Manuscript Details

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Title	A verification study of gastrointestinal motility-stimulating action of guinea-pig motilin using isolated gastrointestinal strips from rabbits and guinea-pigs
Article type	Full Length Article

Abstract

Motilin (MLN), a 22-amino-acid peptide hormone, is generally present in the mucosa of the upper gastrointestinal (GI) tract, mainly the duodenum of mammals, and it regulates GI motility, especially that related to interdigestive migrating contraction. However, MLN and its receptor are absent in mice and rats, and MLN does not cause any mechanical responses in the rat and mouse GI tracts. The guinea-pig is also a rodent, but expression of the MLN gene in the guinea-pig has been reported. In the present study, two guinea-pig MLNs, FIPIFTYSELRRTQEREQNKGL found in the Ensemble Genome Database (gpMLN-1) and FVPIFTYSELRRTQEREQNKRL reported by Xu et al. (2001) (gpMLN-2), were synthesized, and their biological activities were evaluated in the rabbit duodenum and guinea-pig GI tract in vitro. Both gpMLNs showed contractile activity in longitudinal muscle strips of the rabbit duodenum. The EC50 values of gpMLN-1 and gpMLN-2 were slightly higher than that of human MLN (hMLN), but the maximum contractions were as same as that of hMLN. Treatment with GM109 and hMLN-induced receptor desensitization decreased the contractile activity of both gpMLNs, indicating that the two gpMLN candidates are able to activate the MLN receptor (MLN-R) of the rabbit duodenum. In guinea-pig GI preparations, hMLN and gpMLNs did not show any mechanical responses in circular muscle strips from the gastric antrum or in longitudinal strips of the duodenum, ileum and colon although acetylcholine and 1,1-dimethyl-4-phenylpiperazinium (DMPP) caused definite mechanical responses. The DMPP-induced neural responses in the gastric circular muscle and ileal longitudinal muscles were not modified by gpMLN-1. Even in the gastric and ileal strips with intact mucosa, no mechanical responses were seen with either of the gpMLNs. Furthermore, RT-PCR using various primer sets failed to amplify the gpMLN-2 mRNA. In conclusion, gpMLNs including one that was already reported and the other that was newly found in a database were effective to the rabbit MLN-R, whereas they did not cause any contractions or modification of neural responses in the guinea-pig GI tract, indicating that the MLN system is vestigial and not functional in regulation of GI motility in the guinea-pig as well as in other rodents such as rats and mice.

Keywords	guinea-pig, motilin, gastrointestinal tract, contraction, motilin receptor
Taxonomy	Endocrinology, Zoology
Manuscript category	Comparative Molecular Analyses

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Data will be made available on request

7th January, 2018

Dear Editor

General and Comparative Endocrinology

Re:GCE_2018_448R1

We are re-submitting our manuscript (R2 version) to you for further consideration of its possibility to publish in *General and comparative Endocrinology,* TITLE: **A verification study of gastrointestinal motility-stimulating action of guinea-pig motilin using isolated gastrointestinal strips from rabbits and guinea-pigs.**

Thank you for your useful advices and for the reviewer comments. We have agreed and revised the manuscript according to the comments. In the revised manuscript, we checked the data again and found some small mistakes and recalculated some data. Therefore, the values were slightly different from those of R1version but these changes were very small and did not influence the summary of the manuscript. The corrections made were indicated the letters of bold red in the revised version.

Thank you again for considering this paper again for possible publication in *General and comparative Endocrinology,*.

Sincerely,

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Responses to Editor and reviewers comments

-Editor

- Minor revisions are requested by reviewer 2

Answer： Thank you for your reconsideration of the manuscript. We have agreed the all comments and revised the manuscript according to them. During the revision, we have checked the results again and found some small mistakes. We have re-calculated the data and some values were changed. But these changes were very small and do not influence the summary of the manuscript. We inserted the P values in the text if they are necessary according to the comments. The revisions are indicated in red bold letters in the revised manuscript. We are very sorry for the small change of the values in the manuscript.

-Reviewer 1

Thank you for responding so positively to the comments

Answer： Thank you very much for your useful comments on the manuscript. We appreciate your kind efforts.

-Reviewer 2

- Here are still some suggestions to improve the manuscript

Answer: Thank you very much for your useful comments on our manuscript. We responded to your comments one by one. Followings are our responses to the comments.

Line 51: whereas they did not cause any contractions **and modification of neural responses** in. should be

whereas they did not cause any contractions **or modification of neural responses** in

Answer: We have changed the paragraph according to the comment (line 51).

line 174-175: In the guinea-pig, **the responses to ACh (a muscarinic receptor agonist) and DMPP (a neural nicotinic receptor agonist) were observed to confirm**

ACh is also a nicotinic agonist otherwise the enteric cholinergic interneurons could not operate! Better would have been bethanechol a pure M agonist but at least mention the dual agonist actions of ACh on both cholinergic receptors.

Ans: We have changed the paragraph according to the comment. As the reviewer mentioned, ACh is able to act on both muscarinic and nicotinic receptor depending on the concentrations (line 174).

Line 267: **DMPP-induced contractions are the cholinergic neural origin.**

Should read **DMPP-induced contractions are neurally mediated.**

Ans: We have changed the paragraph according to the comment (line 267).

Lines 269-271: if authors give values to control DMPP responses then should also give the values after gpMLN-1 (10^{-9} - 10^{-6} M). Otherwise leave the control values out.

Ans: We have removed the control values (Control=100%) according to the comment (line 268-269).

Lines 277-280. If values to responses in control and after a drug are give and some conclusions drawn, then some statistics are necessary. Calculating shifting of curves of concentration response is the norm.

Ans: We have re-calculated the data concerning the effects of atropine and TTX on the responses to DMPP and the values were changed. We have added P values in the revised manuscript (line 277 and 278).

283-286 *ditto*

Ans: We have removed the control values (Control=100%) according to the comment (line 284).

Line 340-346: and further down 362-371

Are both very contorted and too far from each other; suggested changes starting from line 340:

The potential action of gpMLN on the enteric neurons, was tested in the present study on the responses to DMPP. DMPP caused neurally mediated contraction of ileal longitudinal muscles and of the gastric circular muscles after blocking nitrergic transmission with L-NAME. The lack of action of gpMLN-1 in these preparations indicates are consistent with those of previous studies in the guinea-pig and indicate that gpMLN does not influence the enteric neurons and is not involved in regulation of GI motility in the guinea-pig GI tract.

Ans: As the reviewer mentioned, the related sentences were contorted and too far from each other. We have agreed the comments. We have combined the related sentences and reconstructed the paragraph (line 340-349).

Highlights

- Effects of motilin on contraction of gastrointestinal (GI) tract were examined.
- Two guinea-pig motilins caused contraction of rabbit duodenum by motilin receptor.
- Two guinea-pig motilins did not cause any responses in the guinea-pig GI tract.
- RT-PCR using various primer sets failed to amplify guinea-pig motilin mRNA.
- Motilin system is not functional in regulation of GI motility in the guinea-pig

A verification study of gastrointestinal motility-stimulating action of guinea-pig motilin using isolated gastrointestinal strips from rabbits and guinea-pigs

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Abstract

Motilin (MLN), a 22-amino-acid peptide hormone, is generally present in the mucosa of the upper gastrointestinal (GI) tract, mainly the duodenum of mammals, and it regulates GI motility, especially that related to interdigestive migrating contraction. However, MLN and its receptor are absent in mice and rats, and MLN does not cause any mechanical responses in the rat and mouse GI tracts. The guinea-pig is also a rodent, but expression of the MLN gene in the guinea-pig has been reported. In the present study, two guinea-pig MLNs, FIPIFTYSELRRTQEREQNKGL found in the Ensemble Genome Database (gpMLN-1) and FVPIFTYSELRRTQEREQNKRL reported by Xu et al. (2001) (gpMLN-2), were synthesized, and their biological activities were evaluated in the rabbit duodenum and guinea-pig GI tract *in vitro*. Both gpMLNs showed contractile activity in longitudinal muscle strips of the rabbit duodenum. The EC_{50} values of gpMLN-1 and gpMLN-2 were slightly higher than that of human MLN (hMLN), but the maximum contractions were as same as that of hMLN. Treatment with GM109 and hMLN-induced receptor desensitization decreased the contractile activity of both gpMLNs, indicating that the two gpMLN candidates are able to activate the MLN receptor (MLN-R) of the rabbit duodenum. In guinea-pig GI preparations, hMLN and gpMLNs did not show any mechanical responses in circular muscle strips from the gastric antrum or in longitudinal strips of the duodenum, ileum and colon although acetylcholine and 1,1-dimethyl-4-phenylpiperazinium (DMPP) caused definite mechanical responses. The DMPP-induced neural responses in the gastric circular muscle and ileal longitudinal muscles were not modified by gpMLN-1. Even in the gastric and ileal strips with intact mucosa, no mechanical responses were seen with either of the gpMLNs. Furthermore, RT-PCR using various primer sets failed

to amplify the gpMLN-2 mRNA. In conclusion, gpMLNs including one that was already reported and the other that was newly found in a database were effective to the rabbit MLN-R, **whereas they did not cause any contractions or modification of neural responses** in the guinea-pig GI tract, indicating that the MLN system is vestigial and not functional in regulation of GI motility in the guinea-pig as well as in other rodents such as rats and mice.

Keywords: guinea-pig, motilin, gastrointestinal tract, contraction, motilin receptor

1. Introduction

Motilin (MLN), a 22-amino-acid-peptide, was first discovered from the porcine intestinal mucosa (Brown et al., 1971, 1973) and it has been shown to stimulate gastrointestinal (GI) motility in several mammals through activation of the MLN receptor (MLN-R) which was deorphanized as GPR 38 (Feighner et al., 1999). In humans, dogs and *Suncus*, MLN is considered to be an endogenous regulator of Phase-III activity of the interdigestive migrating contraction (IMC) in the stomach. The following findings support the involvement of MLN in gastric Phase-III activity of IMC: 1) peaks of endogenous MLN levels in plasma are highly associated with gastric Phase-III contractions, 2) exogenously applied MLN causes Phase-III-like gastric contraction, and 3) the Phase-III contraction is disrupted by administration of anti-MLN serum or MLN-R antagonists such as MA-2029 (Itoh et al., 1976, 1978; Peeters et al., 1980; Lee et al., 1983; Ozaki et al., 2009; Ogawa et al., 2012; Mondal et al., 2012).

The effects of MLN on GI motility have been investigated using dogs (*in vivo*) (Itoh, 1997; Ogawa et al., 2012) and rabbits (*in vivo, in vitro*) (Adachi et al., 1981; Kitazawa et al., 1994). However, since the body sizes of these animals are relatively large, they are unsuitable for animal models to investigate of MLN functions in detail. However, small experimental animals such as mice and rats lack genes for both MLN peptide and its receptor (He et al., 2010; Sanger et al., 2011), and hMLN does not affect the GI tract of either mice or rats *in vivo* and *in vitro* (Strunz et al., 1975; Depoortere et al., 2005).

Ghrelin (GHRL) is a MLN-related peptide that has some structural homology with MLN, and the growth hormone secretagogue receptor 1a (GHS-R1a, GHRL receptor) also has some structural similarity to the MLN-R (Asakawa et al., 2001; Peeters, 2005). GHRL stimulates gastric contraction in rats (Masuda et al., 2000; Fujino et al., 2003) and mice (Zheng et al., 2009), and a GHS-R1a antagonist attenuates the appearance of IMC in mice (Zheng et al., 2009), suggesting that GHRL serves as an alternative to MLN with regard to GI motility in MLN-lacking rodents. Recently, MLN and GHRL and their receptors have been identified in the house musk shrew (*Suncus murinus*), which is similar with humans and it has been shown that GHRL and MLN coordinate the IMC of the stomach (Sakahara et al., 2010; Suzuki et al., 2012; Mondal et al., 2012; Kuroda et al., 2015), proposing that *Suncus* is a suitable small laboratory animal for neurogastroenterological study of MLN and GHRL both *in vivo* and *in vitro*.

The guinea-pig (*Cavia porcellus*) belongs to rodentia as do rats and mice, and its GI tract responds to various bioactive substances and has abundant dense networks of enteric neurons. Therefore, the guinea-pig GI tract has been widely used for physiological and pharmacological studies on GI smooth muscle and enteric neurons.

Several findings indicate the possible presence of the MLN system in the guinea-pig. In a previous molecular biological study, a MLN precursor was identified in the duodenal mucosa (GenBank accession number AF323752) (Xu et al., 2001). The results of an immunohistological study using a hMLN antibody and a hMLN-R antibody suggested the presence of a MLN-like peptide and MLN-R protein in the GI tract (Xu et al., 2005). In a functional study, superfusion of hMLN depolarized some of S and AH neurons in the myenteric plexus (Katayama et al., 2005). However, hMLN did not cause any contractions of the intestine either in a non-stimulated or electrically stimulated condition *in vitro* (Strunz et al., 1975; Minocha and Galligan, 1991), which is different from the results in the human gastric strips (Broad et al., 2012). Comparative functional studies using human, canine and chicken MLNs in isolated canine or chicken GI preparations indicated an obvious species-dependent difference in the contractile efficacy of MLNs (Poitras et al., 1987; Kitazawa et al., 1997). Therefore, the actions of MLN in the guinea-pig should be examined using homologous guinea-pig MLN instead of hMLN. In a search of a genome database (Ensemble, http://asia.ensembl.org/Cavia_porcellus/Info/Index), we found a 366-bp guinea-pig MLN cDNA encoding a 121 amino acid precursor (ENSCPOT00000008024), and a mature MLN peptide was deduced to be FIPIFTYSELRRTQEREQNKGL (gpMLN-1). As a result, unique substitutions were characterized in the second amino acid and in the middle part (from positions 11 to 13) and C-terminal part (from positions 16 to 22) of gpMLN-1: seven amino acids at positions 2, 8, 11, 13, 16, 18 and 22 were different when compared with hMLN (FVPIFTYGELQRMQE KERNKGQ). Actually, the primary structure of gpMLN-1 identified by us was similar to that of gpMLN reported by Xu et al. (2000) (gpMLN-2, FVPIFTYSELRRTQEREQNKRL): which has only two

amino acid substitutions at position 2 (from I to V) and 21 (from G to R).

In the present study, the two gpMLNs, identified by us (gpMLN-1) and by Xu et al. (2001) (gpMLN-2), were synthesized, and their biological activities were evaluated both in the rabbit duodenum and the guinea-pig GI tract *in vitro*. Since it has been shown that MLN induces contractions of GI strips of mammals by actions on smooth muscles and on enteric neurons (Adachi et al., 1981; Kitazawa et al., 1994; Broad et al., 2012), the effects of MLN on smooth muscle tonus and on the neural responses were evaluated. Neural responses in the guinea-pig GI tract are evoked by 1,1-dimethyl-4-phenylpiperazinium (DMPP), an agonist for neural nicotinic receptor. Cloning of both gpMLN transcripts was also tried using various primer sets to examine the presence of gpMLN mRNA.

2. MATERIALS AND METHODS

All experiments were performed in accordance with institutional guidelines for animal care at Rakuno Gakuen University (VH25A18).

2.1. Animals and tissue preparations

Hartley guinea-pigs (*Cavia porcellus*) of both sexes (weighing 200–250 g) were obtained from Sankyo Lab Service (Sapporo, Japan). Since the rabbit duodenum is highly sensitive to MLN and has been used to investigate the mechanical responses to MLNs (Adachi et al., 1981; Kitazawa et al., 1994), Japanese white rabbits of both sexes (weighing 3 – 4 kg) were also obtained from Sankyo Lab Service. Both guinea-pigs and rabbits were in stainless steel cages at a regulated temperature (22 ± 2°C) and at 60% –

65% humidity with a normal 12 to 12-h light/dark cycle.

2.2. In vitro contraction study of gastrointestinal strips

Rabbits were deeply anesthetized by intravenous injection of pentobarbital sodium (50 mg/kg) and then killed by bleeding from the carotid vein. After a midline incision, the duodenum (next part of the gastric antrum, approx. 10 cm) was dissected out and longitudinal muscle strips were peeled out using fine forceps as previously described (Kitazawa et al., 1994). Guinea-pigs were also anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg) and killed by the same method as that for rabbits. After a midline incision, smooth muscle strips from different parts of the GI tract (stomach, duodenum, jejunum, ileum, proximal colon and distal colon) were prepared. We used two kinds of GI preparations for the guinea-pigs: muscle strips with the mucosa (whole tubular preparation of the intestine) and muscle strips without the mucosa (longitudinal muscle layers were peeled out as previously described, Kitazawa et al., 2011).

The GI strips of both rabbits and guinea-pigs were suspended vertically in an organ bath (5 ml) containing warmed (37^{o}C) Krebs solution (mM): NaCl, 118; KCl, 4.75; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25 and glucose, 11.5, bubbled with 95%O_2 + 5%CO_2 (pH 7.4). Mechanical activity of longitudinal muscles was measured with an isometric force transducer (SB-612T, Nihon Kohden), recorded on a computer, and analyzed using a computer-aided analysis system (Power Lab 2/25, Japan Bioresearch center, Nagoya, Japan). The initial load was set at 0.5 g for each preparation. The preparations were rinsed with Krebs solution every 15 min and allowed to equilibrate for 1 h. Prior to MLN treatment, each muscle strip was subjected

169 to 3 or 4 continuous stimulations with 10^{-4} M acetylcholine (ACh) (15-min intervals)
170 until a reproducible contraction was obtained.

171 For examining the response to MLN in the rabbit duodenum, MLN was applied
172 cumulatively at concentrations from 10^{-10} M to 10^{-6} M. Since MLN caused distinct
173 contractions, MLN was applied immediately after observing the peak amplitude of each
174 concentration. In the guinea-pig, the responses to ACh (a muscarinic **and nicotinic**
175 receptors agonist) and DMPP (a neural nicotinic receptor agonist) were observed to
176 confirm the responsiveness of smooth muscles and enteric neurons in the GI
177 preparations. MLN was applied cumulatively at 2-3-min intervals, and the mechanical
178 response was observed. Changes in smooth muscle tonus caused by MLN peptides were
179 normalized using 10^{-4} M ACh-induced contraction and indicated as relative change (%
180 response) in smooth muscle tonus. The effects of pretreatment with gpMLN-1(3min) on
181 the neural responses to DMPP were also examined in the gastric circular muscle and
182 ileal longitudinal muscle.
183

184 ***2.3. PCR cloning of guinea-pig MLN***

185 Based on the guinea-pig MLN sequence reported by Xu et al. (2001), we designed
186 several primer sets for PCR cloning of gpMLN-2 (Table 1). Total RNA was extracted
187 from the guinea-pig small intestine using ISOGEN (Nippon Gene) or an RNeasy mini
188 kit (QIAGEN). After DNase I (Promega) treatment of total RNA to remove genome
189 DNA contamination, 1 μg total RNA was reverse-transcribed to cDNA by using
190 Superscript III reverse transcriptase (Invitrogen) at several temperatures (42℃ to
191 50℃)with a random or oligo-dT primer or gene-specific primers. RT-PCR was
192 performed by using Ex taq polymerase (Takara) or AmpliTaq Gold (Thermo Fisher

193 Scientific) with annealing temperature of 50-70℃ in each cDNA and primer sets. In
194 addition, one-step RT-PCR kits (QIAGEN) were also used with each of the primer sets
195 following the manufacture's instructions.
196
197 ***2.4. Chemicals***
198 Two guinea-pig MLNs were custom-synthesized by Scrum Co. Ltd. (gpMLN-1,
199 Tokyo, Japan) and Peptide Institute Inc. (gpMLN-2, Osaka, Japan), and their purity was
200 commercially confirmed by a single peak of high-performance liquid chromatography.
201 Human MLN (hMLN) was purchased from Peptide Institute Inc. Acetylcholine chloride
202 and tetrodotoxin (TTX) were obtained from Wako Co. Ltd. (Tokyo, Japan). Atropine
203 sulfate, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) and L-nitroarginine
204 methylester hydrochloride (L-NAME) were obtained from Sigma-Aldrich (MO, USA).
205 GM109, a MLN-R antagonist was kindly donated by Chugai Co. Ltd. (Tokyo, Japan).
206 All chemicals were dissolved in distilled water and the desired concentration was
207 directly applied to an organ bath using a micropipette. The applied volume was less than
208 0.5% of the bath volume (5 mL).
209
210 ***2.5. Statistical analysis***
211 Data are expressed as means ± S.E.M of more than four experiments. The
212 significance of differences between the values was determined at $P < 0.05$ using
213 Student's t-test (paired and unpaired) for single comparisons or ANOVA followed by
214 Dunnett's test or Bonferroni test for multiple comparison of the mechanical responses.
215
216 ***3. Results***

3.1. Effects of hMLN and gpMLNs in the rabbit duodenum

In the isolated longitudinal muscle strips from the rabbit duodenum, hMLN caused concentration-dependent contraction at concentrations from 10^{-10} M to 10^{-6} M. EC_{50} and relative maximum contraction (% to 10^{-4} M ACh-induced contraction) were 4.2± 0.7x10^{-9} M and 89.3± 3.0% (n=15), respectively (Fig. 1, left panel and Fig. 2). Synthetized gpMLN-1 caused contraction of the rabbit duodenum (Fig. 1, middle panel). The contraction-response curve was located slightly right to that of hMLN, but EC_{50} (**5.3**± 1.0x10^{-9} M, n=**8**) and maximum amplitude of contraction (**81.1±4.3**%, n=**8**) were comparable to those of hMLN (Fig. 2). Only the contraction at 3x10^{-8} M (**71.6± 4.6%,** n=**8**) was significantly smaller (***P*=0.048**) than that of hMLN (83.1±3.0%, n=15). Synthetized gpMLN-2 also showed contractile activities in the isolated rabbit duodenum in a concentration-dependent manner (Fig. 1, right panel and Fig. 2). The EC_{50} value of gpMLN-2 was **10.7± 1.8 x10^{-9} M** (n=**9**), which was significantly higher than those of hMLN (***P*=0.0005**) and gpMLN-1 (***P*=0.012**).

To confirm the involvement of the MLN-R in gpMLN-induced contractions, the effect of a MLN-R antagonist (GM106, Takanashi et al., 1995) was examined. GM109 alone at a dose of 10^{-6} M did not affect the concentration-response curve of ACh as previously reported (Kitazawa et al., 2017), but it inhibited contractile responses to hMLN, gpMLN-1 and gpMLN-2, and the concentration-response curves shifted rightward by the same degrees (Fig. 2).

MLN-R desensitization by exposure for a long time to a high concentration of hMLN has been used to examine the possible involvement of the MLN-R in this response (Kitazawa et al., 1994). Duodenum longitudinal strips were treated with 10^{-6} M hMLN for 20 min and were then washed for 20 – 30 min. During washing, tonus of the

241 preparations decreased but did not completely recover to the level before application of
242 hMLN. In this condition, 10^{-4} M ACh-induced contraction was slightly reduced (**74.6±**
243 **4.8%** of the control, n=7). The contractions induced by hMLN, gpMLN-1 and gpMLN-
244 2 were markedly decreased by the desensitization treatment (Fig. 3). The relative
245 changes in smooth muscle tonus caused by 10^{-9} M, $3x10^{-9}$ M and 10^{-8} M MLN were 0.6
246 ±**0.2**%, **5.0**±**1.2**% and **11.2**±1.6% (n=7), respectively, for hMLN, and 0±1.**4**%, 3.0
247 ±1.**4**% and 10.**9**±1.**6**% (n=4), respectively, for gpMLN-1 and 3.2±**1.0**, 6.8±**1.9**%
248 and 14.3±**3.6**% (n=6), respectively, for gpMLN-2. The degree of inhibition of MLN-
249 induced contraction by desensitization treatment was markedly high compared with that
250 of ACh-induced contraction (**25**%).
251
252 ***3.2. Effects of gpMLN in the guinea-pig GI tract***
253 Gastric circular muscle strips were prepared from the distal part (antral region) of
254 the guinea-pig stomach. The circular muscles showed small spontaneous contractions
255 (Figs. 4a and 5a), and ACh (10^{-4} M) caused a marked contraction (data not shown).
256 Neither gpMLN-1 (Fig. 4a) nor gpMLN-2 (Fig. 5a) applied cumulatively caused
257 mechanical changes in muscle tonus of gastric strips without the mucosa. In the gastric
258 circular muscle with intact mucosa, neither of the gpMLNs caused contraction of the
259 muscle strips (n=2 or 3) (data not shown). The effects of hMLN were also examined in
260 the same gastric strips without mucosa and with mucosa, and hMLN (10^{-6} M) did not
261 cause mechanical responses regardless of the presence of intact mucosa (without
262 mucosa: 3.4±**1.0**%, n=3, with mucosa: 0.84%, n=2). In this experimental condition,
263 DMPP (10^{-5} M) caused a relaxation of gastric circular muscles without mucosa, which
264 was changed into contraction by L-NAME (10^{-4} M). This DMPP-induced contraction

265 was decreased by atropine (10^{-6} M, relative contraction **4.2±3.5** %, n=4, ***P*=0.046**) and
266 was abolished by TTX (10^{-6} M, **1.6±1.6 %**, n=4, ***P*=0.041**), indicating that the DMPP-
267 induced contractions are **neurally mediated.** The DMPP (10^{-5} M)-induced contraction
268 was not affected by treatment of gpMLN-1 (10^{-9} - 10^{-6} M). Relative contractions of
269 DMPP were 96±9% for 10^{-9} M, 89±14% for 10^{-8} M, 98±15% for 10^{-7} M and 105±
270 28% for 10^{-6} M (n=4), respectively.

271 Typical effects of gpMLNs on contractility of the duodenum longitudinal muscle
272 preparations are shown in Figs. 4b (gpMLN-1) and 5b (gpMLN-2). Neither gpMLN-1
273 nor gpMLN-2 caused any mechanical responses in the duodenal preparations as in the
274 case of the gastric antrum. However, in one of six duodenal strips, a small contraction
275 (over 10% of ACh-induced contraction) caused by gpMLN-2 was observed (Fig. 6a).

276 DMPP (10^{-5} M) caused a contraction of the ileal longitudinal muscle and the
277 responses was significantly decreased by atropine (10^{-6} M, **39.6±15**%, n=5, ***P*=0.02**)
278 and abolished by TTX (10^{-6} M, 9±**2.6**%, n=5, ***P*=0.023**), indicating the activation of
279 enteric cholinergic neurons by DMPP (Fig. 7). As in the case of the duodenal
280 preparations, although one of five preparations showed a small response to gpMLN-1
281 causing over 10% of the ACh-induced contraction (Fig. 6b), neither gpMLN-1 nor
282 gpMLN-2 caused mechanical responses in the ileal strips (Figs. 4c and 5c). Treatment
283 of gpMLN-1 did not change the DMPP (10^{-5} M)-induced neural contractions. Relative
284 contractions of DMPP were 100±3% for 10^{-9} M, 101±4% for 10^{-8} M, 95±**9**% for 10^{-7}
285 M and 104±**2**% for 10^{-6} M (n=5), respectively (Fig. 7). In some experiments (n=3), to
286 investigate the responses to gpMLN-2 in the absence of inhibitory nitrergic innervation,
287 the effect of gpMLN-2 in ileal strips treated with L-NAME (10^{-4} M) was examined.
288 gpMLN-2 also caused no contraction of the muscle strips (Fig. 8). Ileal preparations

289 with intact mucosa (n=4) also did not respond to gpMLN-2 (data not shown), and the
290 relative changes in muscle tonus were -0.4±0.4% for 10^{-10} M, -0.3±0.3% for 10^{-9} M, 1.5
291 ±1.2% for 10^{-8} M, 0.5±0.5% for 10^{-7} M and 0.8±0.8% for 10^{-6} M (n=4).
292 Proximal colon longitudinal muscle strips without mucosa were used to examine the
293 responses to gpMLN-1 (Fig. 4d) and gpMLN-2 (Fig. 5d). As in the case of gastric and
294 small intestinal strips, neither gpMLN-1 nor gpMLN-2 caused contraction of colonic
295 strips.
296
297 *3.3.* ***RT-PCR cloning of gpMLN***
298 In the experiments, we used several primer sets (Table 1) to clone the gpMLN-2
299 gene reported by Xu et al. (2001). However, despite various PCR trials, no specific
300 target products were obtained in any of the RT-PCR conditions (data not shown).
301
302 **4. Discussion**
303 The guinea-pig belongs to rodentia as do rats and mice, and its GI tract has been
304 widely used for physiological and pharmacological studies on GI smooth muscles and
305 enteric neurons. It has been reported that rats and mice lack the genes for the MLN
306 system (MLN and MLN-R) (He et al., 2010; Sanger et al., 2011). In previous studies,
307 Xu et al. (2001) determined the cDNA sequence of the MLN precursor from the
308 duodenal mucosa of the guinea-pig (GenBank accession No. AF323752). Furthermore,
309 Katayama et al. (2005) reported functional activity of hMLN in the guinea-pig.
310 However, the effect of gpMLN determined by Xu et al. (2001) has never been examined
311 in the guinea-pig GI tract itself. Previous comparative studies indicated species-
312 dependent efficacy of homologous MLN peptide for eliciting GI smooth muscle

313 contractions (Kitazawa et al., 1997; Poitras et al., 1987). Moreover, we recently found
314 the possible presence of another MLN molecule (ENSCPOT00000008024), which
315 differs from that reported by Xu et al (2001), by searching the Ensemble Genome
316 Database. Therefore, in the present study, we examined the biological activity of these
317 two gpMLNs (gpMLN-1 and gpMLN-2) by investigating GI contractility in the rabbit
318 duodenum, which is known to be contracted by MLN (positive control), and in guinea-
319 pig GI strips, in which the action is unknown.

320 In the rabbit duodenum, both gpMLN-1 and gpMLN-2 caused concentration-
321 dependent contractions. The maximum amplitudes of contractions were comparable to
322 that of hMLN, but EC_{50} of gpMLNs was slightly higher than that of hMLN. The MLN-
323 R antagonist GM109 and MLN-R desensitization significantly decreased the responses
324 to gpMLNs. These results clearly indicate that both gpMLN candidates function
325 through the MLN-R that exists in the rabbit duodenum. The N-terminal region of
326 hMLN, which corresponds to 1–6 amino acids (FVPIFT), has been thought to be
327 essential for eliciting the biological activity of MLN (Peeters et al., 1992; Poitras et al.,
328 1992). In this regard, gpMLN-2 (FVPIFT) is identical to it, and gpMLN-1 (FIPIFT) has
329 only one substitution at position 2 from V to I, suggesting that this substitution of
330 gpMLN-1 did not significantly affect the contractile efficacy. These two gpMLNs are
331 able to bind to the rabbit MLN-R as a full agonist, but different sequences in the middle
332 and C-terminal amino acids might be related to the slight change in affinity (EC_{50}

values).

hMLN caused the region-dependent facilitation of electrically evoked cholinergic contraction and the increase in the tonus of human GI strips through activation of muscle and neural MLN-Rs (Broad et al., 2012), but hMLN was ineffective to cause contraction (Strunz et al., 1975) and to modify the neural responses of guinea-pig GI strips (Minocha and Galligan, 1991). The results of the present study **using gpMLNs** correspond to those of the previous studies in the guinea-pig. **The potential action of gpMLN on the enteric neurons, was tested in the present study examining the responses to DMPP. DMPP caused neurally mediated contraction of ileal longitudinal muscles and of the gastric circular muscles after blocking nitrergic transmission with L-NAME. The lack of action of gpMLN-1 in these preparations is consistent with those of contraction studies in the guinea-pig (Strunz et al., 1975; Minocha and Galligan, 1991) but is different from the actions of MLN in the human stomach (Broad et al., 2012) and chicken stomach (Kitazawa et al., 1995). The present results indicate that gpMLN does not influence the enteric neurons and smooth muscles, and is not involved in regulation of GI motility in the guinea-pig GI tract.** We also examined another GI preparation with intact mucosa because GHRL, a MLN-related peptide, has been reported to act on intrinsic primary afferent neurons in the gastric mucosa of the *Suncus* (Mondal et al., 2013). However, neither of the gpMLNs caused contraction in the gastric circular muscle preparations with mucosa or whole intestinal preparations in this study. Furthermore, chicken MLN-induced contraction was potentiated by L-NAME in chicken gastric preparations, indicating that MLN acts on nitrergic inhibitory nerves in addition to cholinergic excitatory nerves in

356 the chicken stomach (Kitazawa et al., 2002). Xu et al. (2005) detected MLN-R
357 immunoreactivity in neural nitric oxide synthase-positive neurons of the guinea-pig
358 intestine. Therefore, it might be possible that simultaneous stimulation of inhibitory
359 nitrergic neurons could attenuate the contractile responses to MLN in the guinea-pig GI
360 tract. However, no contractile response to gpMLN was seen in L-NAME-treated gastric
361 and intestinal muscle preparations.

362 In the present study, however, some preparations from the small intestine showed a
363 weak contractile response to gpMLNs (Fig. 6). We have observed similar small
364 contractions in the guinea-pig ileum by GHRL (Kitazawa et al., 2011), but we could not
365 conclude whether it is a specific action or an artifact. The mechanisms of contraction
366 caused by gpMLNs were not examined in the present study because of the small
367 amplitude and infrequent appearance of contractile responses.

368 The effect of MLN is elicited through ligand binding to the specific receptor, MLN-
369 R. Is MLN merely ineffective for GI motility? In this regard, it is critical whether a
370 functional MLN-R exists or not. As mentioned before, Xu et al. (2005) detected MLN-
371 R immunoreactivity in the guinea-pig intestine. To explore the possible presence of the
372 MLN system in the guinea-pig, we searched for the MLN-R cDNA sequence in the
373 Ensemble Genome Database. No sequence was hit by a simple search, but we found a
374 candidate cDNA sequence of which the amino acid sequence shares 42.5% homology
375 with human MLN-R when human MLN-R was used as a query for the TBLASTN
376 search. This homology was lower than that of chickens (59.1%, Yamamoto et al., 2008)
377 and was close to that of zebrafish (47%, Liu et al., 2013), and there is a great difference
378 compared with mammalian sequences (rabbit: 84%, *Suncus*: 76%, dog: 71%, Dass et

al., 2003; Ohshiro et al., 2008; Suzuki et al., 2012). We tried to clone the MLN-R candidate that is consistent with the second exon of the MLN-R gene. However, we failed to amplify the correspondent part of exon-1 by 5'RACE PCR. This result suggests a MLN-R gene is not present in the guinea-pig as mentioned by He et al. (2010) and Sanger et al. (2011).

Xu et al. (2001) cloned a cDNA encoding the MLN precursor and deduced a mature 22-amino-acid MLN peptide as FVPIF TYSEL RRTQE REQNK RL (namely gpMLN-2 in this study). However, in the Ensemble Genome Database, we could not find this gpMLN-2 sequence. Instead, we found another cDNA candidate encoding a deduced mature MLN peptide as FIPIF TYSEL RRTQE REQNK GL (gpMLN-1), in which amino acids at positions 2 and 21 differed from those of gpMLN-2. We tried to amplify these two gpMLNs, especially for gpMLN-2 (Xu et al., 2001), using various RT-PCR primer sets (Table 1). However, no specific target product was obtained in any of the PCR conditions, suggesting that gpMLN-2 mRNA reported by Xu et al. (2001) is not expressed in the guinea-pig. In addition, gpMLN-1 could also not be amplified using the duodenal first-strand cDNA as a template by various PCR conditions. We do not know the reason why the target product found in the genomic sequence of the guinea-pig is not amplified. gpMLN-1 has been annotated by *in silico* estimation. Our results indicate the possibility that actual transcription of gpMLN-1 does not proceeds *in vivo*. The results suggest that although MLN gene might be present, a functional MLN peptide is not transcribed and translated in the guinea-pig. Expression of the MLN and MLN-R genes in several rodentia including, kangaroo rat, guinea-pig, mouse and rat have been compared (He et al., 2010). Mouse and rat are thought to be animal species lacking both MLN and MLN-R genes by pseudogenization, but kangaroo rat has an intact open

403 reading frame for the MLN gene but lacks the MLN-R gene. In the case of guinea-pig,
404 the MLN gene is present but the MLN-R gene is degenerated, similar with other rodent
405 species (squirrel, kangaroo rat, mouse and rat) (He et al., 2010). Therefore, guinea-pig is
406 a species in a state of evolution away from a functionally-viable MLN system like a
407 kangaroo rat.

408 In conclusion, two gpMLN candidates caused contractions in the rabbit
409 duodenum but did not show any mechanical response and modification of neural
410 responses in the guinea-pig GI tract, probably due to the lack of a functional MLN-R.
411 The results indicate that the guinea-pig is an animal in the transition period for
412 evolution of the MLN system prior to the loss of the MLN-R gene in rats and mice.

413

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418

419

420 **Conflict of interest**

421 The authors declare no conflict of interest.

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427 **References**

428

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571 **Figure legends**

572 **Fig. 1. Representative contractile responses to human MLN (hMLN), guinea-pig**
573 **MLN-1 (gpMLN-1) and guinea-pig MLN-2 (gpMLN-2) in the rabbit duodenal**
574 **longitudinal muscles.** hMLN, gpMLN-1 and gpMLN-2 were applied cumulatively (10^{-10} M - 10^{-6} M) and evoked contractions were observed. The amplitude of MLN-induced
576 contraction was normalized by acetylcholine (ACh, **10^{-4} M**, ●)-induced contraction.
577 The number under each triangle indicates the concentration of MLNs (log M).
578
579 **Fig. 2. Concentration-contraction curves for hMLN and gpMLNs and effects of**
580 **GM109 on MLN-induced contractions in isolated rabbit duodenal strips.** The
581 symbols indicate the concentration-response curves for hMLN (■), gpMLN-1 (●) and
582 gpMLN-2 (▲) in the normal condition and in the presence of GM109 (10^{-6} M). (hMLN:
583 □, gpMLN-1:○, gpMLN-2:△). The amplitude of MLN-induced contractions (y-axis)
584 was normalized by a standard contraction by ACh (**10^{-4} M**) in the absence of GM109.
585 GM109 did not change the responses to ACh (**10^{-4} M**). The x-axis is the concentration
586 of MLN (log M). Values are means ± S.E.M. (4 experiments or more).
587
588 **Fig. 3. Effects of desensitizing treatment of the MLN receptor on the**
589 **concentration-response curves of hMLN and gpMLNs in isolated rabbit duodenal**
590 **strips.**
591 The symbols indicate the concentration-response curves for hMLN (■), gpMLN-1(●)
592 and gpMLN-2 (▲) in the normal condition and in the condition of hMLN-induced
593 desensitization (see text, hMLN:□, gpMLN-1:○, gpMLN-2:△). The desensitization
594 treatment by hMLN (10^{-6} M for 20 min) decreased the responses to both gpMLNs. The

595 amplitude of MLN-induced contractions (y-axis) was normalized by a standard
596 contraction by ACh (10^{-4} M). The-axis is the concentration of MLN (log M). Values are
597 means ± S.E.M. (4 experiments or more).
598
599 **Fig. 4. Representative responses to gpMLN-1 in various regions of isolated**
600 **gastrointestinal strips from the guinea-pig.** gpMLN-1 applied cumulatively (10^{-10} M-
601 10^{-6} M) did not cause any mechanical changes in gastric circular muscle strips (a),
602 duodenal longitudinal muscle strips (b), ileal longitudinal muscle strips (c) and proximal
603 colon longitudinal strips (d). The number under each triangle indicates the concentration
604 of gpMLN-1 (log M).
605
606 **Fig. 5. Representative responses to gpMLN-2 in various regions of isolated**
607 **gastrointestinal strips from the guinea-pig.** gpMLN-2 applied cumulatively (10^{-10} M -
608 10^{-6} M) did not cause any mechanical changes in gastric circular muscle strips (a),
609 duodenal longitudinal muscle strips (b), ileal longitudinal muscle strips (c) and proximal
610 colon longitudinal strips (d). The number under each triangle indicates the concentration
611 of gpMLN-2 (log M).
612
613 **Fig. 6. Small contraction induced by gpMLN-1 and gpMLN-2 in longitudinal**
614 **muscle of the guinea-pig duodenum and ileum.** Representative contraction caused by
615 gpMLN-2 in the duodenum (a) and caused by gpMLN-1 in the ileum (b). The number
616 under each triangle indicates the concentration of gpMLNs (log M).
617

Fig. 7. Effects of gpMLN-1 on the DMPP-induced contraction of guinea-pig ileal longitudinal muscle. (a) DMPP (10^{-5} M) caused a contraction and atropine (Atr, 10^{-6} M) and tetrodotoxin (TTX, 10^{-6} M) decreased the DMPP-induced responses. (b) The effects of treatment with gpMLN-1 (10^{-9} M– 10^{-6} M for 3 min) on the DMPP-induced (10^{-5} M) contraction of the guinea-pig ileum. Each concentration of gpMLN-1 did not cause any mechanical changes. The number indicates the concentration of each drug (log M).

Fig. 8. Effects of L-NAME treatment on responses to gpMLN-2 in longitudinal muscle of the guinea-pig ileum. Each trace indicates typical mechanical responses to gpMLN-2 (▲) in the absence (upper) and presence of L-nitroarginine methylester (L-NAME, 10^{-4} M for 15 min). ACh (**10^{-4} M**, ●) caused obvious contraction in each condition.

Fig.1

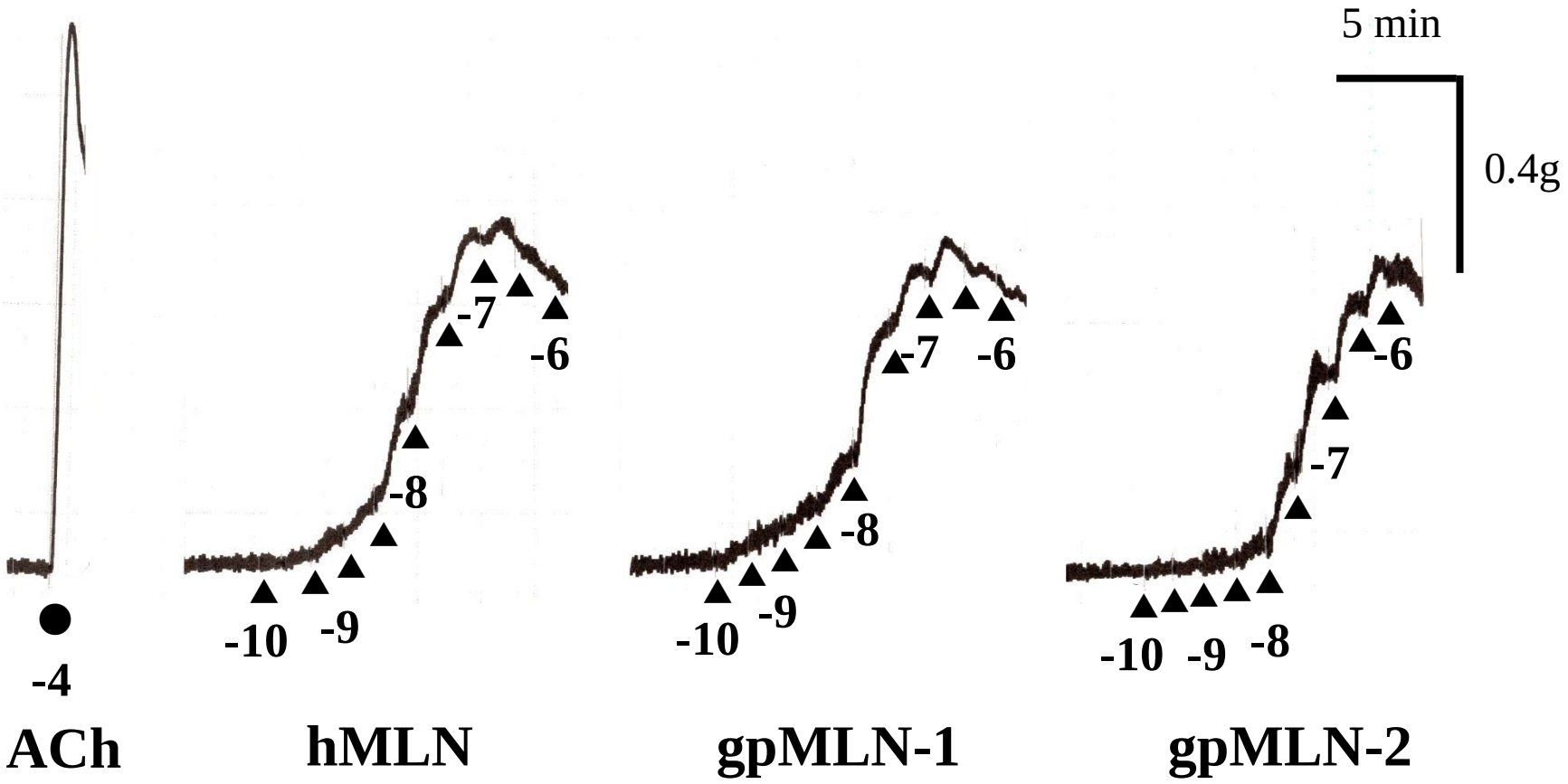

Fig.2

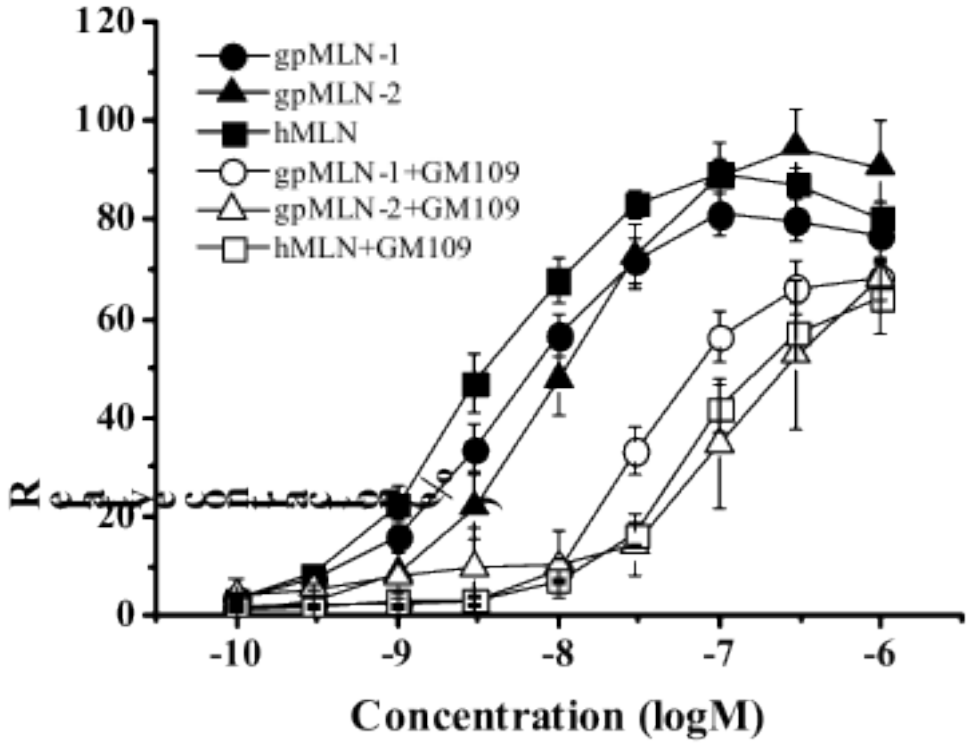
120
100
80
60
40
20
0
-10
-9
-8
-7
-6
Concentration (logM)
gpMLN-1
gpMLN-2
hMLN
gpMLN-1+GM109
gpMLN-2+GM109
hMLN+GM109

Fig.3

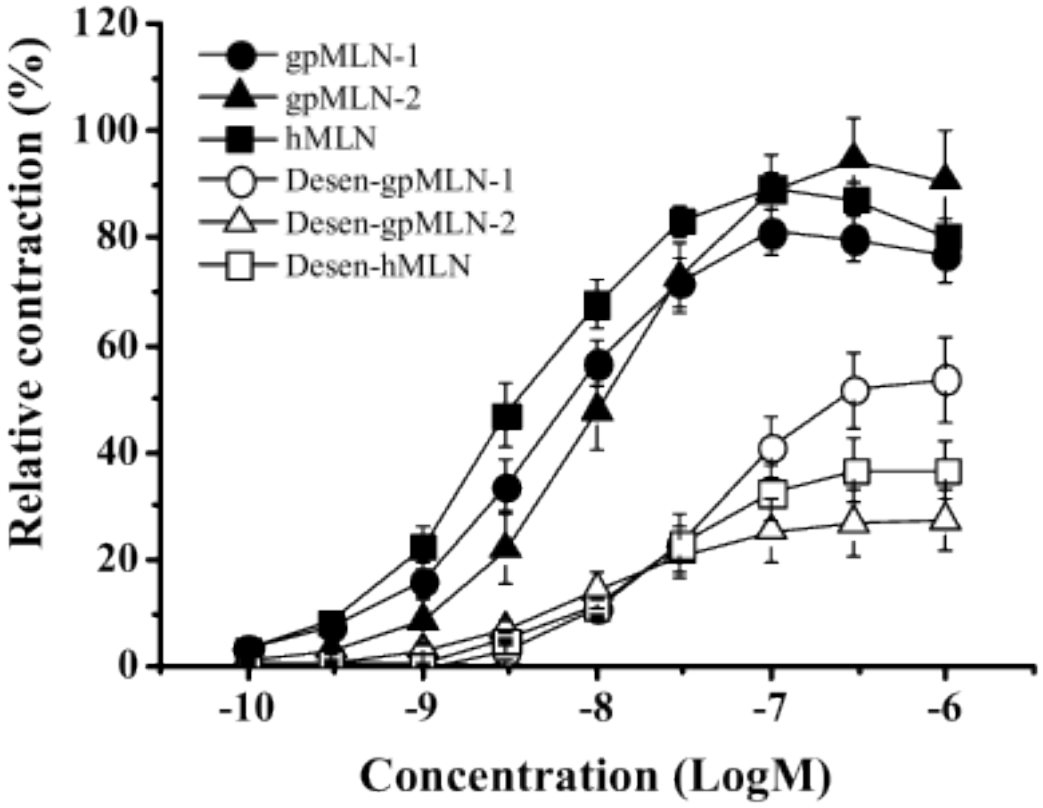
Relative contraction (%)
120
100
80
60
40
20
0
gpMLN-1
gpMLN-2
hMLN
Desen-gpMLN-1
Desen-gpMLN-2
Desen-hMLN
-10
-9
-8
-7
-6
Concentration (LogM)

Fig.4

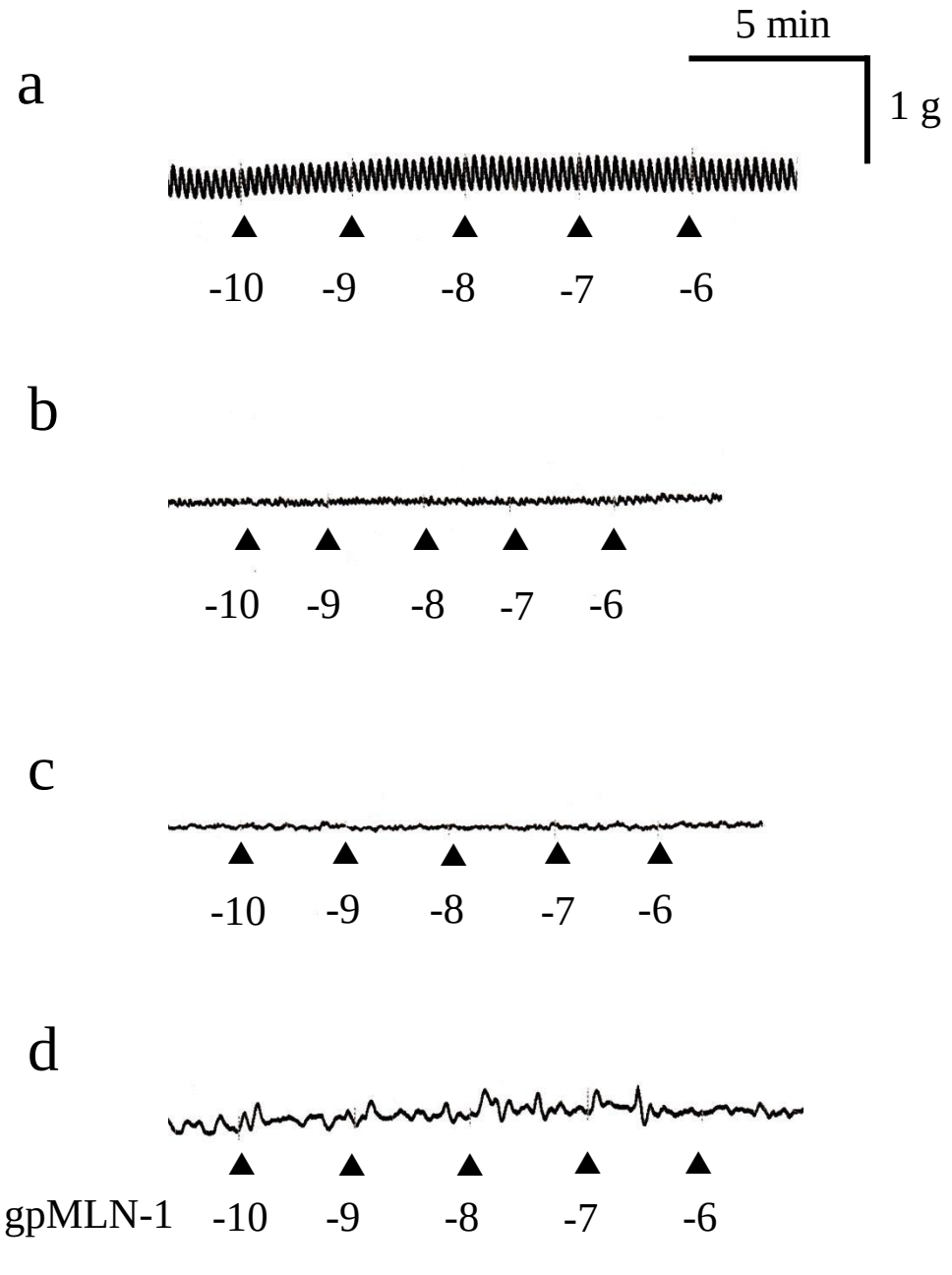

Fig.5

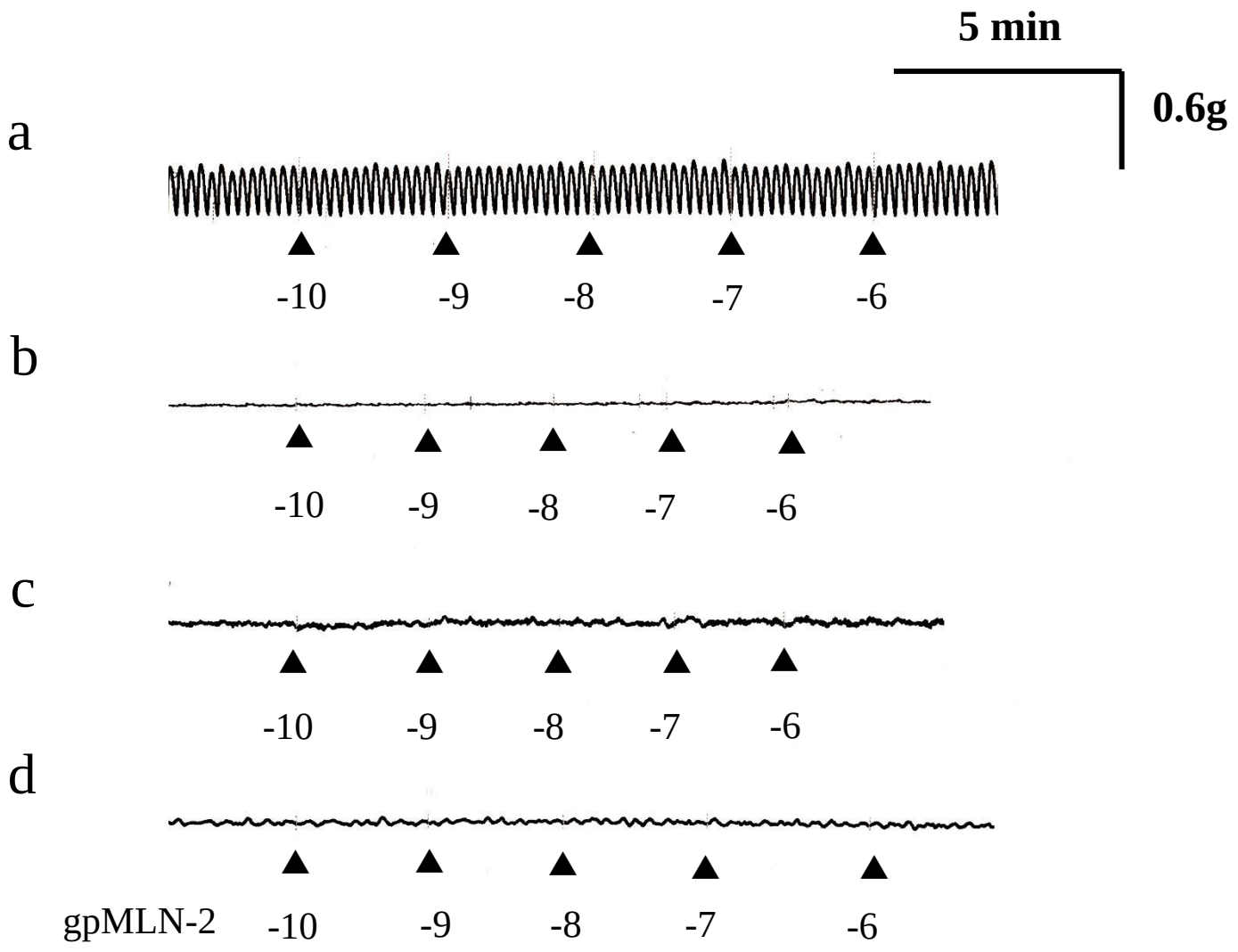

Fig.6

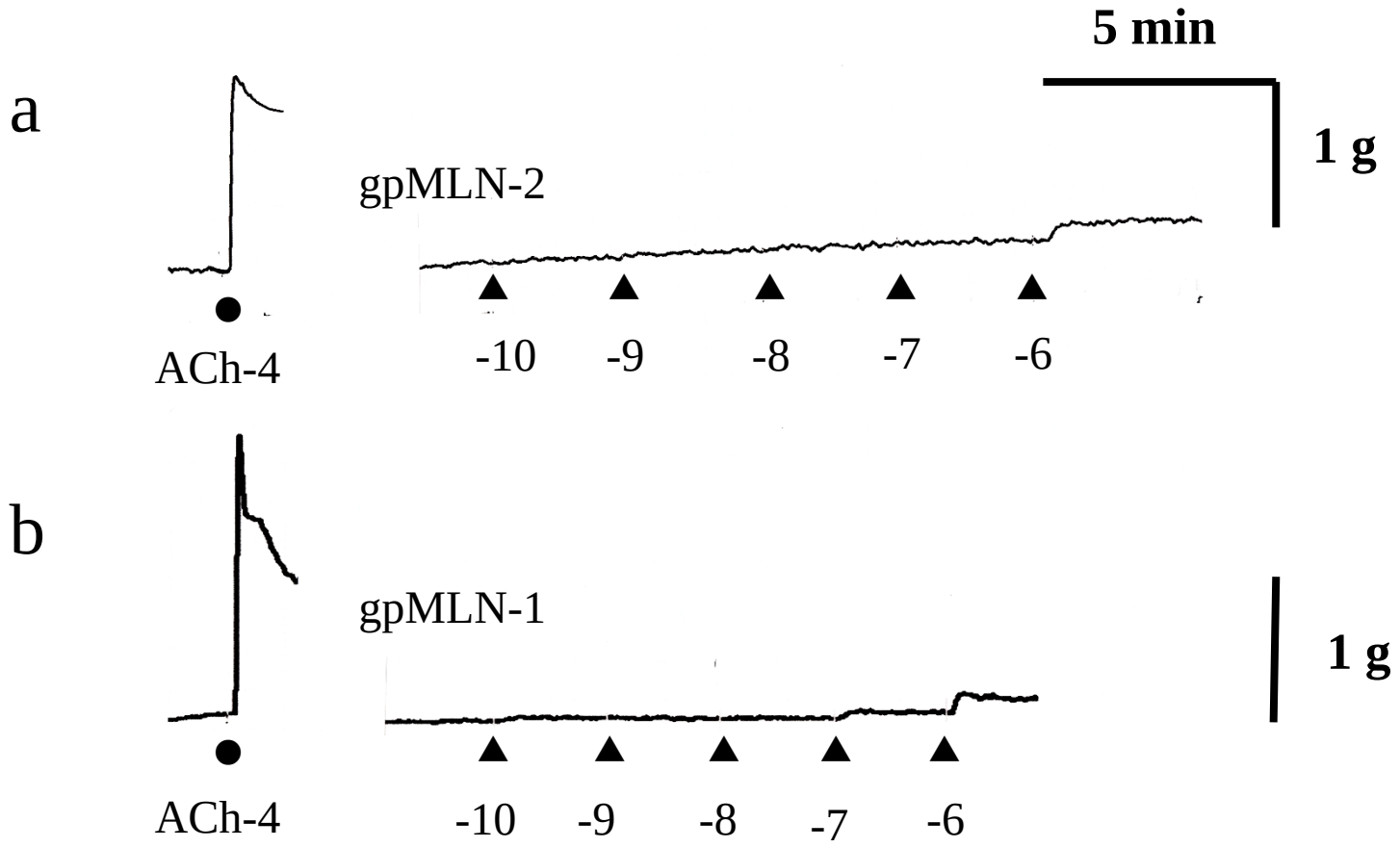

Fig.7

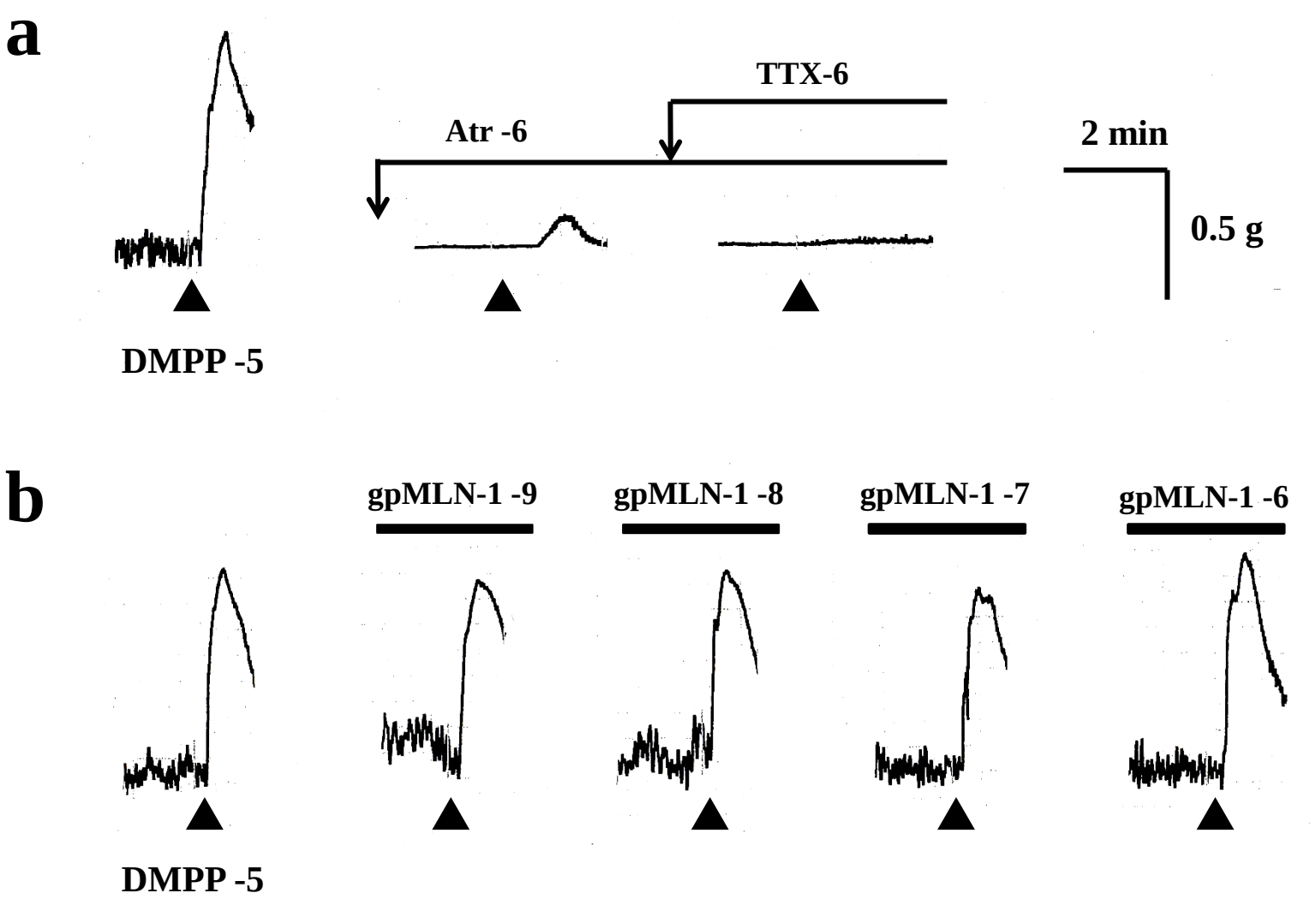
a
TTX-6
Atr -6
2 min
0.5 g
DMPP -5
b
gpMLN-1 -9
gpMLN-1 -8
gpMLN-1 -7
gpMLN-1 -6
DMPP -5

Fig.8

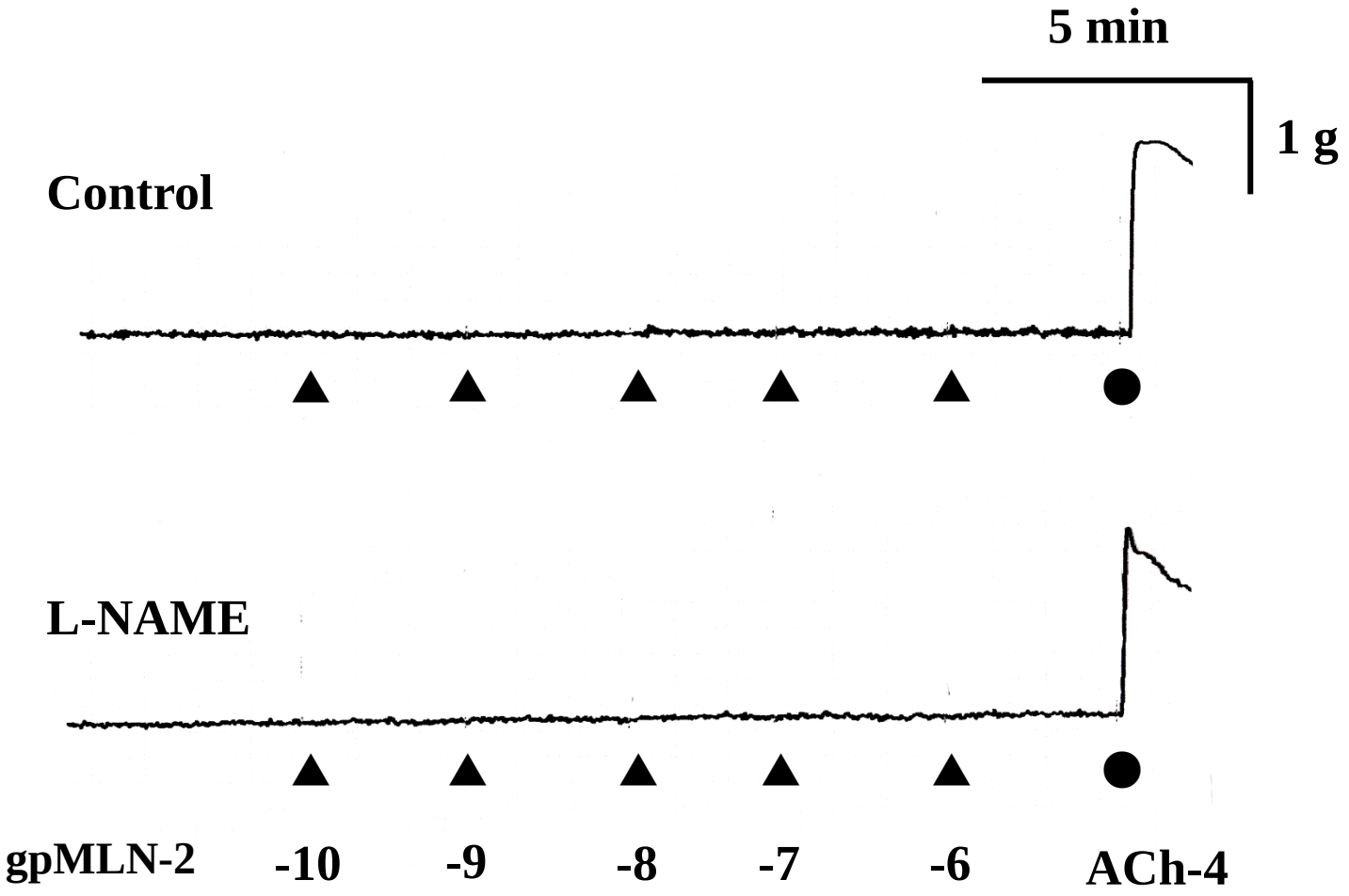

Table 1

Six primer sets for RT-PCR detection of gpMLN-2 in the present experiments

Primer sets for RT-PCR		Direction	Sequence
Set A	guinea pig motilin	FWD	AGAATGCTGTCCCGAAAGG
	guinea pig motilin	BWD	GAGGAGTCTGCCTTGGAGAG
Set B	guinea pig motilin	FWD	GCGTACATCCAGAATGCTGTC
	guinea pig motilin	BWD	CCAATTTCCACTGGAGCAG
Set C	guinea pig motilin	FWD	AGAATGCTGTCCCGAAAGG
	guinea pig motilin	BWD	GAGGAGTCTGCCTTGGAGAG
Set D	guinea pig motilin	FWD	AGAATGCTGTCCCGAAAGG
	guinea pig motilin	BWD	CCAATTTCCACTGGAGCAG
Set E	guinea pig motilin	FWD	TTCCAATCTTCACTTACAGCGAG
	guinea pig motilin	BWD	CCAATTTCCACTGGAGCAG
Set F	guinea pig motilin	FWD	GTCCCTGAGGGTACAGCAGA
	guinea pig motilin	BWD	CCTCACTGAGCAGAGCTTCC