine the regulation of gastrointestinal motility by ghrelin.

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The authors declare no conflict of interest.

Authors contributions:

T.K. and H.K. designed research; T.K., M.S., A.K., N.Y., H.T. and H.K. performed research; T.K., N.Y., H.T. and H.K. analyzed data; and T.K., H.T. and H.K. wrote the paper.

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458 **References**

459 Baudet, M.L., Harvey, S., 2003. Ghrelin-induced GH secretion in domestic fowl in vivo
460 and in vitro. J . Endocrinol. 179, 97-10

461 Dass, N.B., Munonyara, M., Bassil, A.K., Hervieu, G.J., Osbourne, S., Corcoran, S.,
462 Morgan, M., Sanger, G.J., 2003. Growth hormone secretagogue receptors in rat
463 and human gastrointestinal tract and the effects of ghrelin. Neuroscience.120,
464 443-453.

465 De Clercq , P., Depoortere, I., Macielag, M., Vandermeers, A., Vandermeers-Piret, M.C.,
466 Peeters, T.L., 1996. Isolation, sequence, and bioactivity of chicken motilin. Peptides.
467 17, 203-208.

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

471 Depoortere, I., Peeters, T.L., Vantrappen, G. 1993. Distribution and characterization of
472 motilin receptors in the cat. Peptides 14, 1153-1157.

473 Edholm, T., Levin, F., Hellström, P.M., Schmidt, P.T., 2004. Ghrelin stimulates motility
474 in the small intestine of rats through intrinsic cholinergic neurons. Regul Pept. 121,
475 25-30.

476 Fujino, K., Inui, A., Asakawa, A., Kihara, N., Fujimura, M., Fujimiya, M., 2003.
477 Ghrelin induces fasted motor activity of the gastrointestinal tract in conscious fed
478 rats. J. Physiol. 550, 227–240.

479 Fukuda, H., Mizuta, Y., Isomoto, H., Takeshima, F., Ohnita, K., Ohba, K., Omagari, K.,
480 Taniyama, K., Kohno, S., 2004. Ghrelin enhances gastric motility through direct
481 stimulation of intrinsic neural pathways and capsaicin-sensitive afferent neurones
482 in rats. Scand. J. Gastroenterol. 39, 1209-1214.

483 Ghelardoni, S., Carnicelli, V., Frascarelli, S., Ronca-Testoni, S., Zucchi, R., 2006.
484 Ghrelin tissue distribution: comparison between gene and protein expression. J.
485 Endocrinol. Invest. 29, 115-121.

486 Hosoda, H., Kojima, M., Kangawa, K., 2006. Biological, physiological, and
487 pharmacological aspects of ghrelin. J. Pharmacol. Sci. 100, 398-410.

488 Itoh, Z., 1997. Motilin and clinical application. Peptides 18, 593-608.

489 Jönsson, E., Kaiya, H., Björnsson, B.T., 2010. Ghrelin decreases food intake in juvenile
490 rainbow trout (Oncorhynchus mykiss) through the central anorexigenic
491 corticotropin-releasing factor system. Gen. Comp. Endocrinol. 166, 39-46.
492

493 Kaiya, H., Miyazato, M., Kangawa, K., Peter, R.E., Unniappan, S., 2008. Ghrelin: a
494 multifunctional hormone in non-mammalian vertebrates. Comp. Biochem. Physiol
495 A. 140, 109-128.

Kaiya, H., Kojima, M., Hosoda, H., Koda, A., Yamamoto, K., Matusmoto, M., Minamitake ,Y., Kikuyama, S., Kangawa, K., 2001. Bullfrog ghrelin is modified by n-octanoic acid at its third threonine residue. J. Biol. Chem. 276, 40441-40448.

Kaiya, H., Miura, T., Matsuda, K., Miyazato, M., Kangawa, K., 2010. Two functional growth hormone secretagogue receptor (ghrelin receptor) type 1a and 2a in goldfish, *Carassius auratus*. Mol. Cell. Endocrinol. 327, 25-39.

Kaiya, H., Miyazato, M., Kangawa, K., 2011a. Recent advances in the phylogenetic study of ghrelin. Peptides 32, 2155-2174.

Kaiya, H,, Mori, T., Miyazato, M., Kangawa, K., 2009. Ghrelin receptor (GHS-R)-like receptor and its genomic organisation in rainbow trout, Oncorhynchus mykiss. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 153, 438-450.

Kaiya, H., Sakata, I., Yamamoto, K., Koda, A., Sakai, T., Kangawa, K., Kikuyama, S., 2006.Identification of immunoreactive plasma and stomach ghrelin, and expression of stomach ghrelin mRNA in the bullfrog, Rana catesbeiana. Gen. Comp. Endocrinol. 148, 236-244.

Kaiya, H., Van Der Geyten, S., Kojima, M., Hosoda, H., Kitajima, Y., Matsumoto, M., Geelissen, S., Darras, V.M., Kangawa, K., 2002. Chicken ghrelin: purification, cDNA cloning, and biological activity. Endocrinology 143, 3454-3463.

Kaiya, H., Koizumi, Y., Konno, N., Yamamoto, K., Uchiyama, M., Kangawa, K., Miyazato, M., 2011b. Ghrelin Receptor in Two Species of Anuran Amphibian,

517 Bullfrog (*Rana catesbeiana*), and Japanese Tree Frog (*Hyla japonica*). Front
518 Endocrinol (Lausanne). 2, 31.

519 Kaiya, H., Small, B.C., Bilodeau, A.L., Shepherd, B.S., Kojima, M., Hosoda, H.,
520 Kangawa, K., 2005. Purification, cDNA cloning, and characterization of ghrelin in
521 channel catfish, Ictalurus punctatus. Gen. Comp. Endocrinol. 143, 201-210.
522
523 Kaiya, H., Kangawa, K., Miyazato, M., 2013. What is the general action of ghrelin for
524 vertebrates? - comparisons of ghrelin's effects across vertebrates. Gen. Comp.
525 Endocrinol. 181, 187-191.
526
527 Kaiya, H., Kangawa, K., Miyazato M., 2015 Ghrelin receptor in Japanese fire belly
528 newt, Cynops pyrrhogaster. Comp. Biochem. Physiol B. 189, 15-22.
529

530 Kitazawa, T., Ichikawa, S., Yokoyama, T., Ishii, A., Shuto, K., 1994. Stimulating action
531 of KW-5139 (Leu^{13}-motilin) on gastrointestinal motility in the rabbit. Br. J.
532 Pharmacol. 111, 288-294.

533 Kitazawa, T., De Smet, B., Verbeke, K., Depoortere, I., Peeters, T.L., 2005. Gastric
534 motor effects of peptide and non-peptide ghrelin agonists in mice in vivo and in
535 vitro. Gut 54, 1078-1084.

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

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

541 Kitazawa, T., Nakamura, T., Saeki, A., Teraoka, H., Hiraga, T., Kaiya, H., 2011.
542 Molecular identification of ghrelin receptor (GHS-R1a) and its functional role in
543 the gastrointestinal tract of the guinea-pig. Peptides 32, 1876-1886.

544 Kitazawa, T., Itoh, K., Yaosaka, N., Maruyama, K., Matsuda, K., Teraoka, H., Kaiya,
545 H., 2012. Ghrelin does not affect gastrointestinal motility in rainbow trout and
546 goldfish in vitro. Gen. Comp. Endocrinol.178, 539-545.

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

550 Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matuo, H., Kangawa, K., 1999.
551 Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402,
552 656-660.

553 Kojima, M., Kangawa, K., 2005. Ghrelin: Structure and Function. Physiol. Rev. 85,
554 495-522.

555 Liu, Y., Li, S., Huang, X., Lu, D., Liu, X., Ko, W.H., Zhang, Y., Cheng, C.H., Lin, H.,
556 2013. Identification and characterization of a motilin-like peptide and its receptor
557 in teleost. Gen. Comp. Endocrinol. 186, 85–93.

558 Lüdtke, F.E., Müller, H., Golenhofen, K., 1989. Direct effects of motilin on isolated
559 smooth muscle from various regions of the human stomach. Pflugers Arch. 414,
560 558-563.

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

564 Matsuda, K., Miura, T., Kaiya, H., Maruyama, K., Uchiyama, M., Kangawa, K., Shioda,
565 S., 2006. Stimulatory effect of n-octanoylated ghrelin on locomotor activity in the
566 goldfish, *Carassius auratus*. Peptides 27, 1335-1340.

567 McKee, K.K., Tan, C.P., Palyha, O.C., Liu, J., Feighner, S.D., Hreniuk, D.L., Smith,
568 R.G., Howard, A.D., Van der Ploeg, L.H., 1997. Cloning and characterization of
569 two human G protein-coupled receptor genes (GPR38 and GPR39) related to the
570 growth hormone secretagogue and neurotensin receptors. Genomics. 46, 426-434.

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

574 Mondal, A., Aizawa, S., Sakata, I., Goswami, C., Oda, S., Sakai, T., 2013. Mechanism
575 of ghrelin-induced gastric contractions in Suncus murinus (house musk shrew):
576 involvement of intrinsic primary afferent neurons. PLoS One. 8, e60365.

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

580 Nunoi, H., Matsuura, B., Utsunomiya, S., Ueda, T., Miyake, T., Furukawa, S., Kumagi,
581 T., Ikeda, Y., Abe, M., Hiasa, Y., Onji, M., 2012. A relationship between motilin
582 and growth hormone secretagogue receptors. Regul Pept. 176, 28-35.

583 Ogawa, A., Mochiki, E., Yanai, M., Morita, H., Toyomasu, Y., Ogata, K., Ohno, T.,
584 Asao, T., Kuwano, H., 2012. Interdigestive migrating contractions are coregulated
585 by ghrelin and motilin in conscious dogs. Am. J. Physiol. Regul. Integr. Comp.
586 Physiol. 302, R233-241.

587 Olsson, C., Holbrook, J.D., Bompadre, G., Jönsson, E., Hoyle, C.H., Sanger, G.J.,
588 Holmgren, S., Andrews, P.L., 2008. Identification of genes for the ghrelin and
589 motilin receptors and a novel related gene in fish, and stimulation of intestinal
590 motility in zebrafish (*Danio rerio*) by ghrelin and motilin. Gen. Comp. Endocrinol.
591 155, 217-226.

592 Peeters, T.L., 2005. Ghrelin: a new player in the control of gastrointestinal functions.
593 Gut 54, 1638-1649.

594 Riley, L.G., Fox, B.K., Kaiya, H., Hirano, T., Grau, E.G., 2005. Long-term treatment of
595 ghrelin stimulates feeding, fat desposition and alters the GH/GF-I axis in the
596 tilapia, *Oreochromis mossambicus*. Gen. Comp. Endocrinol. 142, 234-240.

597 Saito, E.S., Kaiya, H., Tachibana, T., Tmononaga, S., Denbow, D.M., Kangawa, K.,
598 2005. Inhibitory effect of ghrelin on food intake is mediated by the
599 corticotropin-releasing factor system in neonatal chicks. Regul Pept. 125, 201-208.

600 Shimizu, S., Kaiya, H., Matsuda, K., 2014. Stimulatory effect of ghrelin on food intake
601 in bullfrog larvae. Peptides 51, 74-79.

602 Takeshita, E., Matsuura, B., Dong, M., Miller, L.J., Matsui, H., Onji, M., 2006.
603 Molecular characterization and distribution of motilin family receptors in the
604 human gastrointestinal tract. J Gastroenterol. 41, 223-230.

605 Tsutsui, C., Kajihara, K., Yanaka, T., Sakata, I., Itoh, Z., Oda, S., Sakai, T., 2009.
606 House musk shrew (Suncus murinus, order: Insectivora) as a new model animal for
607 motilin study. Peptides 30, 318-329.
608
609 Yamamoto, I., Kaiya, H., Tsutsui, C., Sakai, T., Tsukada, A., Miyazato, M., Tanaka, M.,
610 2008. Primary structure, tissue distribution, and biological activity of chicken motilin
611 receptor. Gen. Comp. Endocrinol. 156, 509-514.
612
613 Yano, K., Vaudry, H., Conlon, J.M., 1994. Spasmogenic actions of frog urotensin II on
614 the bladder and ileum of the frog, Rana catesbeiana. Gen. Comp. Endocrinol. 96,
615 412-419.
616

Figure Legends

Fig. 1 Typical mechanical responses to high-K^+ (50 mM KCl), carbachol (10 μM), rat ghrelin (1 μM), bullfrog ghrelin (1 μM), neurotensin (1 μM) and substance P (1 μM) in longitudinal muscle strips from the bullfrog stomach. High-K^+-induced contraction was as serving as a standard contraction to normalize the mechanical responses of other substances.

Fig. 2 Typical mechanical responses to high-K^+ (50 mM KCl), carbachol (10 μM), rat ghrelin (1 μM), bullfrog ghrelin (1 μM), neurotensin (1 μM) and substance P (1 μM) in the circular muscle strips from the bullfrog stomach. High-K^+-induced contraction was as serving as a standard contraction to normalize the mechanical responses of other substances.

Fig. 3 Typical mechanical responses to high-K^+ (50 mM KCl), carbachol (10 μM), rat ghrelin (1 μM), bullfrog ghrelin (1 μM), neurotensin (1 μM) and substance P (1 μM) in longitudinal muscle strips from the bullfrog upper intestine. High-K^+-induced contraction was as serving as a standard contraction to normalize the mechanical responses of other substances. Neurotensin caused contraction of intestinal strips different from that of gastric muscle strips.

Fig. 4 Comparison of mechanical responses to ghrelin peptides with those to other bioactive stimulants in the gastric longitudinal muscle (A), gastric circular muscle (B), upper intestinal longitudinal muscle (C) and lower intestinal longitudinal muscle (D). The mechanical changes induced by carbachol (1 and 10 μM), rat ghrelin (100 nM and

1 μM), bullfrog ghrelin (100 nM and 1 μM), neurotensin (1 μM) and substance P (1 μM) were normalized by the amplitude of standard contraction induced by high-K^+ (50 mM) and are shown as relative changes in muscle tonus. The number following each substance indicated concentration (log M). Columns are means ± SE of more than 3 experiments.

Fig. 5 Expression of GHS-R1a mRNA in gastrointestinal tracts of the bullfrog and Japanese fire belly newt. A: The gastrointestinal tract of the bullfrog was divided into three regions: stomach, upper small intestine and lower small intestine, **and expression of GHS-R1a mRNA was compared with that in the stomach (Whole).** The mucosal layer **(Mucosa)** and smooth muscle layer **(Muscle)** were separated **from each whole preparation** by microscopic dissection, and **the expression level of GHS-R1a mRNA was compared among three regions.** B: The gastrointestinal tract of the newt was divided into five regions: stomach, upper, middle and lower small intestine, and large intestine (whole preparations). * $P<0.05$ significantly different from **respective** gastric **values** using one-way ANOVA followed by Dunnett's test. Values are means ± SE of 4 (bullfrog) and 5 (newt) preparations.

Fig. 6 Human motilin (1 μM)-induced responses in longitudinal (LM) and circular muscle strips (CM) from the stomach (A) and upper, middle and lower small intestinal LM strips (B) of bullfrog. Motilin caused contraction only in the upper small intestinal strips.

Fig. 7 Typical mechanical responses to high-K^+ (50 mM KCl), carbachol (10 μM), rat

665 ghrelin (1 μM), bullfrog ghrelin (1 μM), motilin (1 μM) and substance P (1 μM) in
666 longitudinal muscle strips from the stomach of the Japanese fire belly newt.
667 High-K^+-induced contraction was as serving as a standard contraction to normalize the
668 mechanical responses of other substances.
669
670 Fig. 8 Typical mechanical responses to high-K^+ (50 mM KCl), carbachol (10 μM), rat
671 ghrelin (1 μM), bullfrog ghrelin (1 μM), motilin (1 μM) and substance P (1 μM) in
672 longitudinal muscle strips from the upper intestine of the Japanese fire belly newt.
673 High-K^+-induced contraction was as serving as a standard contraction to normalize the
674 mechanical responses of other substances.
675

Figures

Fig.1

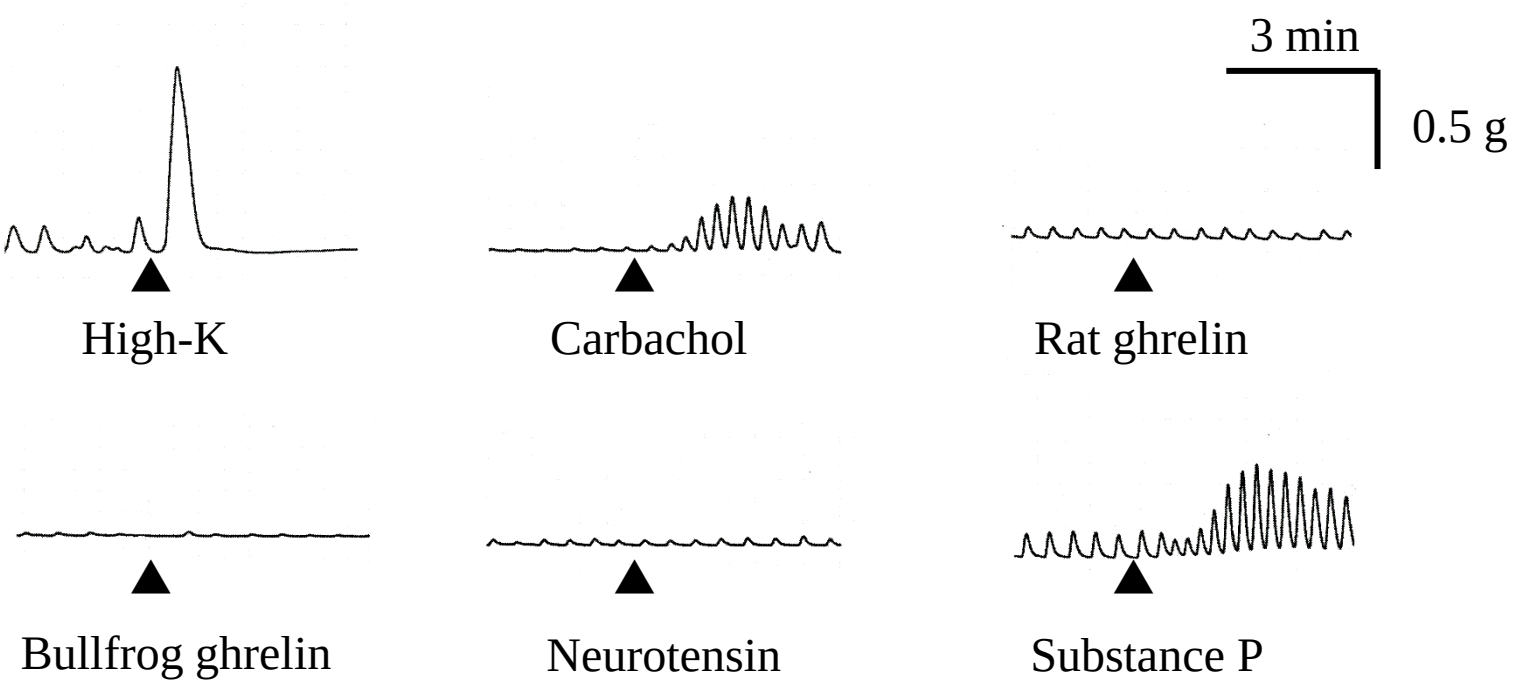
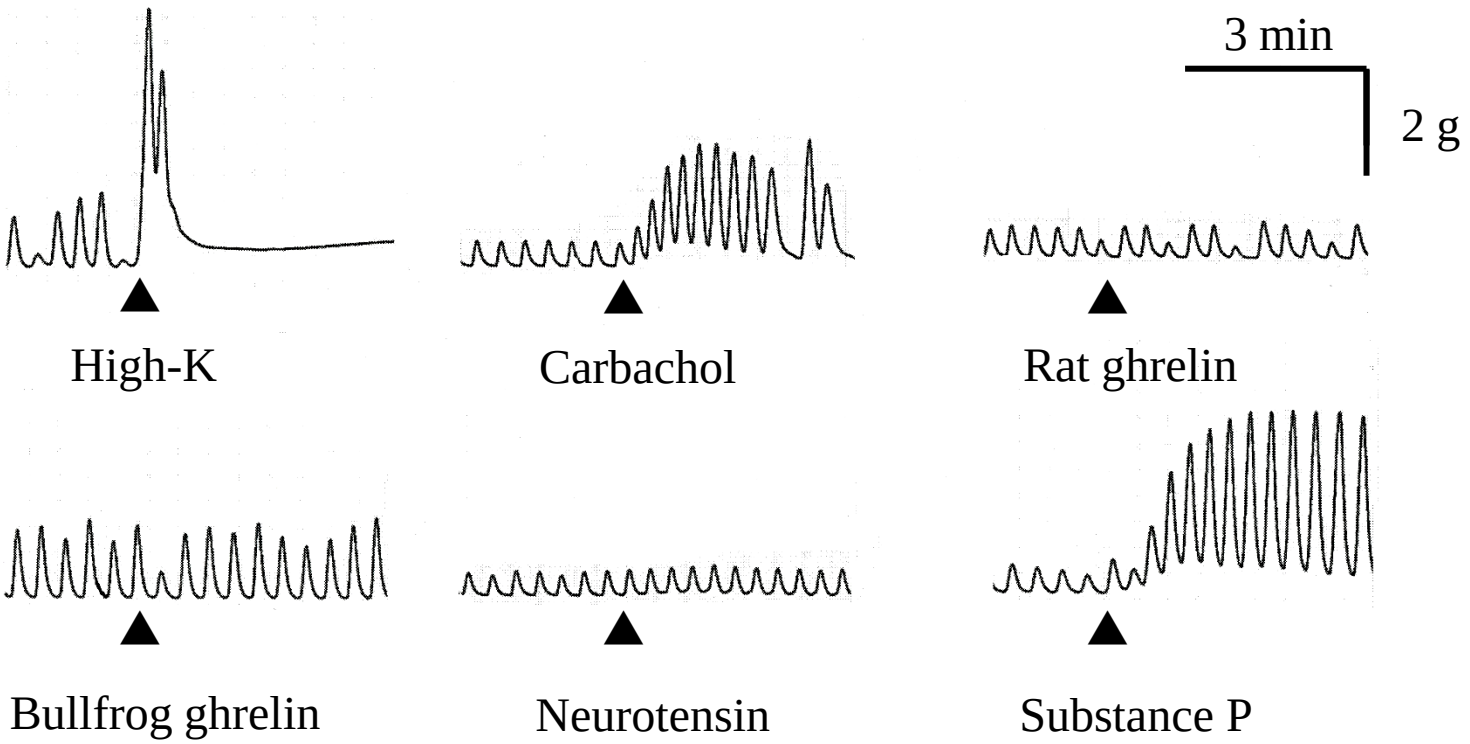


Fig.2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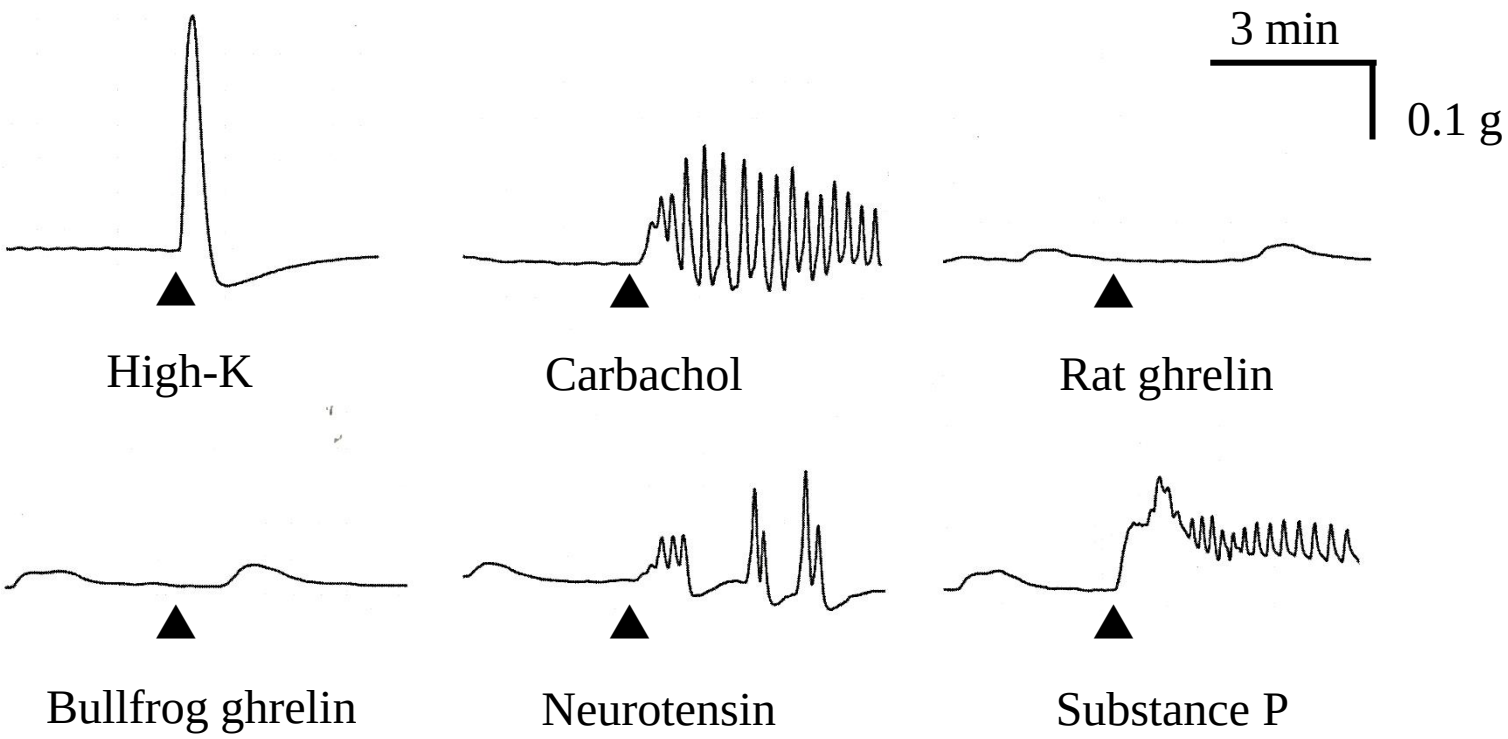
Bullfrog ghrelin
Neurotensin
Substance P

Fig.3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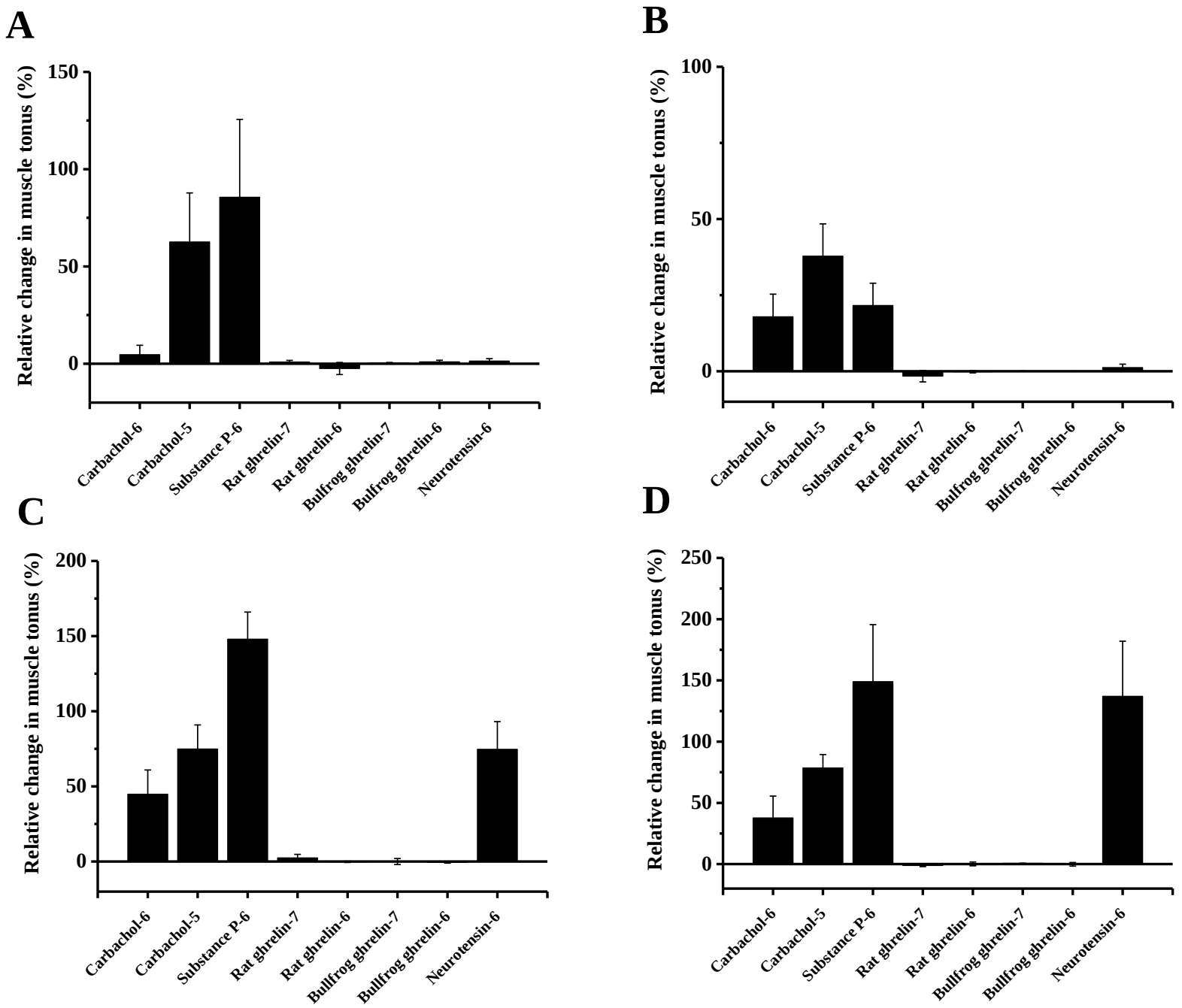
Rat ghrelin
Bullfrog ghrelin
Neurotensin
Substance P

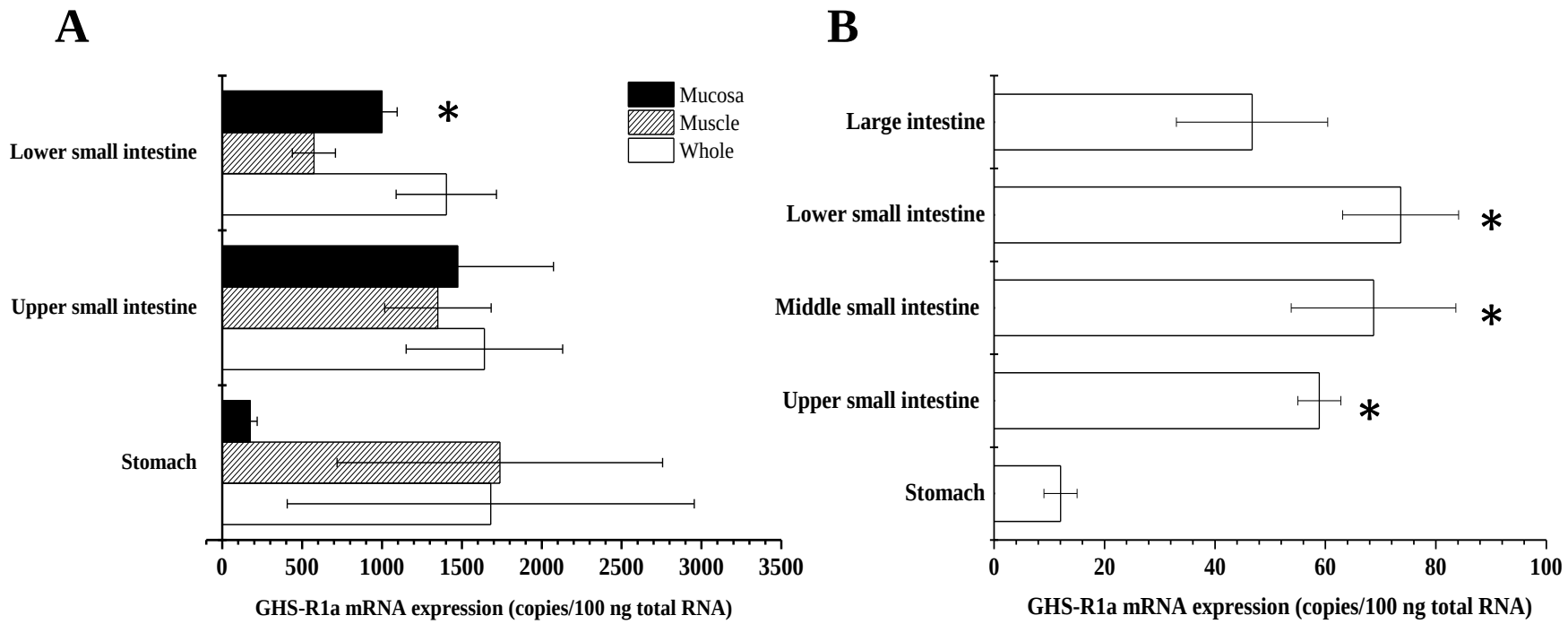
Figures

Fig.4



Figures

Fig.5



Figures

Fig.6

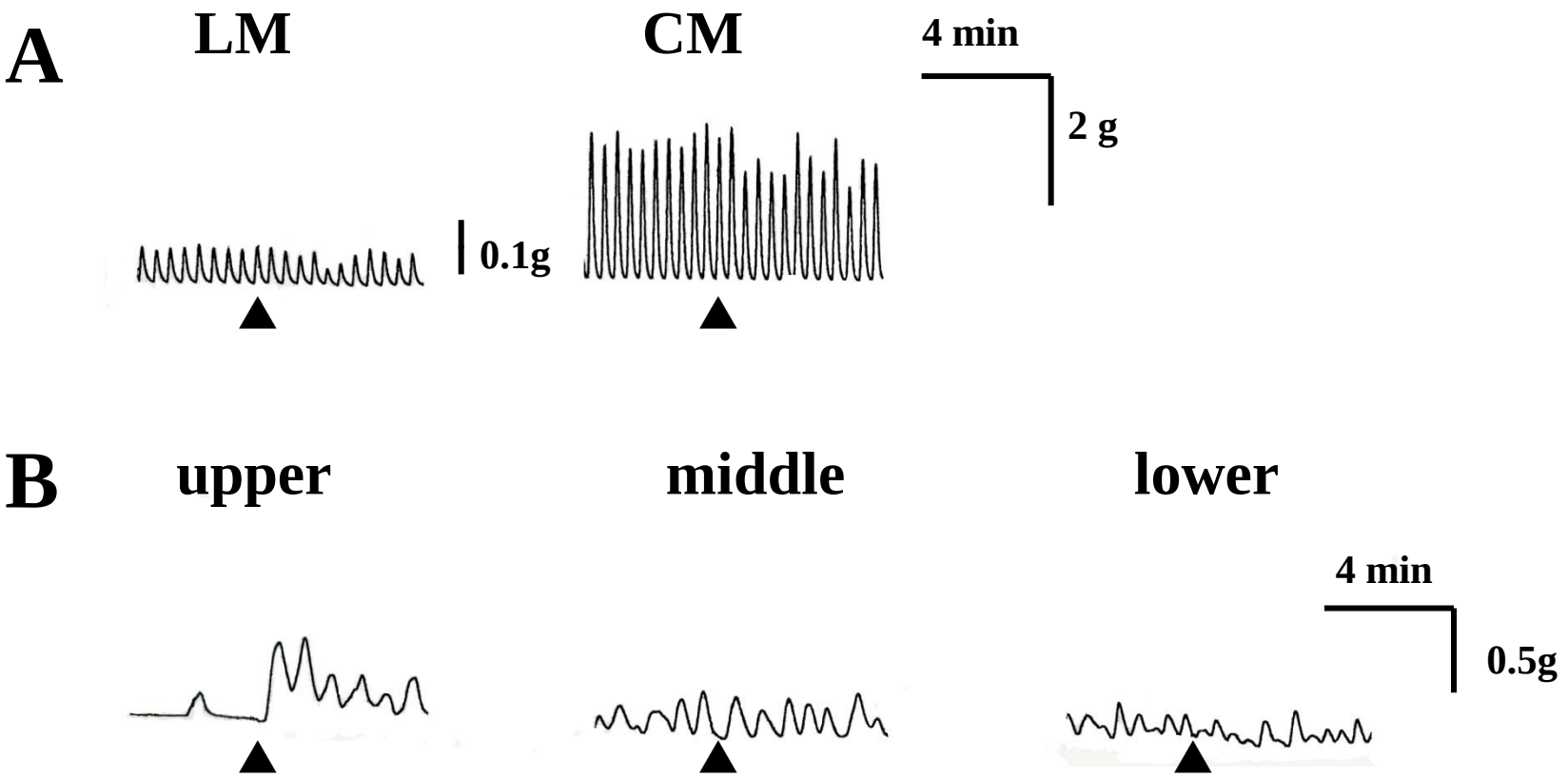
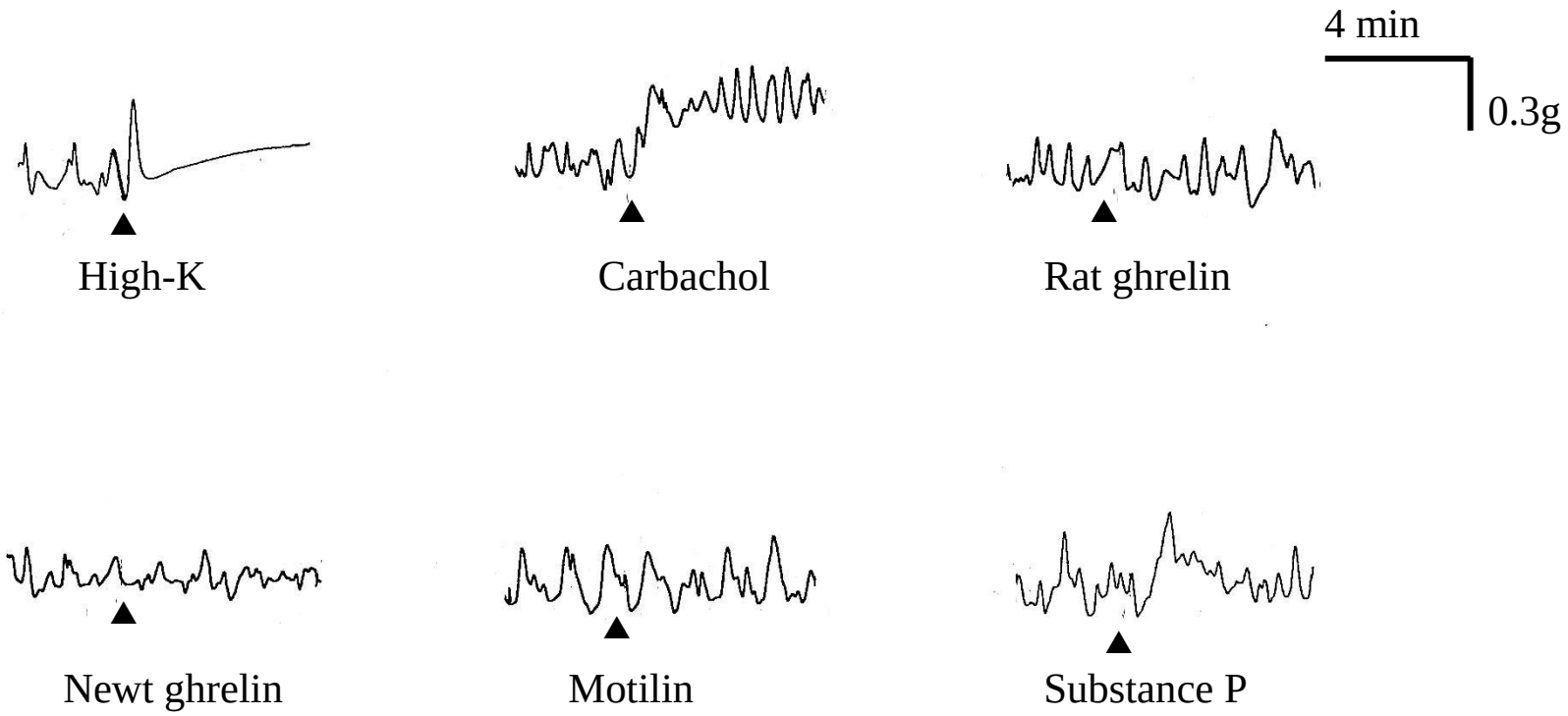


Fig.7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0.3g
High-K
Carbachol
Rat ghrelin
Newt ghrelin
Motilin
Substance P

Figures

Fig.8