Title: Role of neurotensin in the regulation of gastric motility in healthy conscious sheep

Short running title: Neurotensin inhibits ovine ruminal contractions

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Abrrebiations: neurotensin, NT; neurotensin receptor, NTR; gastrointestinal, GI, bethanechol, BCh; electromyographic, EMG; electric field stimulation, EFS

Abstract

The goal of present study was to determine the effects of the intravenous (i.v.) administration of neurotensin (NT) on the ovine forestomach and abomasal motility in conscious sheep. NT injection at 0.3 nmol/kg slightly raised abomasal pressure, although the effect was not dose-dependent. A bolus i.v. injection of NT at 1 or 3 nmol/kg significantly inhibited the amplitude of cyclic ruminal contractions. NT injection did not alter omasal motility. Pre-injection of an NT receptor subtype-1 antagonist, SR 48692, at 60 nmol/kg immediately before NT injection did not block the inhibitory effect of NT. In an *in vitro* study using smooth muscle strips of the rumen dorsal sac, NT application at 0.3-10 μmol/L did not inhibit the bethanechol (BCh, 10 μmol/L)-induced tonic contractions of either the longitudinal and circular muscle strips, nor did NT inhibit the electrical field stimulation (EFS)-induced phasic contractions of the muscle strips. The results suggest that circulating NT selectively inhibits the amplitude of cyclic rumen contractions presumably by inhibiting the gastric center in the medulla oblongata and/or the vagus nerves, but not through its peripheral action. An elevation in the plasma concentration of NT appears able to exert the ileal brake on ruminal motility in sheep.

Keywords: neurotensin, forestomach, rumen, contraction, inhibition, ileal-brake, sheep

1. Introduction

Neurotensin (NT) is a tetradecapeptide (Carraway and Leeman, 1975), and 85 % of NT in the body in rats is distributed in the gastrointestinal (GI) tract (Carraway and Leeman, 1976). NT-positive endocrine cells are primarily distributed in intestinal crypts in the caudal small intestine (Helmstaedter et al., 1977; Kitamura et al., 1985; Reinecke, 1985), whereas NT-containing neurons are also localized in the submucosal and myenteric nerve plexuses throughout the GI tract in humans, rats, guinea pigs, cats, and rabbits (Carraway and Leeman, 1976; Holzer et al., 1982). NT exerts a variety of biological effects in the GI tract (Ferris, 1989) including the inhibition of gastric acid, pepsinogen secretion and gastric blood flow (Blackburn et al., 1980; Fletcher et al., 1985), the stimulation of pancreatic exocrine secretion (Konturek et al., 1983), and the modulation of pacemaker activity in the interstitial cells of Cajal (Lee et al., 2012).

With regard to GI motility, the administration and application of NT exerts both excitatory and inhibitory effects on the same tissues. In the stomach, NT application causes smooth muscle contraction of the rat gastric fundus (Huidobro-Toro and Kullak, 1985), whereas i.v. infusion of NT inhibits gastric motility in rats and humans (Blackburn et al., 1980; Hellstrom et al., 1982), indicating the indirect predominant inhibitory action of NT. Due to this inhibitory action on the upper GI tract, NT has been considered as a possible mediator of the ileal brake induced by the caudal intestinal hormones involving peptide YY and glucagon-like peptide-I (Onaga et al., 2002; Barreto and Windsor, 2017). In contrast, NT application leads to contractions of ileal muscle strips, in part through the neural release of acetylcholine and substance P in guinea pigs (Kitabgi and Freychet, 1978), indicating the peripheral indirect excitatory

64 action of NT. In terms of colonic motility, application of NT induced muscle strip contractions in the rat
65 and human colon (Mule et al., 1995; Croci et al., 1999), whereas NT application directly induced muscle
66 relaxation in the guinea-pig colon (Kitabgi and Vincent, 1981), indicating region- and species-specific
67 differences in the action of the peptide.

68 Most studies on the physiological roles of NT in the regulation of GI motility have been
69 performed using monogastric species. However, unlike monogastric species, ruminant species have a
70 characteristic forestomach. The rumen, which acts as a huge fermentation chamber, plays a crucial role in
71 the microbial digestion of feed, particularly dietary fibers. Some immunohistochemical studies reported
72 that NT is abundantly distributed in the ileum in cattle (Kitamura et al., 1985) and that NT-containing
73 neurons and fibers are localized in the myenteric region of the forestomach and abomasum in Karakul lambs
74 (Groenewald, 1994). However, the effects of NT on forestomach and abomasal motility have not been
75 examined in sheep or other ruminant species *in vivo*, and it remains unknown whether NT can serve as a
76 mediator of the ileal brake from the hindgut to the stomach in ruminant species. We previously
77 demonstrated that peptide YY did not exert any inhibitory effect on gastric motility in sheep, implying that
78 peptide YY does not play a role as a mediator of the ileal brake in sheep (Onaga et al., 1997a; Onaga et al.,
79 2000). We, then, hypothesized that NT might play a role as a mediator of the ileal brake on gastric motility
80 in sheep. Therefore, the present study was designed to determine the role of NT in the regulation of gastric
81 motility in sheep. We examined the effects of i.v. injection of human NT, which is identical to ovine NT, on
82 forestomach and abomasal motility in conscious healthy adult sheep. As NT exerted an inhibitory effect on

83 only ruminal contractions, we also examined the peripheral effects of NT on BCh- and EFS-induced
84 contractions of smooth muscle strips of the rumen in an *in vitro* experiment.
85
86 **2. Materials and methods**
87 *2.1 Drugs*
88 The nucleotide sequence coding ovine NT was translated into the amino acid sequence
89 QLYENKPRRPYIL (GenBank accession No. XM_004006237). The deduced sequence completely
90 coincided with the amino acid sequence of human NT (BC010918). Therefore, we used commercial
91 human NT for all the experiments. Human NT was purchased from the Peptide Institute (Product No.
92 4029, molecular weight 1672.9, Osaka, Japan). NT was dissolved at 100 μmol/L in a sterilized
93 physiological saline solution (NaCl 150 mmol/L), and frozen at -35° C until use. Bethanechol
94 hydrochloride (BCh) was purchased from Sigma Chemicals (St. Louis, MO, USA). NT receptor
95 subtype-1 (NTR-1) antagonist, SR 48692, was purchased from Santa-Cruz Biotechnology Inc. (sc-
96 363290, Santa Cruz, CA, U.S.A.) (Gully et al., 1993). SR 48692 was dissolved at 28.4 mmol/L in
97 dimethyl sulfoxiside as a stock solution, and diluted immediately before administration with a
98 commercial electrolyte solution. The final concentration of dimethyl sulfoxiside in the infusion was
99 approximately 3.5%. Pentobarbital sodium (Somuno pentil injection®) and atropine sulfate were
100 purchased from Schering-Plough Animal Health Corp. (NJ, USA) and Wako Pure Chemical Industries
101 (Osaka, Japan), respectively. Halothane and buprenorphine hydrochloride were purchased from

Takeda Pharmaceutical (Osaka, Japan) and Ohtsuka Pharmaceutical (Tokyo, Japan), respectively.

2.2 Animals

Twelve male Suffolk sheep weighing 37.5-57.0 kg (mean ± SD: 43.3 ± 5.9 kg) were used for the experiment. The experiment was performed under the Laboratory Animal Control Guidelines of our institution, which conform to the Guide for the Care and Use of Laboratory Animals of the NIH in the USA. The experimental protocol used in the present study was approved by the Ethics Committee for Animal Experiments in the School of Veterinary Medicine, Rakuno Gakuen University (H17C17, VH25C11, VH14C6). Sheep were kept in individual cages in an experiment room and trained to maintain a standing position for 2 hours before the onset of experiment. The animals were fed lucerne hay (400 g) and lucerne pellets (1.6 % of body weight) once a day at 18:00. Water was freely available except during the experiment.

Seven sheep were used for the first experiment of the series. Before surgery, animals were fasted for one day. After i.v. administration of atropine sulfate (0.2 mg/kg) and pentobarbital sodium (12 mg/kg), animals were anesthetized by inspiration of halothane-oxygen gas through an intratracheal cannula. Animals were laid down on their right side and were fitted with a plastic ruminal cannula (I. D. 20 mm, O. D. 25 mm, length 70 mm, Fujiya Rika Instruments, Sapporo, Japan) in the left flank. The cannula was fixed at the gastric wall and skin by a double purse-string ligature. During laparotomy on the right flank, bipolar silver electrodes (0.5 mm in diameter, fixed 10 mm apart in a sheet of nylon

mesh and covered with epoxy and silastic resin) were sutured onto the center of the greater curvature of the omasum. The electrodes were handmade as described previously (Onaga et al., 1997b). Wires from the electrodes were exteriorized from the right flank and fixed by a purse-string suture on the skin. Flexible polyethylene cannulae (Multipurpose tube, 7 Fr., 2.35 mm O.D., 750 mm length, Atom Medical Inc.,, Tokyo, Japan) with a silicon rubber guard attached 30 mm from the tip were inserted into the lumen of the omasal canal, abomasal corpus, and abomasal antrum. The cannulae were fixed on the gastric wall by double purse-string ligatures. After surgery, buprenorphine hydrochloride (5 μg/kg) was injected intramuscularly (i.m.), and administration of an antibiotic (benzylpenicillin, 1,500 U/kg, i.m., Meiji-Seika, Tokyo, Japan) was continued for 3 days. Animals were allowed a recovery period of 1 week before the start of experiments.

Five sheep were used in the second experiment of the series, which focused on ruminal motility. Sheep were equipped with only a ruminal cannula in the left flank using a similar surgical procedure to that described above for the first experiment.

2.3 The effects of NT on in vivo *motility in the rumen, omasum, and abomasum in conscious sheep*

Before the initiation of the experiments, animals were trained to maintain a standing position for at least 3 hours. The experiments were carried out between 9:00 and 15:00. An indwelling catheter was inserted into the jugular vein before the experiment and filled with sterilized sodium chloride solution (150 mmol/L) containing heparin (10 U/ml). Frozen stock solutions of NT were melted at

140 room temperature and then diluted with a commercial isotonic electrolyte solution, Solulact (mmol/L:
141 Na^+ 131.0, K^+ 4.0, Ca^{2+} 3.0, Cl^- 110.0, $Lactate^-$ 28.0; Terumo, Japan). Solulact was used as the vehicle
142 solution for the drugs.

143 Animals were kept in a standing position in the cages during the experiment. In the first
144 experiment of the series, after a control period of 40 minutes, NT was injected (i.v.) at bolus doses of
145 0.3, 1.0, and 3.0 nmol/kg at 0 minutes. Recording was continued until 50 minutes after NT injection.
146 In the second experiment of the series, after a control period of 20 minutes, NT was injected (i.v.) at
147 a bolus dose of 1.0 nmol/kg at 0 minutes with and without an i.v. pre-injection of SR 48692 at 60
148 nmol/kg at -5 minutes. In addition, SR 48692 was solely injected at -5 minutes without NT injection
149 as a control.

150 Changes in intraluminal pressure in the rumen dorsal sac, omasal canal, abomasal corpus, and
151 abomasal antrum were measured using the intraluminal cannulae, which were connected to pressure
152 transducers (Becton Dickinson, Franklin Lakes, NJ, USA) (Onaga et al., 1998). The ruminal cannula
153 had a balloon (thin gum balloon, 6 ml volume) on the tip to avoid clogging of the tip. The balloon was
154 placed 2-3 cm forward of the inner opening of the ruminal cannula. The cannulae in the omasum and
155 abomasum were infused with physiological saline at 15 μl/kg/min using a four-channel peristaltic
156 pump (SJ-1220, Atto, Tokyo, Japan) to avoid clogging of the tip. With regard to ruminal basal pressure
157 and contractions, tonic and phasic changes in intraluminal pressure were recorded through a transducer
158 amplifier (NEC San-ei, Tokyo, Japan) using a digital data acquisition systems consisting of MacLab

(AD Instruments, Castle Hill, Australia) and a Macintosh computer (Apple, Cupertino, CA, U.S.A.), and PowerLab (AD Instruments) and an Windows computer (Hitachi, Tokyo, Japan). In the omasum, motility of the omasal greater curvature was measured by electromyographic (EMG) techniques, as it is very difficult to measure the luminal pressure of the omasum on account of the multiple layers of omasal leaves and fibrous digesta in their tight interspaces. Omasal EMG activities were recorded through the bipolar silver electrodes and bioelectric amplifiers (time constant 0.1 msec, high cut frequency 30 Hz, NEC San-ei) using the same digital data recording system (Onaga et al., 1997a).

2.4 The in vitro *effects of NT application on muscle strips of the rumen dorsal sac*

Eight sheep were used for the *in vitro* experiment which was performed after the *in vivo* experiments. After euthanasia under pentobarbital anesthesia (25 mg/kg i.v.), the cranial left wall of the rumen dorsal sac was excised and washed with ice-cooled Krebs-Henseleit solution (mmol/L); Na^+ 137.80, K^+ 5.90, Ca^{2+} 1.25, Mg^{2+} 1.20, Cl^- 122.20, $H_2PO_4^-$ 1.20, HCO_3^- 22.00, SO_4^{2-} 1.2, Glucose 5.5, acetic acid 0.8 (pH 7.40 under 95 % O_2 + 5 % CO_2). The mucosa was immediately removed from the specimens, and the muscle layer was kept in ice-cooled Krebs-Henseleit solution.

Longitudinal and circular muscle strips involving the myenteric nerve plexus (length 10 mm, width 1.0-1.5 mm) were excised from the ruminal specimen and incubated in Krebs-Henseleit solution in warmed organ baths (9 mm I. D. x 24 mm depth, volume 2 ml) maintained at 37°C. In the first experiment, an initial tension of 1.0 g was loaded onto the muscle strips. Isometric tension was

recorded using force transducers, transducer amplifiers (type 45196A and polygraph system 366, NEC San-ei) and the PowerLab system. After an equilibration period of 30 minutes, bethanechol hydrochloride (BCh, 10 μmol/L) was applied to the muscle strips for 6 minutes (Onaga et al., 2009). After washing with fresh Krebs-Henseleit solution and an interval of 30 minutes, BCh (10 μmol/L) was again applied. Two minutes later, NT (0.3-10 μmol/L) was simultaneously applied in an accumulative manner at 1-minute intervals. The viability and recovery of muscle strips was confirmed by a third application of BCh alone.

In the second experiment, isotonic tension was recorded under a tension of 1.0 g using displacement transducers, transducer amplifiers (type 45347 and polygraph system 366, NEC San-ei) and the PowerLab system as it was difficult for the isotonic transducer to record steady contractions by repeated electric field stimulation (EFS) over a long period. After preincubation for 30 minutes, muscle contractions were induced by EFS (duration 0.5 msec, frequency 20-40 Hz, voltage 80-100 V, stimulator type 2907, polygraph system 366, NEC San-ei) for 10 seconds at 2-minute intervals. After recording four EFS-induced contractions as a control, NT (0.1-10 μmol/L) was cumulatively applied to the muscle strips at 8-minute intervals and three EFS-induced contractions were recorded for each concentration of NT. As the amplitude of the EFS-induced contractions tended to slightly lower without NT application, the contractions were compared with and without NT application. After washing, the inhibitory effect of lidocaine at 1 mmol/L on the EFS-induced contractions was confirmed.

197

198 *2.5 Statistical analysis*

199 Experimental data were analyzed for every 5-minute period and shown as the mean ± SEM. In
200 the rumen, contractions were analyzed for three parameters; i.e., mean intraluminal pressure involving
201 cyclic phasic rumen contractions, and the frequency and mean amplitude of primary contractions. We
202 did not analyze secondary contractions of the rumen as they occurred irregularly. Omasal EMG
203 activity was analyzed for frequency (spikes/5 minute). EMG recordings involving a lot of continuous
204 or repeated electric noises higher than 20 Hz. (n = 2) were excluded from the analysis. Mean
205 intraluminal pressure in the omasum, abomasal corpus and antrum was calculated for every 5-minute
206 period. Statistical significance of the temporal changes was determined by one-way repeated
207 measurements analysis of variance (ANOVA) and Tukey's multi-comparison test using Prism
208 commercial software (GraphPad, San Diego, CA, USA). The value at every time point was compared
209 to the value immediately before the injection (Fig. 2, Fig. 3, and Fig. 5). In addition, integrated change
210 was calculated for the increment of 15 minutes between before and after the injection (Fig. 2, Fig. 4,
211 and Fig. 5) and compared by one-way factorial ANOVA using Prism. Differences were considered to
212 be statistically significant at a *p*-value of less than 0.05.

213 In the *in vitro* study, the amplitude of the contractile responses to BCh and EFS varied in muscle
214 strips. Therefore, we calculated the contractile ratio of the BCh-induced tonic contraction after NT
215 application against the tension immediately before application of NT in the first application of BCh.

Likewise, we calculated the contractile ratio of the EFS-induced phasic contractions after NT application against the tension of the first EFS-induced contraction without NT application. In both cases, changes in the contractile ratio were compared by one-way factrional ANOVA using Prism and differences were considered to be statistically significant at a *p*-value of less than 0.05.

3. Results

3.1 Effects of the intravenous injection of NT on forestomach and abomasum motility in sheep

Rumen: The rumen exhibited regular phasic contractions (Fig. 1). During the control period, the mean intraluminal pressure was 51.7 ± 2.4 mmHg and the rumen showed cyclic contractions at a stable frequency and amplitude of 4.3 ± 0.2 times/5 min and 8.5 ± 0.9 mmHg, respectively (Fig. 2, left). Bolus i.v. injections of NT at all doses did not alter the mean intraluminal basal pressure of the rumen (Fig. 2, left), whereas NT injection at 1 and 3 nmol/kg inhibited cyclic ruminal contractions, with NT significantly decreasing the amplitude of ruminal contractions in particular (Fig. 2, $p < 0.001$). The frequency of ruminal contractions showed a tendency to increase shortly after the NT injection at 0.3 nmol/kg, whereas it significantly decreased after the NT injection at 3 nmol/kg ($p < 0.05$). As regards the total response for 15 minutes, the increment in mean intraluminal pressure of the rumen did not change, whereas the frequency and amplitude of cyclic rumen contractions were significantly inhibited by NT injections (Fig. 2, right).

Omasum: Intraluminal pressure in the omasal canal was stable during the control period (Fig. 1) with

a mean value of 7.75 ± 1.73 mmHg (Fig. 3). EMG activity of the omasal greater curvature was also stable before and after the control saline injection (Fig. 1) with a mean value of 227.3 ± 76.1 spikes/5 min (Fig 3). Although the values in the control period tended to increase at the beginning of the NT experiments, they became stable before the NT injections. Intravenous injection of NT did not alter the increment of the mean intraluminal pressure in the omasal canal or the spike frequency in the EMG activity in the omasal greater curvature at any dose (Fig. 4). The spike frequency of the omasal EMG significantly declined at the end of the experiment after NT injection at 1 and 3 nmol/kg.

Abomasum: Intraluminal pressure in the abomasal corpus and antrum was stable during the control period (Fig. 1) with a mean value of 26.8 ± 3.9 and 6.7 ± 0.9 mmHg, respectively (Fig. 3). Intravenous infusion of NT at 0.3 nmol/kg raised the intraluminal pressure in the antrum, though the increase did not proceed in a dose-dependent manner (Fig. 3). NT injection at 1 and 3 nmol/kg did not significantly alter the increment in intraluminal pressure for the 15-minute period in either segment (Fig. 4), although the intraluminal pressure slightly but significantly rose at around 30 minutes in both the corpus and antrum (Fig. 3).

3.2 The influence of pre-administration of SR 48692 on the inhibitory effect of NT on ruminal contractions.

The bolus i.v. injection of saline did not significantly change either the amplitude or frequency of the ruminal contractions, while NT injection at 1 nmol/kg significantly inhibited the amplitude of

254 the ruminal contractions for 15 minutes (Fig. 5). The bolus i.v. administration of SR 48692 at 60
255 nmol/kg at -5 minutes did not change either the amplitude or frequency of the ruminal contractions.
256 Although the amplitude of the contractions varied slightly before NT injection, NT injection similarly
257 and significantly decreased the amplitude of the contractions after pre-administration with the
258 antagonist. The increment in the amplitude over the 15-minute period significantly declined for NT
259 injection after pre-administration of the antagonist in comparison with the value for pre-treatment with
260 the antagonist alone. The inhibitory effect of NT on the increment of the amplitude did not significantly
261 differ from the inhibitory effect of NT without the antagonist (Fig. 5).
262
263 *3.3 The* in vitro *effects of NT application on smooth muscle tension in the ruminal dorsal sac.*
264 BCh application induced tonic and long-lasting contractions in the longitudinal and circular
265 muscle strips (Fig. 6). Repeated application of BCh induced stable tonic contractions of a similar
266 amplitude. NT application to the muscle strips did not significantly alter the amplitude of the second
267 BCh-induced contractions of either type of muscle strip at any of the NT concentrations tested (Fig. 6).
268 EFS induced stable mono-phasic contractions without relaxation of the longitudinal or circular
269 muscle strips of the ruminal dorsal sac (Fig. 7). Repeated application of EFS tended to gradually and
270 slightly decrease the amplitude of the phasic contractions of the muscle strips to 62.2±8.5 % in the
271 longitudinal muscle and 71.2±13.4 % in the circular muscle without NT application, and 68.2±13.7 %
272 and 72.4±7.5 % with NT application, respectively. However, these changes were not statistically

273 significant. A comparison of the time-course of EFS-induced contractions with and without NT
274 application did not show any significant alteration in the amplitude of the EFS-induced contractions in
275 either the longitudinal or circular muscle strips due to NT application (Fig. 7). Application of SR 48692
276 slightly recovered the mean contractile ratio in the circular muscle from a mean value of 72.4 to 87.4 %;
277 however, the change was not significant. EFS-induced contractions in both types of muscle strip were
278 almost abolished by the application of lidocaine at 1 mmol/L ($p < 0.001$, Fig. 7), indicating that the
279 contractile responses to EFS were mediated by intrinsic neurons.
280

281 **4. Discussion**

282 The present study demonstrated for the first time that exogenous human NT exerted a selective
283 and significant inhibitory effect on the amplitude of cyclic ruminal contractions in conscious sheep as
284 measured by change in the intraluminal pressure of the rumen, but not on motility in the omasum or
285 abomasum. NT was shown to inhibit pacemaker current in the interstitial cells of Cajal in mice mediated
286 through NTR-1 (Lee et al., 2012). However, as cyclic ruminal contractions are regulated by vagal efferent
287 input from the gastric center (Ruckebusch, 1989), the inhibitory effect of NT on the rumen does not account
288 for the inhibition on the pacemaker cells in the rumen. The inhibitory effect was not blocked by the pre-
289 administration of SR 48692, implying that the effect is not mediated through NTR-1. On the other hand,
290 NT application to isolated ruminal muscle strips did not significantly inhibit BCh-induced tonic or EFS-
291 induced phasic contractions of either the longitudinal and circular muscles, suggesting that the inhibitory

292 effect of NT on the ruminal contractions is neither a direct action on the smooth muscles nor an action
293 mediated by peripheral intrinsic motor neurons. Moreover, as application of SR 48692 did not alter the
294 EFS-induced contractions of the smooth muscles, NTR-1 does not seem to be involved the regulation of
295 contractile responses in the gastric wall in either an excitatory or inhibitory manner. The lack of local effect
296 of NT on both types of muscle strips in the rumen implies that the inhibitory effect of NT on cyclic rumen
297 contractions is mediated through the central nervous system (CNS) or the extrinsic efferent nervous system
298 associated with the rumen; i.e., the vagal motor nerve.

299 With regard to the mechanism underlying the inhibitory action, NT-immunopositive neurons are
300 located not only in the peripheral nervous system but also in the brain (Uhl et al., 1979; Reinecke, 1985;
301 Papadopoulos et al., 1986), and the central administration of NT has been shown to produce several actions
302 (Hernandez et al., 1982; Nemeroff et al., 1982a; Nemeroff et al., 1982b; Tyler-McMahon et al., 2000).
303 Although the effect of the peptide was contrary to our results, the local application of NT was shown to
304 increase the firing rate of neurons in a sliced preparation of the nucleus of the solitary tract in rats (Ogawa
305 et al., 2005). In addition, inconsistencies in the other effects of NT between central and peripheral
306 administration have been reported in rats and dogs (Sumners et al., 1982; Bueno et al., 1985; Zhang et al.,
307 1989). A comparison of difference in the actions suggests that permeability of the blood-brain barrier is
308 probably a key to solving the discrepancies in the NT actions, as it has been suggested that NT as a natural
309 form of tridecapeptide does not cross the blood-brain barrier (Vincent, 1995). In sheep, however, the gastric
310 center was reported to be outside the blood-brain barrier (Ruckebusch, 1989) and is considered to be

311 responsible to circulating peptides such as cholecystokinin. Further, it has been hypothesized that both the
312 amplitude and frequency of the reticulo-ruminal contractions are regulated in the gastric center in the
313 medulla oblongata (Ruckebusch, 1989). In the present study, NT injection preferentially inhibited the
314 amplitude of the cyclic ruminal contractions in sheep. Accordingly, NT seems likely to primarily act on the
315 amplitude circuit of the gastric center or the efferent presynaptic neurons of the vagus nerves to inhibit
316 ruminal contractions. Such a selective action of the peptide is different from the inhibitory action of
317 cholecystokinin (Onaga et al., 1995) as i.v. infusion of cholecystokinin inhibits both the amplitude and
318 frequency of cyclic ruminal contractions in sheep.

319 On the other hand, the omasum and abomasum are also innervated by the motor neuron of the
320 vagus nerve (Ruckebusch, 1989). However, their motilities were not altered in the period immediately after
321 the i.v. injection of NT. They later decreased in the omasum and increased in the abomasum, although it is
322 not clear if the changes were due to the NT injection as with the rapid inhibitory effect on the rumen.
323 Although the reason remains unclear, the differences in the effect of NT may be accounted for by differences
324 in the dependency of these organs on vagal input.

325 The NTR-mediating action of NT has been classified into three subtypes in mammals; NTR-1,
326 NTR-2, and NTR-3. The first two subtypes are seven-transmembrane receptors in the cell membrane
327 (Vincent et al., 1999). In our study, i.v. injection of the NTR-1 antagonist, SR 48692, 5 minutes before NT
328 injection did not alter the inhibitory effect of NT, implying that NTR-1 is not involved in the *in vivo* action
329 of NT. The dose of the antagonist (60 nmol/kg) is close to the dose employed in mice to block increases in

vascular permeability (50 μg/kg; equivalent to 85.2 nmol/kg) (Donelan et al., 2006), and the dose of 60 nmol/kg would afford an initial plasma concentration of 300 nmol/L (if the extracellular fluid is 20% of body weight), which was shown to be sufficient to inhibit the contractile effect of NT (1-100 nmol/L) in the rat duodenal and colonic muscles (SR 48692, 30-300 nmol/L) (Mule et al., 1996; Li et al., 2016). Accordingly, it does not appear that the dose of the antagonist used in the sheep was too low to block the effect of NT. Indeed, a higher dose (100 nmol/kg, n = 2, data not shown) of the antagonist yielded similar results in the sheep. The possible role of the other subtypes of NTR in the inhibitory effect of NT remains to be determined. We cannot completely exclude the possibility that SR 48692 does not bind to ovine NTR-1 due to the possible species difference in the receptor molecules. Also, we should consider other possible indirect influences of NT on ruminal motility similar, for example, to the effects on heart rate and systemic blood pressure observed in rats (Kubo and Kihara, 1990; Ciriello and Zhang, 1997).

Although NT did not exert any peripheral inhibitory effect on the ovine rumen in the present study, localization of NT nerves was demonstrated in the forestomach wall of Karakul lambs in the past (Groenewald, 1994). Accordingly, NT neurons in the ruminal myenteric plexus possibly release NT to inhibit ruminal contractions. However, the results of the *in vitro* experiment showed that NT did not inhibit BCh- or EFS-induced contractions, which is not consistent with the previous study in Karakul lambs. It remains unknown whether breed and/or age differences in sheep account for the different results between the immunohistochemistry in Karakul lambs (Groenewald, 1994) and in adult Suffolk sheep. It was demonstrated in rats that the NT gene was expressed in the stomach in the early postnatal period, but not in

adult rats (Wang and Evers, 1999; Evers, 2002), and such developmental changes in NT gene expression might explain the different results observed in the two sheep studies. In pigs, likewise, NT-positive cells appear in the entire intestine at 6- to 8-weeks of gestation, while NT-positive cells are restricted to the small intestine throughout development (Alumets et al., 1983). Developmental changes in neural NT expression in the ovine GI tract remain to be determined.

Extra-gastric sources of NT also have to be taken into account when considering the inhibition of cyclic ruminal contractions by circulating NT. Mucosal endocrine cells in the caudal small intestine seem a likely candidate as a peripheral source of NT in sheep, as with peptide YY (PYY) (Onaga et al., 2000; Onaga et al., 2002). Ileal hormones; i.e., PYY, glucagons-like peptides (GLPs), and NT, are released through the luminal response to long-chain fatty acids and are considered to be mediators of the negative feedback regulation to the upper GI tract, termed the “ileal brake” (Read et al., 1984; Wen et al., 1995; Barreto and Windsor, 2017), which results in inhibition of motor and secretory functions in the stomach and upper small intestine in humans and dogs. The present study also suggests the possibility that NT plays the role of mediator of the ileal brake on ruminal motility in sheep. If the ileal brake were activated in the rumen, ruminal contractions would be inhibited or abolished. As the result, weak mixing causes mal-fermentation and the particle size of the contents remains large. In addition, stasis of the rumen can inhibit entry of ruminal digesta into the omasum and abomasum through the reticulo-omasal orifice, which would greatly alter digestion in the intestine and presumably elicit malnutrition in the affected animals. However, a blood concentration of NT of 5.0-12.5 nmol/L immediately after the injection is estimated from the effective dose

368 of 1 nmol/kg. Although a nano mol level of 50 % effective dose of NT was shown in rats (Huidobro-Toro
369 and Kullak, 1985) and a similar plasma concentration of NT (6.7 nmol/L) was reported after fat ingestion
370 humans (Rosell and Rokaeus, 1979), the fat content in common feeds for domestic ruminants are not so
371 high. With regards to the response to high fat diet, the postprandial concentration of plasma NT was reported
372 to be 44-48 pmol/L after milk ingestion in calves (Blackburn et al., 1981) and 26-91 pmol/L after fat
373 ingestion in humans (Theodorsson-Norheim and Rosell, 1983; Walker et al., 1985; Feurle et al., 1986)..
374 Thus, the effective plasma concentration of NT seems to be fairly high unless NT has the ability to surge.
375 However, we do know that some sheep were fed residual pellets from food factories, such as those
376 producing potato chips, in a suburban area, and in some countries cattle are fed tallow and palm oils as a
377 fat supplement (Shibata, 1988; Tomkins and Drackley, 2010). Such feeds have a higher fat content and
378 could possibly cause a high fat concentration in the lower intestine that would activate the ileal brake. On
379 the other hand, a 25-amino acid peptide, xenin, has high homology with NT in terms of the N-terminal
380 amino acid residues (Huidobro-Toro and Kullak, 1985), and xenin was shown to relax rat ileal muscle via
381 muscular NT receptors (Clemens et al., 1997). Also, the contractile and inhibitory effects of xenin on the
382 intestine in guinea pigs were inhibited by a NTR-1 antagonist (Feurle et al., 1996). Xenin is also found in
383 the human gastric mucosa (Feurle et al., 1992). Accordingly, it is possible that xenin is endogenously
384 released from the mucosa of the GI tract where it inhibits ruminal contractions in sheep. The physiological
385 role of endogenous NT and xenin in the inhibitory regulation of ruminal contractions in sheep remains to
386 be determined.

5. Conclusion

In conclusion, the present study demonstrated that intravenous injection of NT inhibited the amplitude of cyclic ruminal contractions in sheep, whereas it did not inhibit omasal or abomasal motility in a dose-dependent manner. However, pre-injection of SR 48692 immediately before NT injection did not block the inhibitory effect of NT, implying that NTR-1 is not involved in the inhibitory effect of NT. In the *in vitro* study, NT application did not inhibit BCh-evoked smooth muscle contractions of the rumen, and NT did not alter the basal tension or EFS-induced contractions, implying that NT does not inhibit ruminal muscle contractions through the local action. Hence, the present study suggests that circulating NT selectively inhibits the amplitude of cyclic ruminal contractions by inhibiting the gastric center in the CNS and/or excitatory presynaptic motor neurons of the vagus nerve in sheep. An elevation in the plasma concentration of NT appears likely activate the ileal brake on ruminal motility in sheep, but not that on the omasum or abomasum.

Author contributions

T. Onaga contributed to all aspects of this work. T. Shimoda and T. Oh-ishi contributed to the animal surgery and experiments, and data analysis. Y. Yasui and H. Hayashi contributed to the experimental design, manuscript preparation, and provided technical expertise for animal surgery.

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Figure captions

Fig. 1 Representative records of the effects of i.v. injection of NT on ovine gastric motility.

The intraluminal pressure of the caudal dorsal blind sac of the rumen, omasal canal, abomasal corpus and antrum and EMG activity in the greater curvature of the omasum were recorded in seven conscious sheep. After a control period for 40 minutes, NT was injected into the jugular vein at 0.3, 1, and 3 nmol/kg. Horizontal and vertical bars indicate scales for time and pressure or voltage, respectively.

Fig. 2 Effects of intravenous injection of NT on motility in the rumen in sheep.

After a control period for 40 minutes, a bolus of NT was injected into the jugular vein at 0.3, 1, and 3 nmol/kg, and recording was continued for 50 min. Data indicate changes in the raw value in mean basal pressure of the rumen (top), and frequency (middle) and mean amplitude (bottom) of the cyclic primary contractions of the rumen. Significant differences from the value immediately before NT injection are indicated by asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p< 0.001$). Bar graphs indicate the increments for the 15 minutes between before and after the injection, and significant changes against a control saline injection are indicated by asterisks (*, $p < 0.05$; **, $p < 0.01$). Mean $\pm$ SEM ($n = 7$).

Fig. 3 Effects of intravenous injection of NT on motility in the omasum and abomasum in sheep.

The intraluminal pressure of the omasal canal, abomasal corpus and antrum was recorded. The motility of the greater curvature of the omasum was measured by EMG. After a control period of 40

577 minutes, a bolus of NT was injected into the jugular vein at 0.3, 1, and 3 nmol/kg, and recording was
578 continued for 50 minutes. Data indicate temporal changes per 5 minutes in intraluminal pressure in the
579 omasal canal and the corpus and antrum of the abomasum, and spike frequency in EMG activity of the
580 greater curvature of the omasum. Mean ± SEM (intraluminal pressure; n = 7, EMG; n = 5). Significant
581 differences are shown by closed markers with asterisks (*, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$) for
582 each time point.
583
584 Fig. 4 Increments in motility indices after intravenous injection of NT in the omasum and abomasum.
585 Integrals of motility indices as an increment for the 15 minutes before after the NT injection are
586 indicated. Mean ± SEM (intraluminal pressure; n = 7, EMG; n = 5).
587
588 Fig. 5 Effect of SR 48692 on NT-induced inhibition of cyclic ruminal contractions.
589 After a control period of 40 minutes, a bolus of NT was injected into the jugular vein at 1 nmol/kg
590 with and without pre-administration of SR 48692 (SR) at 60 nmol/kg (i.v.). SR 48692 alone was injected
591 as the second control experiment. Data in the line graphs indicate changes in mean amplitude (top) and
592 frequency (bottom) of the cyclic primary contractions of the rumen. Significant differences from the
593 value immediately before NT injection are indicated by asterisks (*, $p < 0.05$; **, $p < 0.01$). Data in the
594 bar graphs indicate changes in the parameters as an increment for the 15 minutes against the pre-
595 injection period. Bars marked with different letters indicate significant differences ($p < 0.05$). Mean ±

596 SEM (n = 5).

597

598 Fig. 6 Effects of NT application on BCh-induced contractions of the ruminal muscle strips.

599 Two minutes after BCh application at 10 μmol/L to longitudinal (LM) and circular muscle strips

600 (CM) of the rumen dorsal sac, NT was cumulatively applied at 0.3-10 μmol/L at 1-minute intervals.

601 Changes in isometric contractions were compared with (B) and without NT application (A). Horizontal

602 and vertical bars indicate scales for time and tension, respectively. Changes in isometric contraction at

603 the end of each application period were compared against tension before NT application for the first

604 application of BCh (C). Mean ± SEM (n = 6-7).

605

606 Fig. 7 Effects of NT application on EFS-induced contractions of the ruminal muscle strips.

607 A: Representative records of the effects of the cumulative application of NT on EFS-induced

608 contractions of smooth muscle strips of the ovine rumen. EFS was applied to longitudinal (LM) and

609 circular smooth muscle (CM) strips of the ovine rumen dorsal sac for 10 seconds at 2-minute intervals.

610 After four control EFS-evoked contractions, NT was cumulatively applied at 0.1-10 μmol/L at 6-minute

611 intervals and three EFS-evoked contractions were recorded for each NT concentration. After NT

612 application, SR 48692 was applied at 100 μmol/L. As a control experiment, EFS were performed

613 without NT application following the above procedure. Failure of EFS due to shortened duration is

614 shown by artifact (af). EFS-evoked contractions were inhibited by the final application of lidocaine at

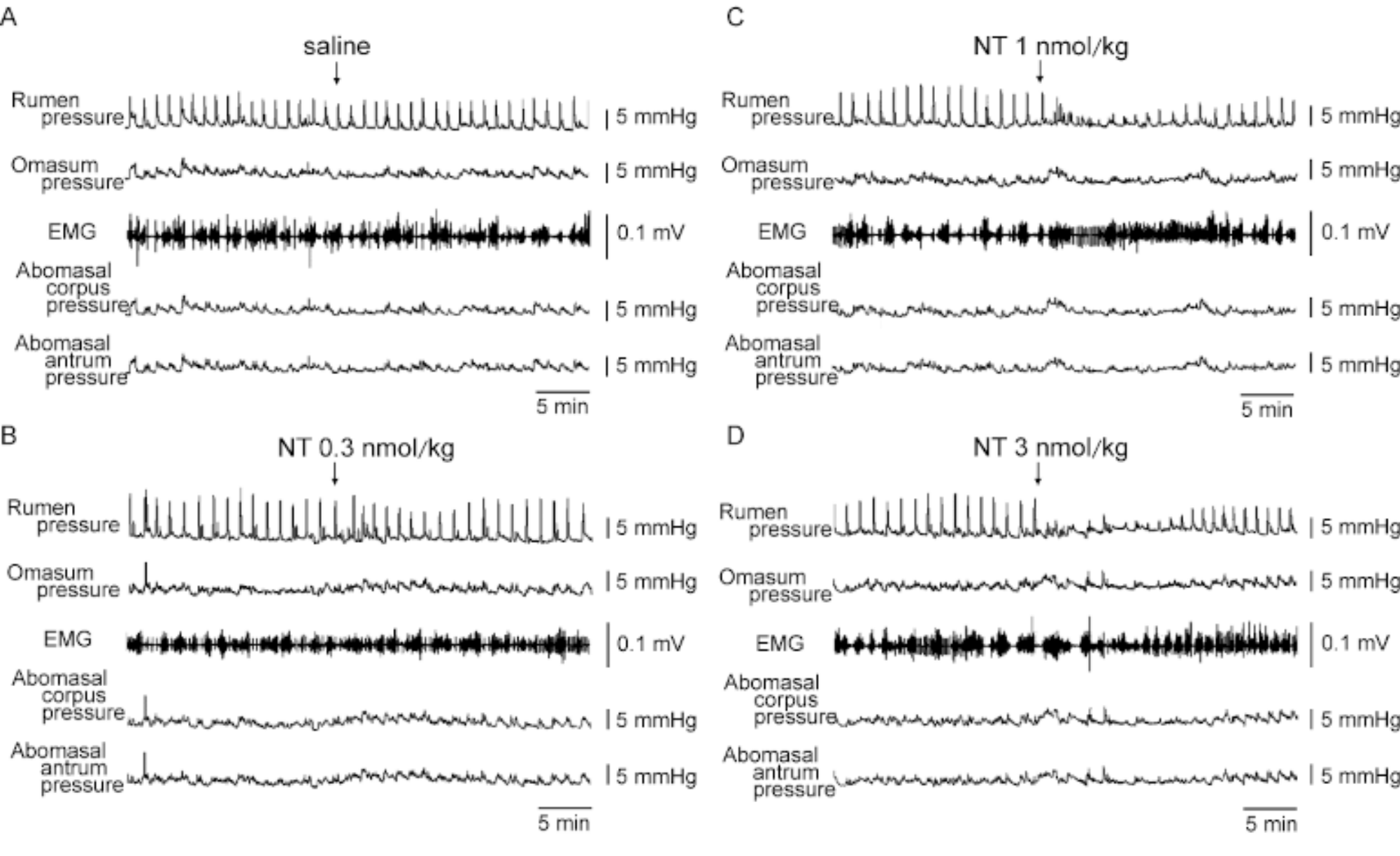
615 1 mmol/L. B: Changes in isotonic contraction were compared against the first EFS-evoked contraction

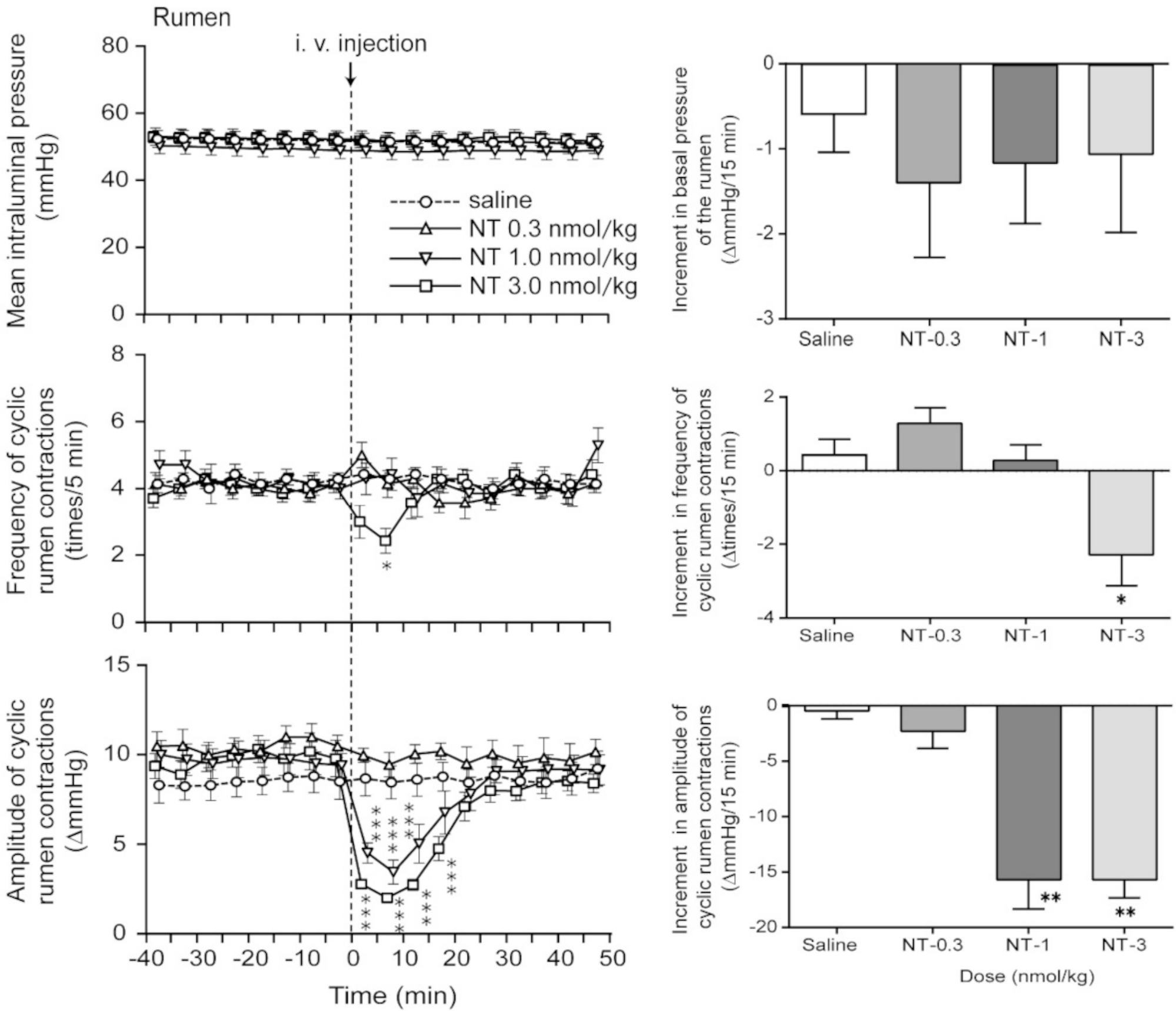
616 without NT. Horizontal and vertical bars indicate scales for time and contraction, respectively.

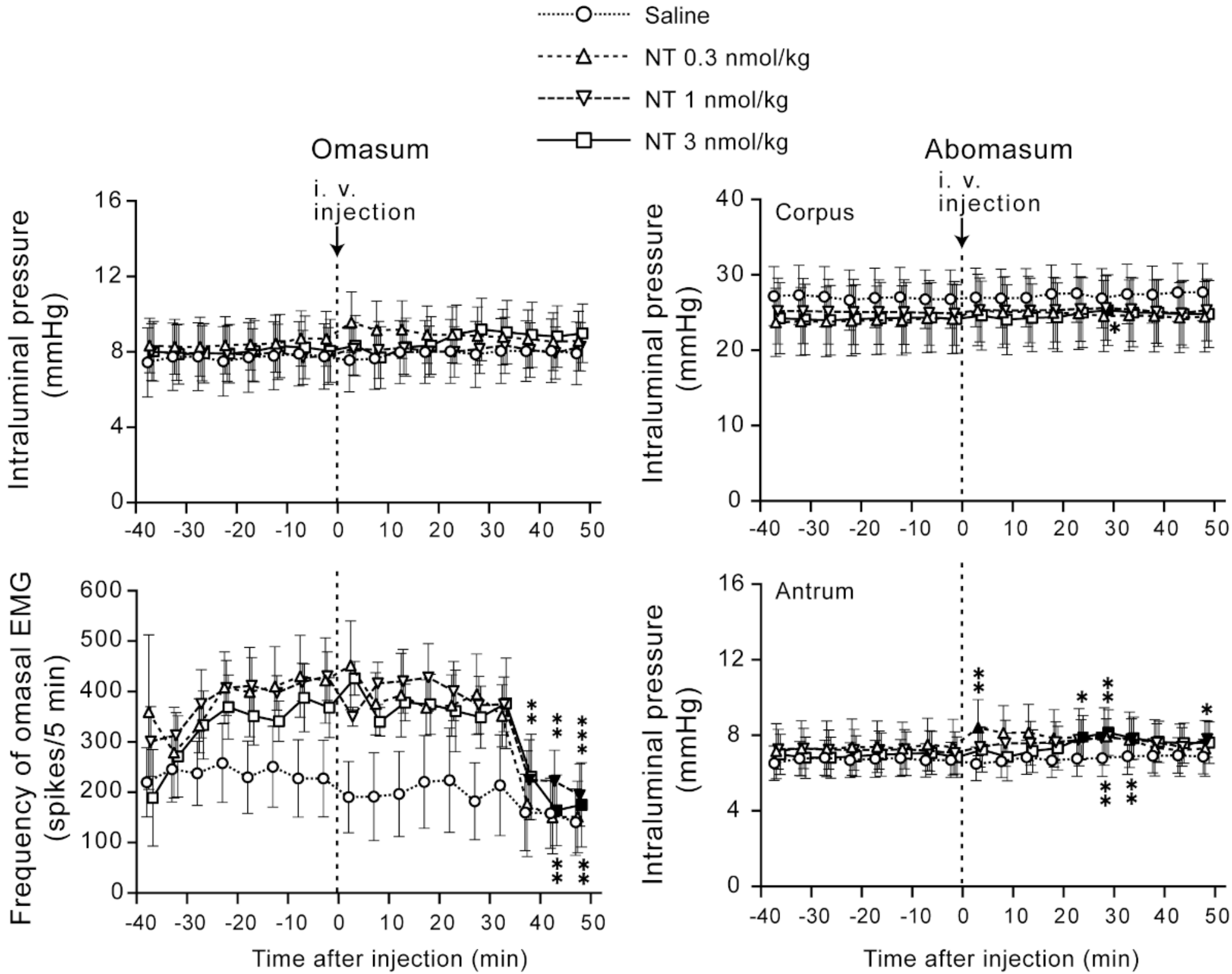
617 Significant differences from the control value are indicated by asterisks (*, $p < 0.05$; ***, $p < 0.001$).

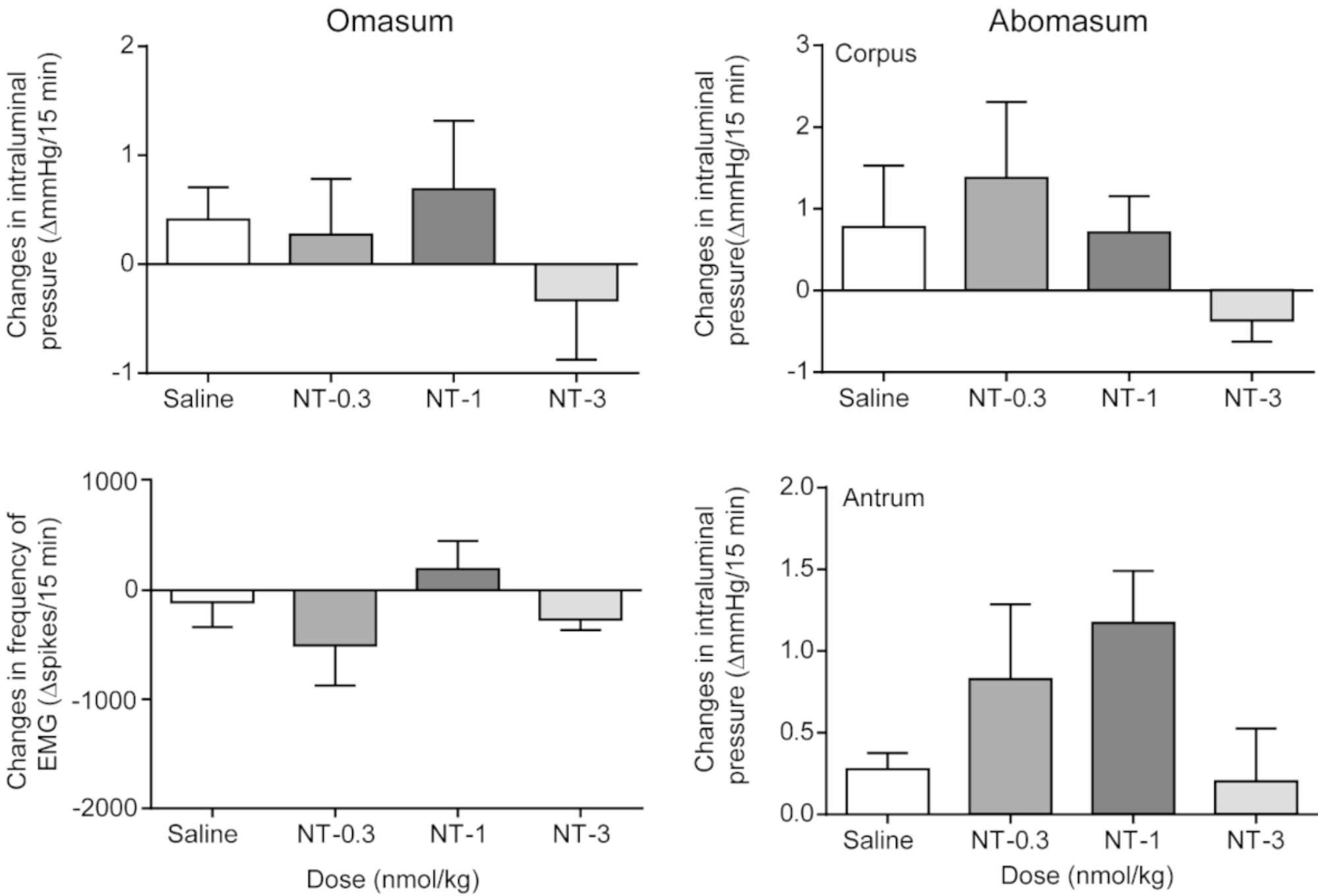
618 Mean ± SEM (n = 6-8).

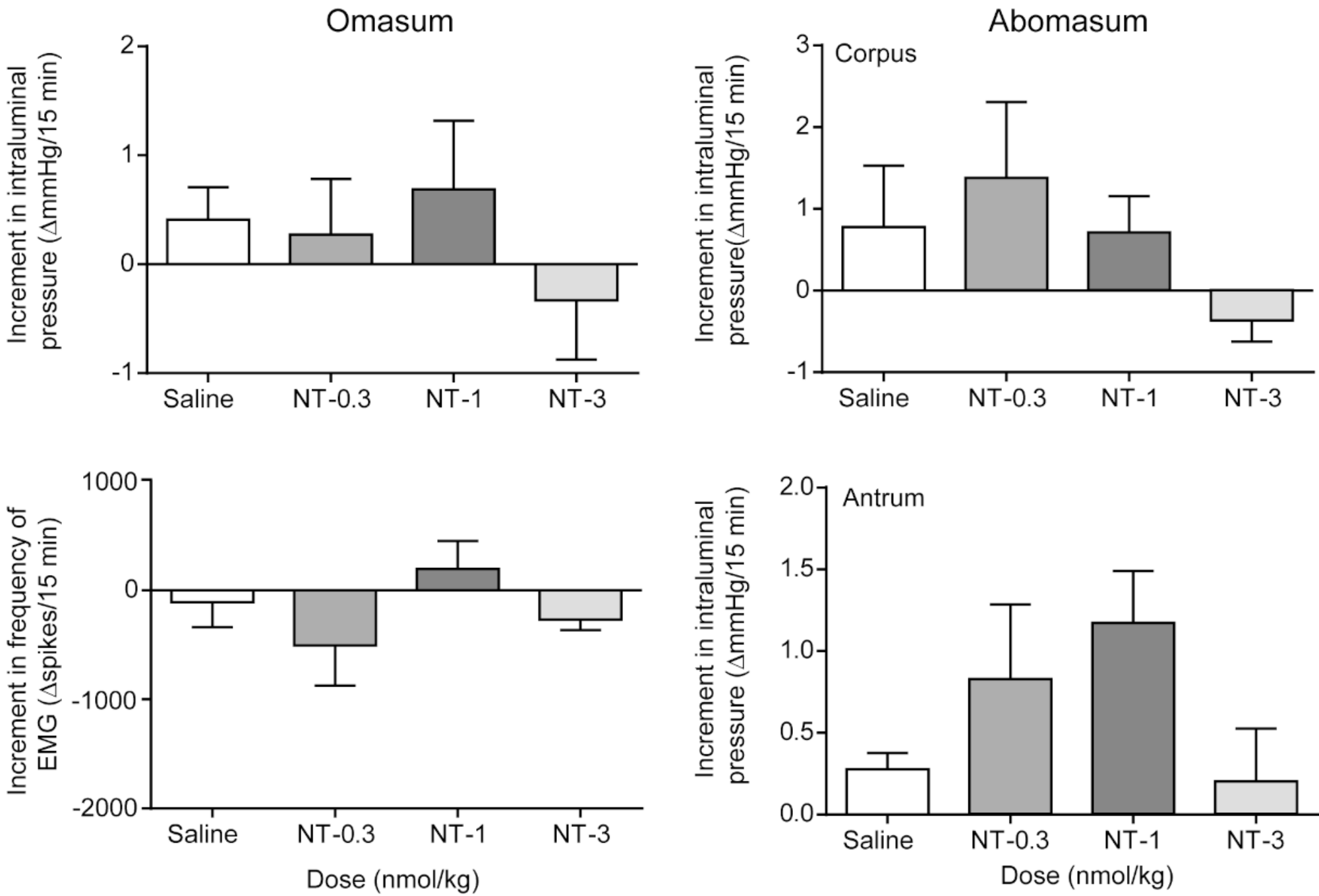
619

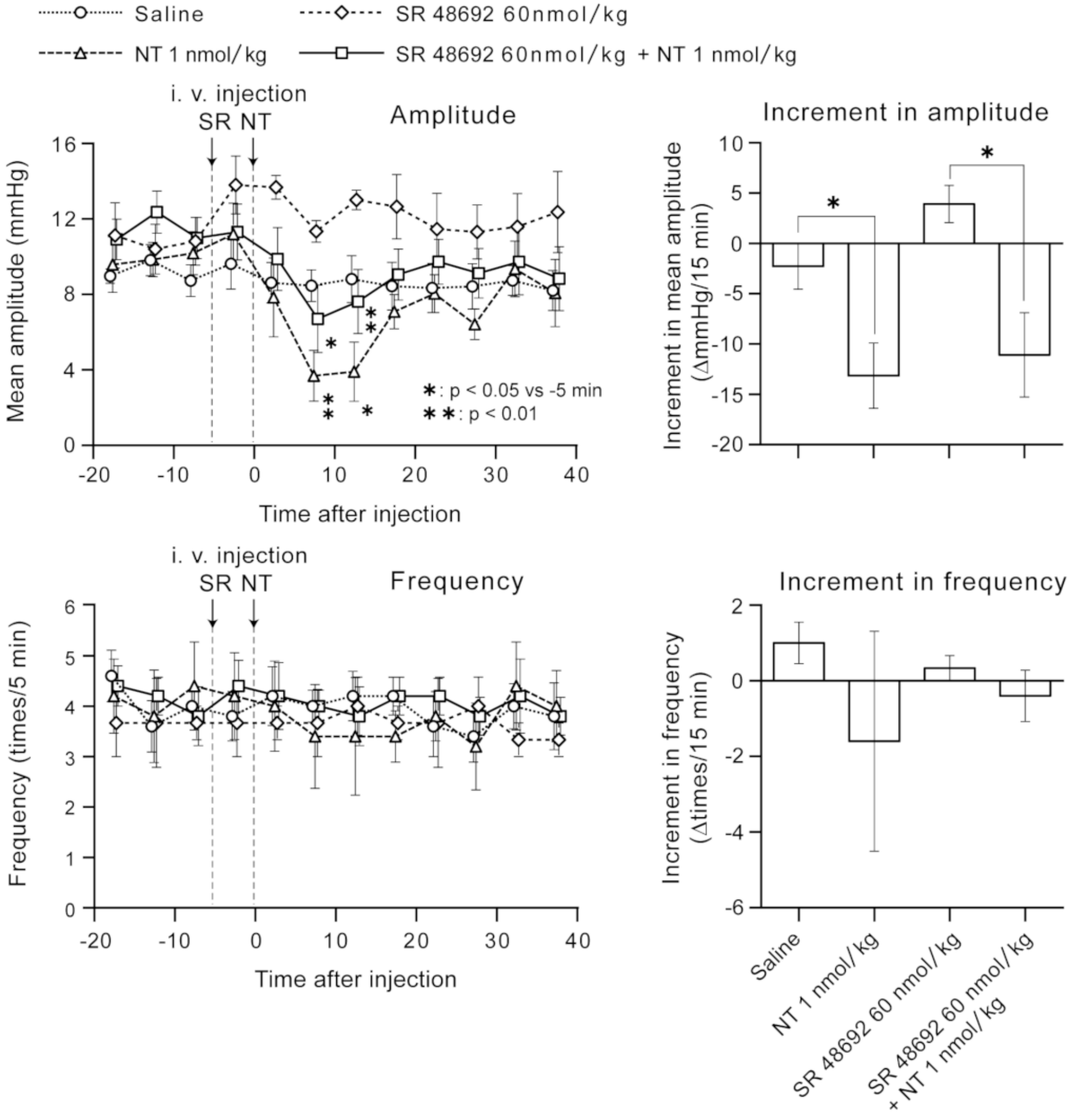

A
saline
Rumen pressure
5 mmHg
Omasum pressure
5 mmHg
EMG
0.1 mV
Abomasal corpus pressure
5 mmHg
Abomasal antrum pressure
5 mmHg
5 min
B
NT 0.3 nmol/kg
Rumen pressure
5 mmHg
Omasum pressure
5 mmHg
EMG
0.1 mV
Abomasal corpus pressure
5 mmHg
Abomasal antrum pressure
5 mmHg
5 min
C
NT 1 nmol/kg
Rumen pressure
5 mmHg
Omasum pressure
5 mmHg
EMG
0.1 mV
Abomasal corpus pressure
5 mmHg
Abomasal antrum pressure
5 mmHg
5 min
D
NT 3 nmol/kg
Rumen pressure
5 mmHg
Omasum pressure
5 mmHg
EMG
0.1 mV
Abomasal corpus pressure
5 mmHg
Abomasal antrum pressure
5 mmHg
5 min

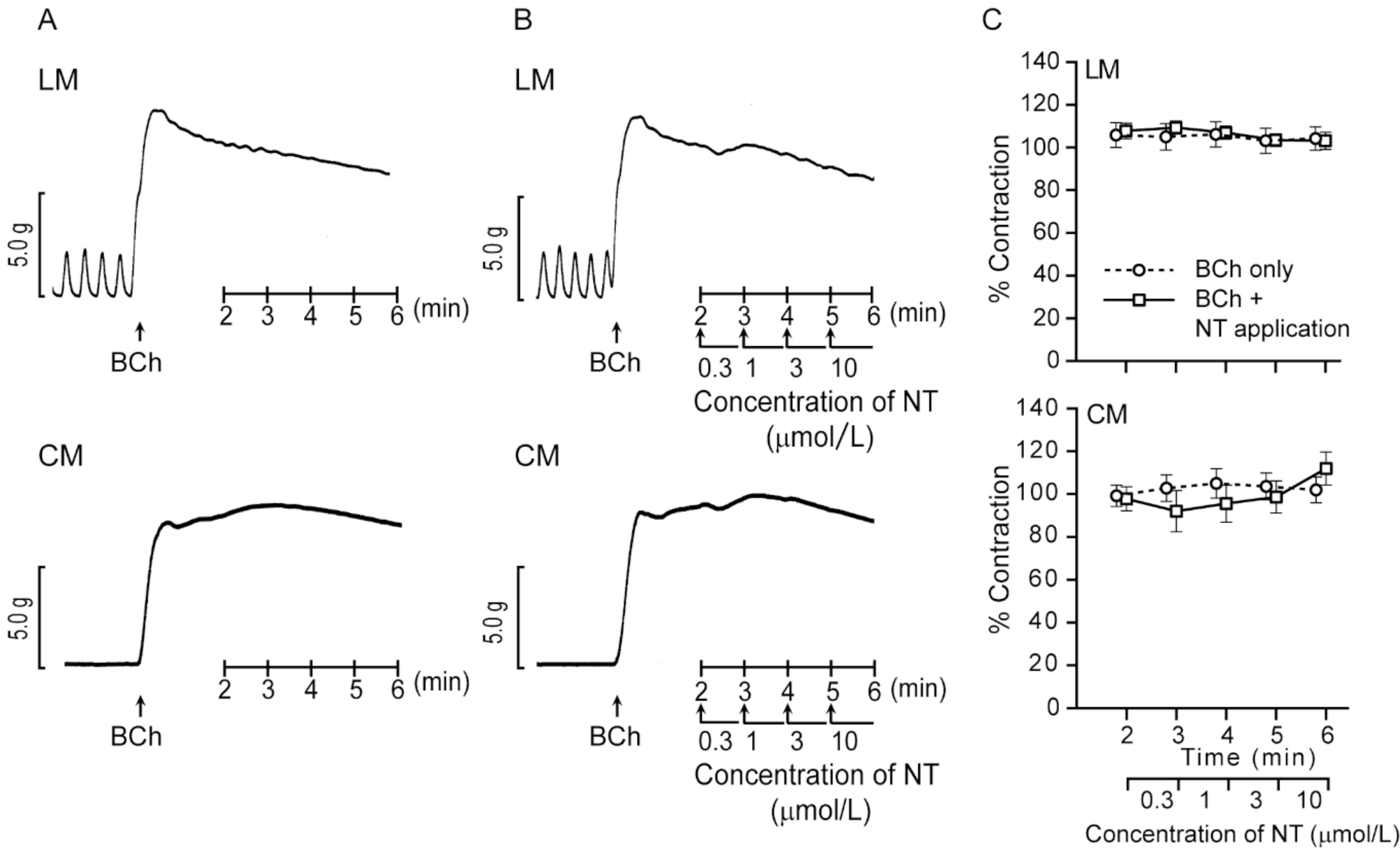

Rumen
i. v. injection
Mean intraluminal pressure (mmHg)
saline
NT 0.3 nmol/kg
NT 1.0 nmol/kg
NT 3.0 nmol/kg
Frequency of cyclic rumen contractions (times/5 min)
Amplitude of cyclic rumen contractions (ΔmmHg)
Time (min)
Increment in basal pressure of the rumen (ΔmmHg/15 min)
Increment in frequency of cyclic rumen contractions (Δtimes/15 min)
Increment in amplitude of cyclic rumen contractions (ΔmmHg/15 min)
Saline
NT-0.3
NT-1
NT-3
Dose (nmol/kg)

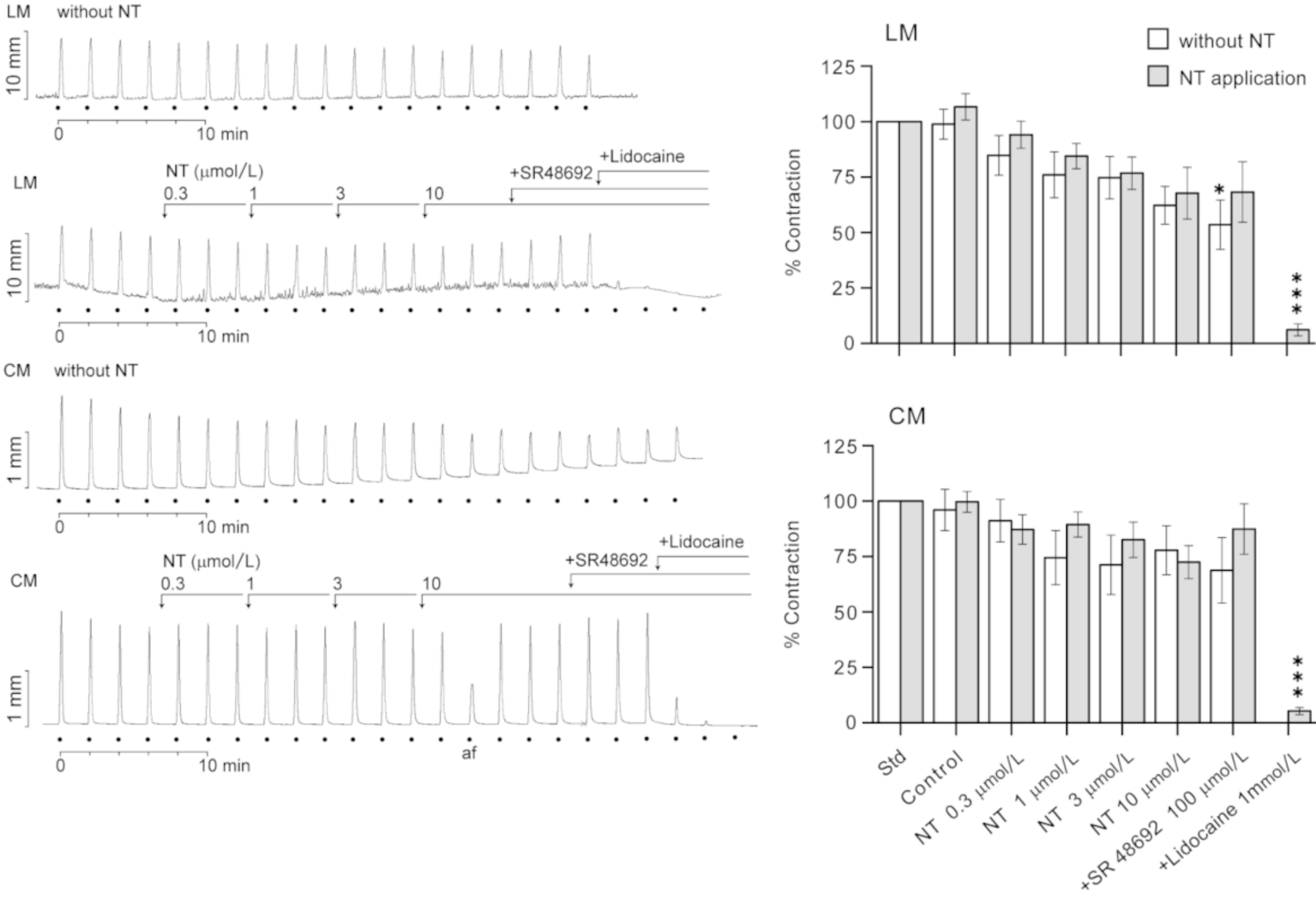

Saline
NT 0.3 nmol/kg
NT 1 nmol/kg
NT 3 nmol/kg
Omasum
i. v. injection
Abomasum
Corpus
Antrum
Intraluminal pressure (mmHg)
Frequency of omasal EMG (spikes/5 min)
Time after injection (min)


Omasum
Changes in intraluminal pressure (ΔmmHg/15 min)
2
1
0
-1
Saline
NT-0.3
NT-1
NT-3
Abomasum
Corpus
Changes in intraluminal pressure(ΔmmHg/15 min)
3
2
1
0
-1
Saline
NT-0.3
NT-1
NT-3
Changes in frequency of EMG (Δspikes/15 min)
1000
0
-1000
-2000
Saline
NT-0.3
NT-1
NT-3
Dose (nmol/kg)
Antrum
Changes in intraluminal pressure (ΔmmHg/15 min)
2.0
1.5
1.0
0.5
0.0
Saline
NT-0.3
NT-1
NT-3
Dose (nmol/kg)


Omasum
Abomasum
Corpus
Antrum
Increment in intraluminal pressure (ΔmmHg/15 min)
Increment in intraluminal pressure(ΔmmHg/15 min)
Increment in frequency of EMG (Δspikes/15 min)
Increment in intraluminal pressure (ΔmmHg/15 min)
2
1
0
-1
3
2
1
0
-1
1000
0
-1000
-2000
2.0
1.5
1.0
0.5
0.0
Saline
NT-0.3
NT-1
NT-3
Dose (nmol/kg)


Saline
SR 48692 60nmol/kg
NT 1 nmol/kg
SR 48692 60nmol/kg + NT 1 nmol/kg
i. v. injection
SR NT
Amplitude
Mean amplitude (mmHg)
Time after injection
*: p < 0.05 vs -5 min
**: p < 0.01
Increment in amplitude
Increment in mean amplitude (ΔmmHg/15 min)
i. v. injection
SR NT
Frequency
Frequency (times/5 min)
Time after injection
Increment in frequency
Increment in frequency (Δtimes/15 min)
Saline
NT 1 nmol/kg
SR 48692 60 nmol/kg
SR 48692 60 nmol/kg + NT 1 nmol/kg


A
LM
5.0 g
2 3 4 5 6 (min)
BCh
CM
5.0 g
2 3 4 5 6 (min)
BCh
B
LM
5.0 g
2 3 4 5 6 (min)
BCh
0.3 1 3 10
Concentration of NT (μmol/L)
CM
5.0 g
2 3 4 5 6 (min)
BCh
0.3 1 3 10
Concentration of NT (μmol/L)
C
LM
% Contraction
140
120
100
80
60
40
20
0
BCh only
BCh + NT application
CM
% Contraction
140
120
100
80
60
40
20
0
2 3 4 5 6
Time (min)
0.3 1 3 10
Concentration of NT (μmol/L)


LM
without NT
10 mm
0
10 min
LM
NT (μmol/L)
0.3
1
3
10
+SR48692
+Lidocaine
10 mm
0
10 min
CM
without NT
1 mm
0
10 min
CM
NT (μmol/L)
0.3
1
3
10
+SR48692
+Lidocaine
1 mm
0
10 min
af
LM
without NT
NT application
% Contraction
125
100
75
50
25
0
*
***
CM
% Contraction
125
100
75
50
25
0
***
Std
Control
NT 0.3 μmol/L
NT 1 μmol/L
NT 3 μmol/L
NT 10 μmol/L
+SR 48692 100 μmol/L
+Lidocaine 1mmol/L