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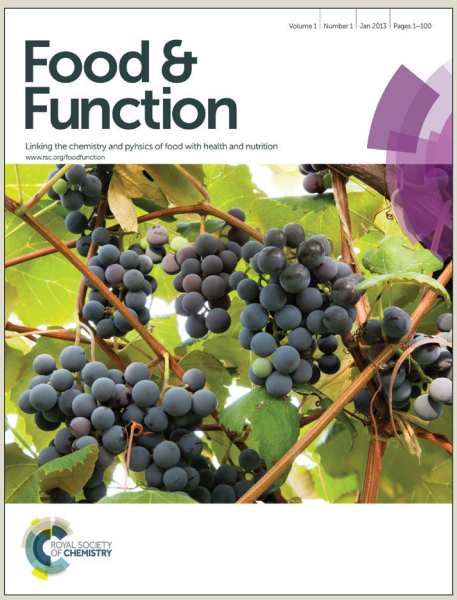

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Title: An *in-vitro* rat model of colonic motility to determine the effect of β-casomorphin-5 on propagating contractions.

Running title: β-casomorphin-5 inhibits propagating colonic contractions.

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This work was supported by the Ministry of Business, Science & Innovation, New Zealand (C10X1003).

Keywords: opioid agonist, casomorphin, colon, gastro-intestinal motility, transit, casein, milk

Abstract

Beta-casomorphin-5 (βCM-5) is a milk-derived bioactive peptide that slows gastro-intestinal transit (GIT) *in vivo* and blocks the peristaltic reflex in the guinea pig colon *in vitro*. We wanted to establish an *in vitro* model system in which effects of dairy-derived substances containing opioid peptides on intestinal motility can be assessed and used to predict *in vivo* outcomes. Because βCM-5 is an opioid agonist that acts on enteric neurons, we used this substance to compare two different isolated colonic tissue preparations to determine which would more closely mimic the *in vivo* response previously reported in the literature. We compared and characterized the effects of βCM-5 on spontaneous contractions in isolated segments of distal colon (1 cm length) compared with propagating contractions along the isolated intact large intestine (22 cm length). In short segments of distal colon, βCM-5 increased the tension and frequency of spontaneous contractions in a concentration-dependent manner. At 20 μM βCM-5 tension increased by 71 ± 17 % and doubled the frequency (n=9), an effect inhibited by naloxone (n=7) and therefore mediated by opioid receptors. In contrast 20 μM βCM-5 disrupted propagating contractions in the large intestine preparation. At 20 μM βCM-5 reduced the proportion of contractions initiated in the proximal colon reaching the rectum by 83 ± 11 % (n=5) and this effect was also inhibited by naloxone, consistent with altered GIT reported *in vivo*. Our results demonstrate that the isolated whole large intestine provides an ideal preparation that mimics the reduced propagation of GIT *in vivo* in response to an opioid agonist, whereas short colon segments did not. The findings of the current study reveal that preserving large segments of intact large intestine, and hence intact enteric neural circuitry provides an ideal *in vitro* model to investigate the effect of opioid receptor modulators on intestinal transit.

Introduction

The major protein in milk, casein, is known to slow GIT in rats (relative to whey protein suspension) [1] and to decrease motility in the canine small intestine (relative to soy) [2]. In both studies, casein-induced slowing of GIT was prevented by the opioid antagonist naloxone, implicating opioid receptors in mediating the effect [1, 3]. When young rats were given native casein powder small intestine transit was slower relative to those given extensively hydrolysed casein. The opioid antagonist naloxone prevented the slower transit for native casein but did not affect transit for hydrolysed casein treated animals [3]. This suggests that during digestion the native casein released peptides with opioid agonist activity, but that the extensively hydrolysed casein did not.

Hydrolysis of milk casein during digestion produces bioactive peptides such as β-casomorphins that are derived from fragments between the 60th and 70th amino acid residues of β-casein and act as opioid agonists [4]. Bovine β-casomorphin 5 (βCM-5) is particularly resistant to proteolytic degradation and is a potent opioid agonist relative to other β-casomorphins [1]. βCM-5 slows GIT in young rats [1]. It also blocks the peristaltic reflex in 10 cm long isolated segments of guinea pig colon and this effect is reversed by naloxone [5].

One of the primary aims of this study was to develop an *in vitro* model system in which effects of dairy-derived substances containing opioid peptides on intestinal motility can be assessed and used to predict effects on *in vivo* large intestine transit. Muscle contraction assays are in routine use in the pharmaceutical industry to identify active compounds, but have not been widely used for food research. Studies to investigate the enteric nervous system regulation of intestinal motility measure propagating contractions in the isolated whole large intestine preparation, but this methodology has not been applied to food research. We used a rat model because many *in vivo* studies are done in rats and this would enable comparison with other rat studies. βCM-5 was chosen as an example of a bioactive milk

peptide that alters intestinal motility by a known mechanism. Based on competitive binding studies from rat and guinea pig brain homogenates it is known that βCM-5 has a high degree of selectivity for μ opioid receptors [6, 7]. From muscle contractility studies in tissues expressing different opioid receptor subtypes it has been inferred that βCM-5 binds predominately to the μ2 rather than the μ1 subtype [8]. βCM-5 has been reported to alter cardiac contractility in the isolated guinea pig heart at nanomolar concentrations via a non-opioid mediated pathway [9, 10].

Since βCM-5 acts as an opioid agonist on enteric neurons, the length of the colon preparation, and therefore the integrity of the enteric neural network would likely be a major factor in preserving complex propagating motor patterns and characterising the impact on intestinal motility *in vitro* [11]. To determine whether a short or long length of a colon preparation are better to record effects of substances on smooth muscle contraction we compared effects of βCM-5 on spontaneous contractions in 1 cm short distal colon pieces with effects on spontaneous propagating contractions in a 22 cm large intestine preparation from caecum to anus. The effect of βCM-5 on contractile tension and frequency was measured for both preparations and the pattern and velocity of contractions measured in the large intestine.

Materials and Methods

This study was carried out in strict accordance with the recommendations of the New Zealand Animal Welfare Act 1999. The protocol was approved by the AgResearch Limited (Grasslands) Animal Ethics Committee (Ethics Approval No.: AE98) Animals were euthanized by CO_2 inhalation overdose. Male adult Sprague Dawley rats, 3-9 months of age, weighing 250-550 g were obtained from AgResearch Ruakura (Hamilton, NZ) were housed under a 12 hour light/dark cycle, and fed Sharpes Diet 86 (Sharpes Stockfeeds Ltd, Carterton, New Zealand).

Distal colon segment

Methods were similar to those described previously for the short distal colon [12]. After euthanasia, a 4 cm piece of distal colon was removed 1 cm distal to the striations of the mid colon. The tissue was placed in Kreb's buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 $MgSO_4$ mM, 2.6 mM $CaCl_2$, 25 mM $NaHCO_3$, 11 mM glucose, pH 7.4) oxygenated with 95% O_2/5% CO_2. The preparation was divided into four pieces and each distal colon piece was mounted longitudinally on holders in an isolated tissue bath system (SI-MB4, SI-Heidelberg, World Precision Instruments, Sarasota, FL, USA), at 37 °C (Fig. 1a).

Tissues were suspended under 1 g of tension and equilibrated for one hour in Krebs buffer during which time the bath solution was exchanged every 15 min. Spontaneous muscle contractions prior to any treatments were measured as controls during the last 15 min of a two hour exposure to Krebs buffer and compared with the response to 15 min exposure to βCM-5. Muscle contraction data were measured using BAM21-E amplifiers integrated using Lab-Trax 4/24T hardware and acquired and analysed using Labscribe 2 software (iWorx Inc., Dover, NH, USA). To allow for recovery from the solution exchange, data from the last 10 min of a 15 min recording period were analysed and the mean for each parameter determined from the contractile responses recorded. Contractile tension or amplitude (g) was measured from the baseline to the maximum peak of the contractile response. Contractile frequency was measured as the number of contractions counted per min. The maximum tension response was compared to that for 1 μM acetylcholine, and preparations that gave little or no response to acetylcholine were excluded.

Whole large intestine

This method for measuring whole large intestine contraction was adapted from that previously reported for the mouse [13]. After euthanasia, the colon was removed from the rat (22 cm in length) from 3-5 cm below the caecum. The tissue was placed in oxygenated Kreb's buffer in a perfusion bath (210 ml volume) and externally perfused at 10 ml/min at 35 ± 1 °C. The colon was gently flushed with Krebs solution using a syringe to expel faecal pellets. A stainless steel rod (approximately 21 cm long and 4 mm diameter) was inserted through the lumen and the entire colon then mounted in an organ bath (approximately 200 ml capacity) perfused at 20 ml/min with Krebs' buffer at 35 ± 1 °C. The lumen was also perfused with Krebs' buffer at 0.5-1 ml/min which was pumped aborally using a constant flow pump because this provided the pressure required to record consistent propagating contractions. Changes in circular muscle tension were recorded from four sites simultaneously along the length of large intestine, using four custom-made metal hooks anchored 3 cm from each end of the preparation and evenly spaced at approximately 4 cm intervals (Fig. 1b). These hooks were connected via silk thread to force transducers and contractions measured as described for the short colon segments.

A spontaneous propagating contraction was defined as a spontaneous contraction that occurred at four recording sites, and was generated in the proximal colon and migrated aborally to the distal recording site. The contractions that propagated from the proximal colon to the distal colon were analysed and statistically compared. The interval between first and fourth contractions (taken from the time between 50% peak amplitude, on the rising phase, of four successive contractions) was measured. Propagation velocities were calculated by taking the distance between these recording hooks and dividing by the time taken for each

spontaneous propagating contraction. Modulators were applied to the external side of the preparation via the perfusion tube supplying the bath.

Data are expressed as mean ± standard errors of the mean (SEM). Data from each animal were normalised (as a percentage of control) within each animal to take into account the variability between animals.

Modulators

Bovine βCM-5 (Tyr-Pro-Phe-Pro-Gly) was purchased from the Peptide Institute (Osaka, Japan) and stored as a 32 mM frozen stock at -20 °C. Naloxone hydrochloride dihydrate was purchased from Sigma-Aldrich (St. Louis, MO, USA) and stored as a 10 mM frozen stock -20 °C. Both were diluted in Krebs buffer immediately prior to use. Acetylcholine chloride was purchased from Sigma-Aldrich (St. Louis, MO, USA) and diluted from a 1 mM stock.

Statistical analysis

The analyses were carried out using GenStat version 15.1 (VSN International Limited, Hemel Hempstead, UK).

Distal colon segment. Results are expressed as the mean ± SEM from ≥ 5 preparations (from ≥ 3 animals). Ratios for each time point were calculated by dividing the tension or frequency during a treatment by the initial time point prior to the addition of the treatment compound. ANOVA was carried out on the log-ratios because the assumptions of normality for ANOVA were not met by the raw data. Statistical differences were assessed using Studentised Maximum Modulus confidence intervals for the βCM-5 concentration experiments or repeated measures ANOVA for the naloxone experiments.

Whole large intestine. Results are expressed as the mean ± SEM from 3-8 preparations. Differences in the proportion of propagating contractions between control and treatments were analysed by ANOVA. Difference in tension between control and treatments were compared using Mixed Model analysis with AR(1) correlations among the transducers for the comparison of percent difference from control.

Results

Distal colon segment

Spontaneous contractions were measured in the rat distal colon *in vitro* (Fig. 1a) and effects of βCM-5 on contractile tension and frequency measured. Spontaneous contractile activity recorded from the isolated distal colon before and after addition of βCM-5 is shown in Fig. 2a.

The effect of 15 min of βCM-5 treatment on spontaneous muscle contractions was measured relative to that in the last 10 min of the control recording. To determine the most effective concentration of βCM-5, we tested concentrations in the range of 0.2, 2, or 20 μM on individual preparations because concentrations in this range were reported to be effective in the guinea pig colon [5]. The most pronounced response was found to occur at 20 μM βCM-5. To determine the optimal application time for maximal effect, 20 μM βCM-5 was applied for one hour and the contractile response monitored. Data comparing the effect of βCM-5 on contractile tension and frequency over time are shown in Fig. 2b. An increase in tension and frequency occurred that was maximal after 15 min of exposure to βCM-5. The increase in tension reduced slowly over an hour duration of exposure to βCM-5, but the increase in frequency was short-lived and after 30 min of exposure the frequency returned to levels similar to the control. We therefore applied βCM-5 to the short colon preparation for 15 min in subsequent experiments to observe the maximal effect.

To investigate the concentration-response relationship, βCM-5 (0.2, 2, or 20 μM) was applied to separate preparations for 15 min, followed by 45 min in Krebs buffer (n = 7-8) (Fig 2a). βCM-5 increased the tension and frequency of spontaneous contractions in a concentration-dependent manner (Fig. 2c). A concentration of 0.2 μM had no effect, 2 μM increased frequency by 99 ± 20 % (mean ± SEM) but had no effect on tension, and 20 μM increased tension by 71 ± 17 % and frequency by 96 ± 26 %. Muscle contractions were not different from control levels after 45 min wash out in Krebs buffer. Pre-exposure to 1 μM naloxone inhibited the βCM-5-induced increase in spontaneous contraction tension and frequency in the short rat distal colon (Fig.3). Naloxone applied alone had no effect.

Whole large intestine:

Propagating contractions were recorded from the rat large intestine preparation *in vitro* (Fig. 1b) before and after addition of βCM-5 (Fig. 4a) and effects on ability to propagate, direction of propagation and contractile tension were measured. Control recordings had 4.6 ± 0.6 (n=8) forward propagating contractions during a 30 min period, after the preparation had been exposed to Krebs buffer for 1-2 h. Spontaneous propagating contractions were recorded from the rat large intestine for which forward propagations had an interval between contractions of 5:42 (min:s) and a velocity of 10.8 ± 2.2 s (n=8) from proximal colon to rectum, traversing a distance of 13.0 ± 0.7 cm (n=8). Contractions that propagated either forward, mid or backward in direction were referred to as 'synchronous contractions' and were measured as a proportion of all contractions. When βCM-5 was applied to the external side of the whole large intestine preparation via the bath it inhibited the propagation of contractions from the proximal colon to the rectum by 81 ± 6 % (n=5) (Fig. 4b). Following addition of βCM-5,

only four out of eight preparations had some contractions present that were initiated in the proximal colon and propagated to the rectum. Exposure to βCM-5 decreased the tension of the propagating contractions by at least 54 ± 19 % (n=4) ($p < 0.01$) (Fig. 4c), an effect that was equal along the length of the large intestine as there was no significant difference among the transducers ($p > 0.05$).

Since βCM-5 is considered to be an opioid agonist, the antagonist naloxone was used to investigate the mechanism of action. It was found that 20 nM or 100 nM naloxone did not prevent inhibition by 20 μM βCM-5, yet 10 μM naloxone inhibited propagating contractions when applied alone (data not shown). We therefore used 1 μM naloxone which had minimal effects when applied alone, sometimes producing a ripple pattern to contractions. After 20 μM βCM-5 had been applied for 15-30 min and then washed off for 60 min, 1 μM naloxone was applied alone for 15-30 min, then co-applied with 20 μM βCM-5 for 15-30 min. The results showed that 1 μM naloxone prevented the inhibitory effect of βCM-5 on synchronised propagating contractions (Fig. 4b) and the decrease in tension (Fig. 4c), such that it was not significantly different from controls. The forward propagating contractions did not appear to fully recover from the inhibitory effect of βCM-5, giving variable responses in latter treatments. To rule out possible repeated exposure effects of βCM-5, it was applied alone for 15-30 min, then immediately co-applied with naloxone for 15-30 min. Synchronised contractions as a proportion of the total number of contractions were partially inhibited by βCM-5 (control; 87.4 ± 12.6, βCM-5; 59.5 ± 9.5, $p < 0.01$) and this effect was prevented by naloxone when co-applied with βCM-5 (91.1 ± 4.5), which was not significantly different from the control, n=3. Forward propagations however were completely inhibited by βCM-5 (control; 39.6 ± 5.2 %, βCM-5; 0, $p<0.001$) and this effect was prevented by naloxone when

co-applied with βCM-5 (54.2 ± 4.2) which was in fact increased by 36.9% compared with the control, n=3 ($p < 0.05$).

To further reduce βCM-5 exposure (and possible desensitisation with time), an experiment was also done pre-exposing the preparation to 1 μM naloxone for 30 min followed by 1 μM naloxone together with 20 μM βCM-5 for 30 min, which showed no change in contractions compared to the control, further confirming inhibition of the βCM-5 inhibitory effect by naloxone. In preparations with propagating contractions these were inhibited by 1 μM TTX, n=2.

Discussion

The main finding of this study was that βCM-5 disrupted intestinal motility in the rat colon *in vitro*. It produced different effects on muscle contraction properties depending on the length of the colonic preparation used. In the distal colon segment βCM-5 increased motility whereas in the whole large intestine it decreased motility. Motility changes in both tissue preparations occurred at concentrations of βCM-5 (2 - 20 μM) that are similar to that reported to alter motility in the guinea pig ileum [7, 14, 15].

The increase in motility produced by βCM-5 in the distal colon segment preparation was contrary to that expected because it is reported to slow GIT *in vivo*. We therefore examined the effect of βCM-5 in the whole intact large intestine *in vitro* to determine whether a preparation with greater preservation of the enteric nervous system would more closely mimic the effects known to occur *in vivo.* The whole rat large intestine preparation was used because it has been shown to have neurogenic peristaltic activity [16]. Inhibition of synchronous

contractions by the voltage-gated sodium channel inhibitor TTX indicated that this was also the case in this preparation under the current experimental conditions. The concentration of 20 μM βCM-5 was used to produce a maximal inhibitory effect in the whole large intestine preparation because in guinea pig colon 15 μM βCM-5 inhibits the peristaltic reflex by 85% (dePonti 1989 add).In this preparation we found that the predominant effect of βCM-5 was to inhibit propagation of the spontaneous contractions initiated in the proximal colon from reaching the rectum. Inhibition of the βCM-5 response by naloxone indicated that this effect was likely to be mediated by opioid receptors. Forward propagating contractions are considered to be important for normal colonic transit in humans [17], therefore the strong inhibitory effect of βCM-5 on these long distance propagations would explain the slowed gastro-intestinal transit reported in rats *in vivo* [1].

The forward propagating contractions recorded were approximately three times less frequent (0.17 contractions/min) compared with other recent reports in rats and were slightly faster (11 mm/s compared with 3.5 - 3.7 mm/s) [11, 16]. On some occasions the propagating contractions occurred as series of repeated contractions in control recordings or after addition of naloxone. This pattern of contraction has been described as 'myogenic ripples' [16]. The observation that non-propagating contractions occurred with increased frequency in some whole large intestine preparations in the presence of βCM-5 was similar to our results for the short distal colon segment preparation. However because the proportion of these contractions that propagated to the rectum was decreased by βCM-5, this effect would not be expected to speed GIT but instead to produce dysmotility and contribute to slowed GIT.

Our results showed that the inhibitory effect of βCM-5 on synchronous contractions could be reversed upon wash out with Kreb's buffer. Our observations are consistent with reports that

tolerance to opioids upon repeated exposure has been shown to occur in the rat proximal colon (and ileum) *in vivo* [10] but not the mouse distal colon [9].

The increased motility observed in the distal colon segment preparation in response to βCM-5 was unexpected because it is inconsistent with previous reports showing that this compound slows GIT in young rats *in vivo;* and inhibits colonic propagations in the guinea pig *in vitro* [1, 5]. However, the inhibitory effect of naloxone on the βCM-5 response that we observed suggests that the increase in contraction frequency produced by βCM-5 is due to activation of opioid receptors, which is similar to previous reports. We demonstrated previously that spontaneous phasic contractions in the short distal colon are not initiated by enteric neurons as they are not inhibited by tetrodotoxin (TTX) [12]. These contractions are therefore of myogenic rather than neural origin and therefore not initiated by enteric nerves. Furthermore, TTX increases the tension and frequency of contractions [12] suggesting that inhibitory inputs predominate in the enteric neuronal network of this short segment distal colon preparation. The antagonism of the βCM-5 effect by naloxone in this preparation might have occurred through an effect on the remaining inhibitory enteric neurons in this preparation, by supressing the inhibitory inputs resulting in excitation and enhanced motility. We note that a study using a similar preparation, 4 cm rat distal colon segments in which spontaneous contractions were also of myogenic origin (1 μM TTX enhanced rather than inhibited contractions) found that opioid agonists induced contractions in a dose-dependent manner that was inhibited by naloxone [18]. Similar effects have been reported for opioid agonists in isolated rat colon studies [19-21]. Our findings in the short colon segment preparation are consistent with evidence that myogenic mechanisms alone are not considered sufficient to propel lumen contents along the colon, but that such motility patterns require neuronal activity [11, 16]. However, the presence of μ opioid receptors in interstitial cells means that their

involvement cannot be ruled out [22]. It was therefore anticipated that in a longer tissue preparation, where there is a greater proportion of intact enteric nervous system, an opioid agonist may have a greater potential to mimic the inhibitory effect on motility reported *in vivo*.

By comparing two methods used to study intestinal motility we have demonstrated that both short distal colon segments and whole large intestine are able to detect opioid agonist activity that alters colonic motility. The distal colon segment preparation would be useful to screen for substances with opioid agonist activity and an intact colon useful to determine the inhibitory effects of opiates on the propagation of contractile activity along the colon. This is because the whole large intestine *in vitro* preparation reveals effects of opiates that are consistent with *in vivo* data using opioid agonists. The isolated intact whole large intestine provides an ideal preparation to identify other opioid peptides in dairy-derived substances likely to alter GIT time that may improve comfort. These may include paediatric-related problems such as colic and constipation in infants, cramps associated with diarrhea/constipation sensitivity, and constipation in adults and the elderly.

Acknowledgements

We thank Jason Peters and Lee Travis for technical assistance, Ric Broadhurst and Bobby Smith for animal breeding, Hailey Gillespie for animal care, and Catherine Lloyd-West for statistical analysis.

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Figure legends

Figure 1. Schematic showing the large intestine regions for: (a) where the short segments of distal colon preparation originate and the orientation used for recording muscle contraction, and (b) the location of the hooks attached to the whole large intestine preparation to record muscle contraction in the circular orientation at four locations to measure propagation of contractions.

Figure 2. Effect of β-casomorphin 5 (βCM-5) on spontaneous muscle contraction in the short colon preparation. (a) Examples of raw data recordings show changes in muscle tension over time for the control, after addition of βCM-5 at 0.2 μM, 2 μM, or 20 μM, and following 45-60 min in Krebs buffer (recovery), in three separate preparations. (b) Time-dependence of 20 μM β-casomorphin 5 (βCM-5) effect on spontaneous muscle contraction in a distal colon preparation. Changes in tension; g, and frequency; number of contractions per min, were averaged over the preceding 10 min for each time point and are shown as effect of a treatment divided by the pre-treatment control. Treatment with 20 μM βCM-5 was applied for 1 h. (c) Summary of β-casomorphin 5 (βCM-5) effects on contractile parameters of spontaneous muscle contraction in the distal colon segment preparation. Changes in tension and frequency were averaged over the preceding 10 min and are shown as a percent of the control. Each βCM-5 concentration was applied to only one tissue preparation for 15 min each at either: 0.2 μM; n=8, 2 μM; n=7, or 20 μM; n=9. Asterisks indicate the significance of each treatment relative to controls or between treatments as indicated (* $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$). Data show mean ± SEM.

Figure 3. Effect of naloxone (nal) on the β-casomorphin 5 (βCM-5)-induced increase in spontaneous muscle contraction in the short colon preparation. (a) A representative example from one experiment shows changes in muscle tension over time for: the control, and after addition of 20 μM βCM-5, 1 μM naloxone, or 20 μM βCM-5 and 1 μM naloxone, applied consecutively to one preparation. (b) Summary data show mean change in tension and frequency over the preceding 10 min for each condition as a percent of the control. All treatments were applied to each preparation for 15 min each for: 20 μM βCM-5, 1 μM naloxone, or 20 μM βCM-5 and 1 μM naloxone; n=7. Data show mean ± SEM. Asterisks indicate the significance of each treatment relative to controls (* $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$).

Figure 4. Effect of β-casomorphin 5 (βCM-5) on spontaneous propagating muscle contractions in the whole large intestine preparation. (a) A representative example from one experiment shows changes in muscle tension at each of the four recording locations over time for the control, after addition of 20 μM βCM-5, and following 60 min of perfusion with Krebs buffer (recovery). (b) Summary of the effects of β-casomorphin 5 (βCM-5) on propagating contractions in the whole large intestine preparation. The proportion of contractions propagated between the proximal colon and the rectum were measured during a 30 min control recording and compared with that after 15 min of exposure to 20 μM βCM-5 (n=5), 1 μM naloxone (nal) (n=3), both applied together, or following 60 min of perfusion with Krebs buffer (recovery) (n=3). Forward propagations (black bars) and those that were temporally coordinated (forward, mid, or reverse in direction) (grey bars) were calculated as a percent of the total number of contractions recorded in the proximal colon during each treatment condition. Note error bars for naloxone are not visible for synchronised contractions. Asterisks indicate the significance of each treatment relative to controls (* $p <$

0.05; ** $p < 0.01$). Data show mean ± SEM. (c) The effect of 15-30 min of exposure to 20 μM βCM-5 alone (filled bars) (n=4) or 20 μM βCM-5 and 1 μM naloxone (grey bars) (n=3) on contractile tension for contractions that propagated from the proximal colon to the rectum (forward direction) were compared with a control recording at all four locations, and data calculated as a percent of the control in each experiment. Data show mean change in tension ± SEM. Asterisks indicate the significance of each treatment relative to controls (** $p < 0.01$, *** $p < 0.001$).

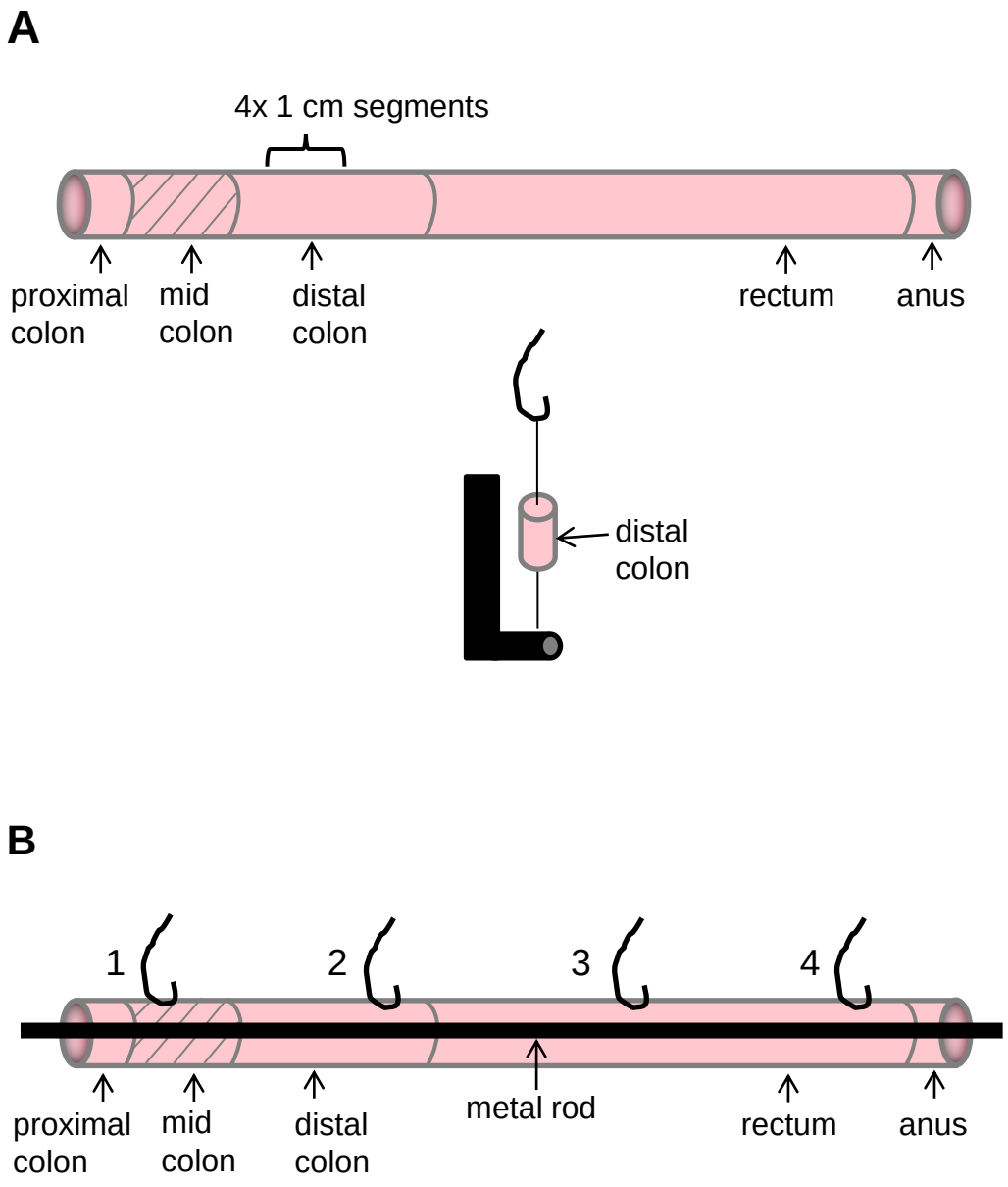


Fig. 1

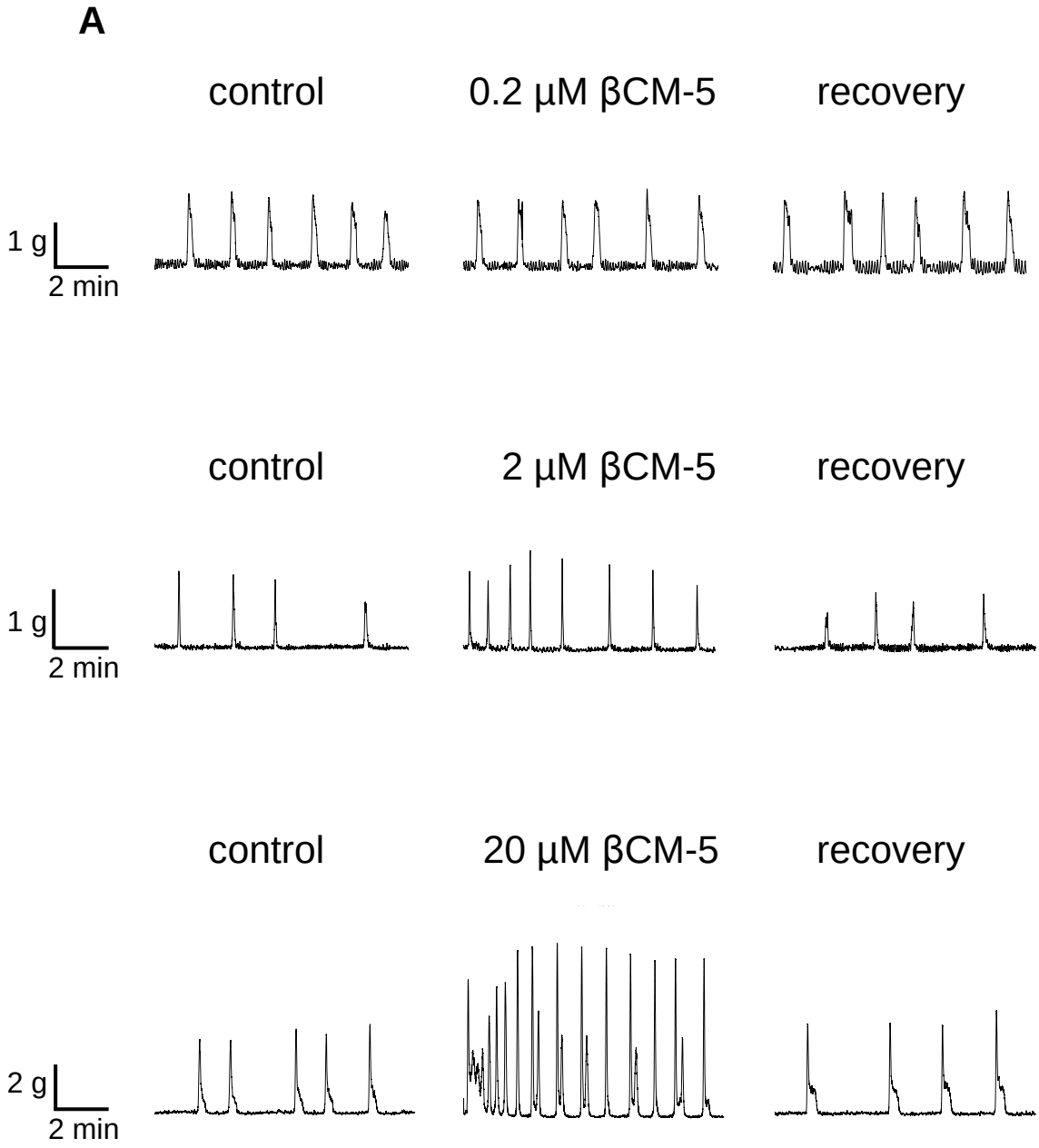


Fig. 2

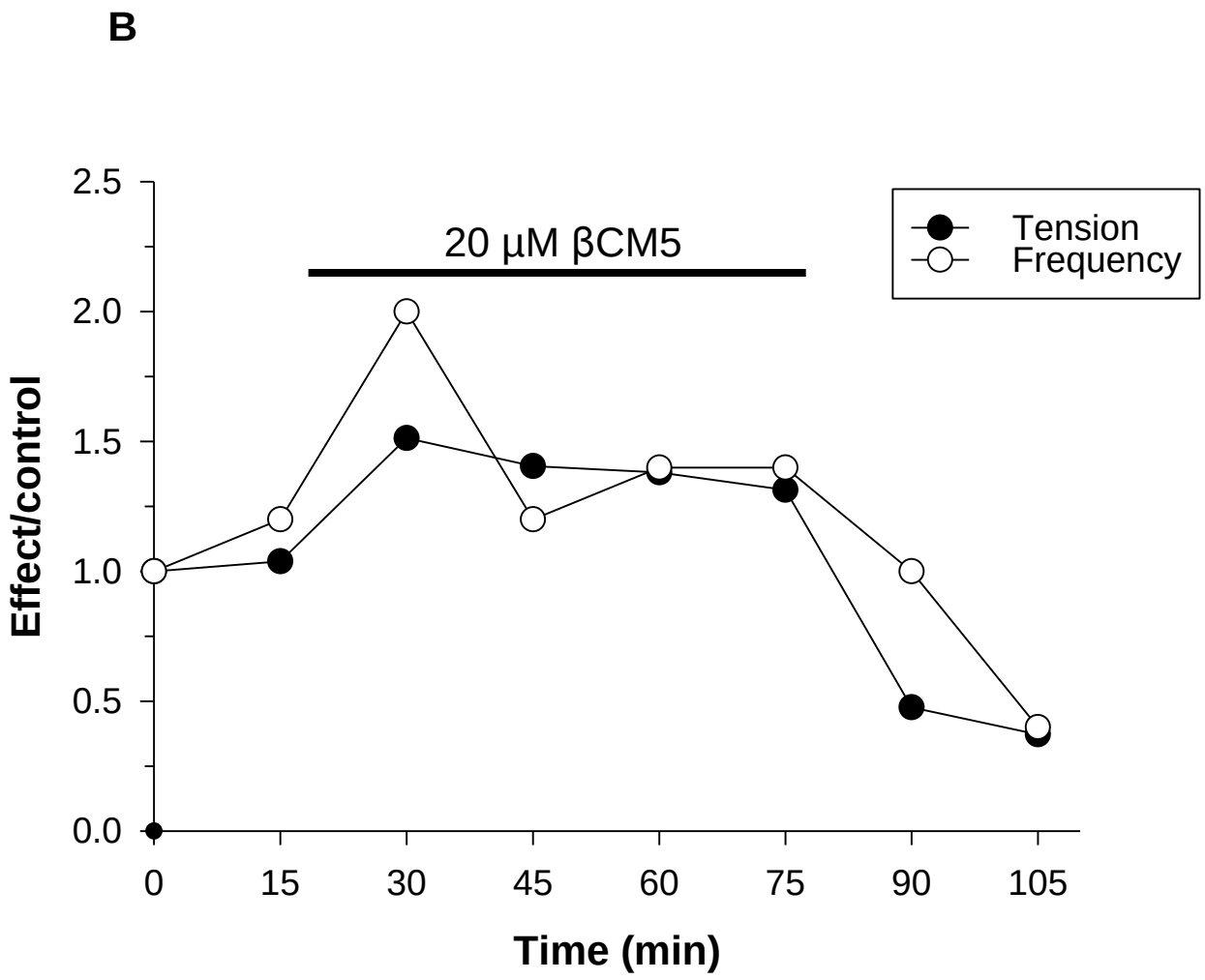


Fig. 2

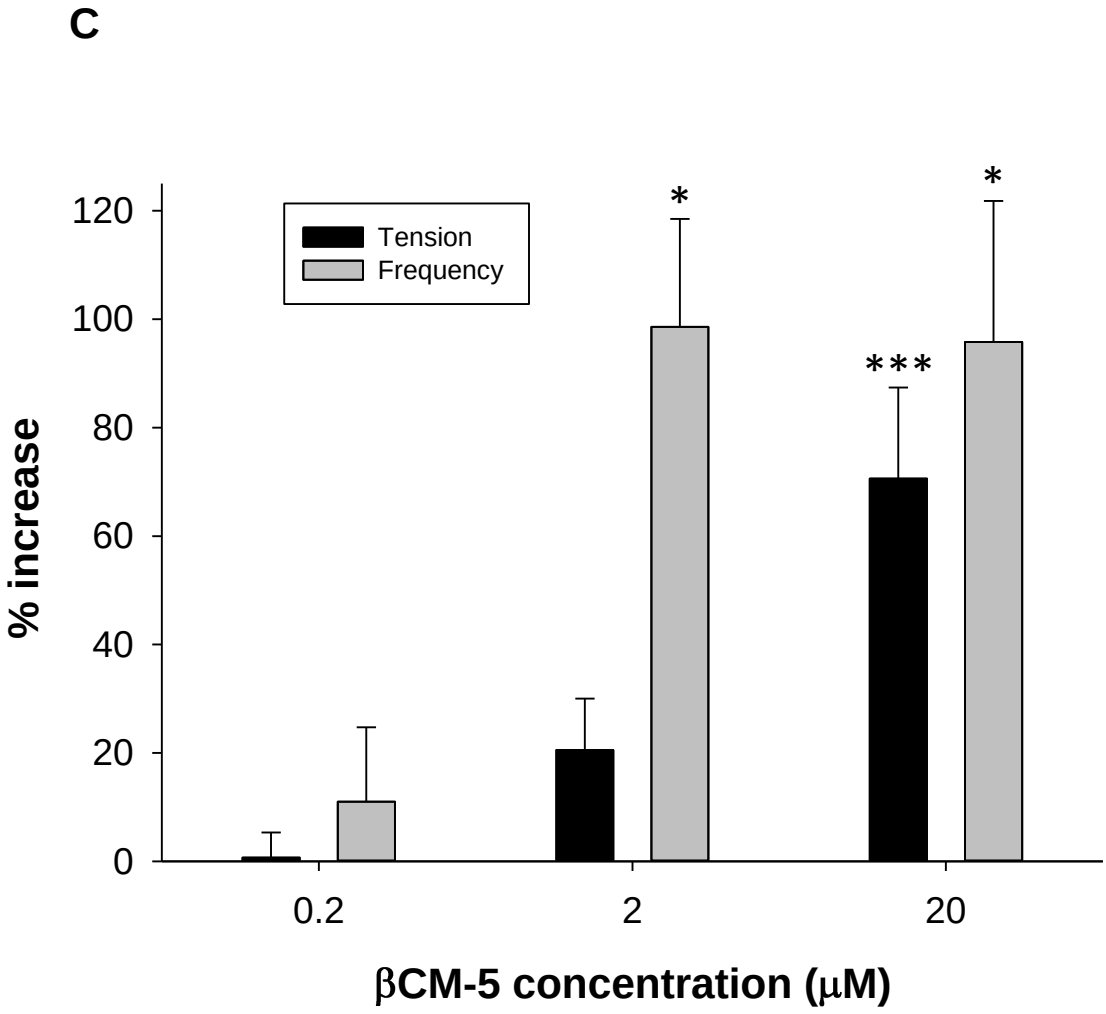


Fig.2

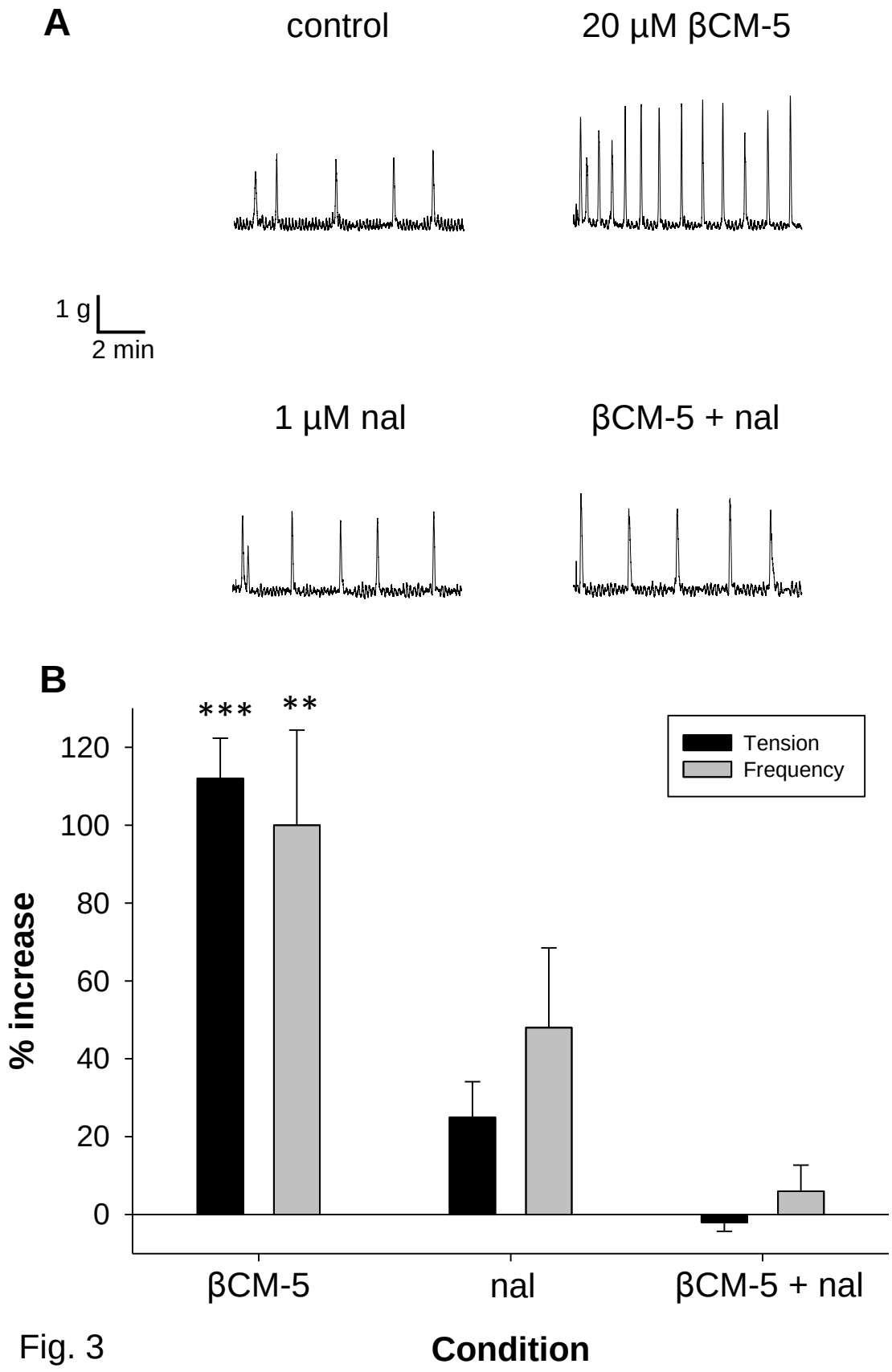


Fig. 3

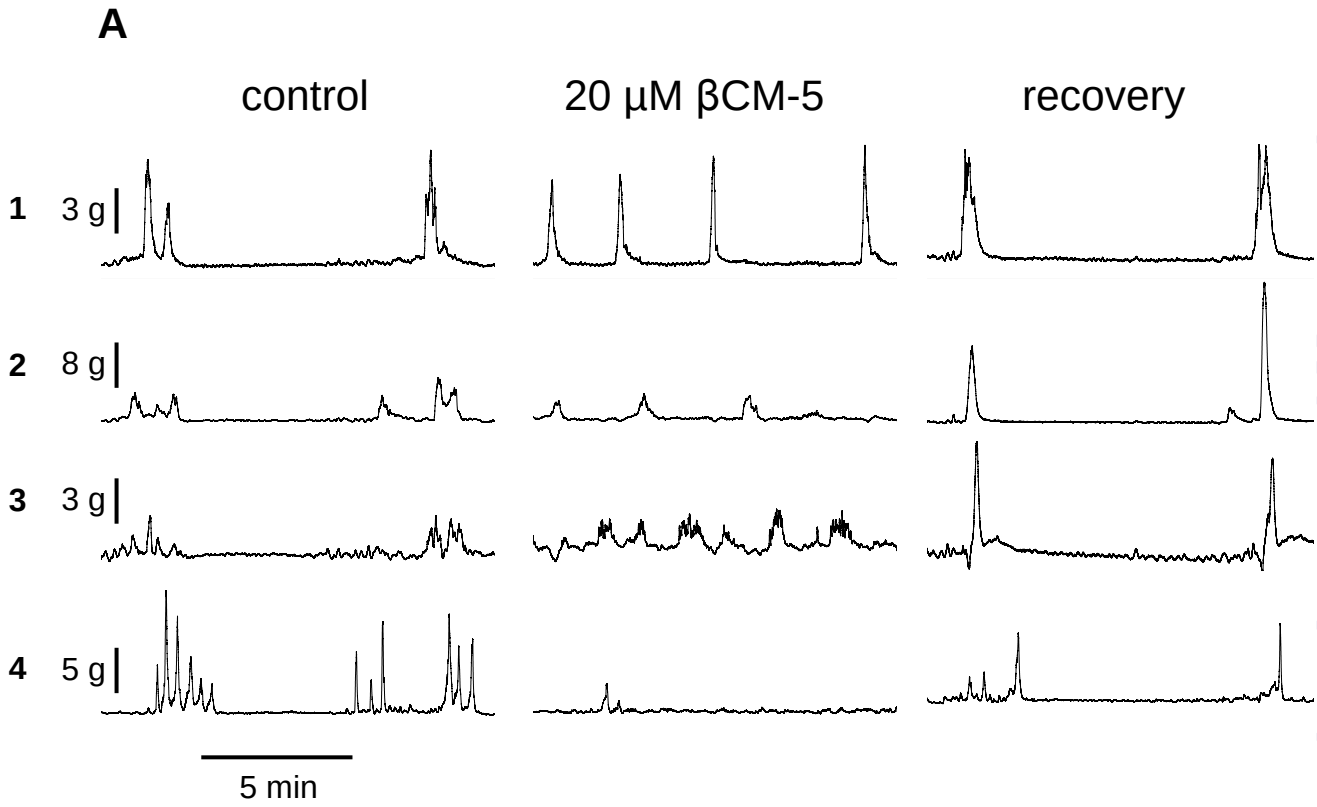
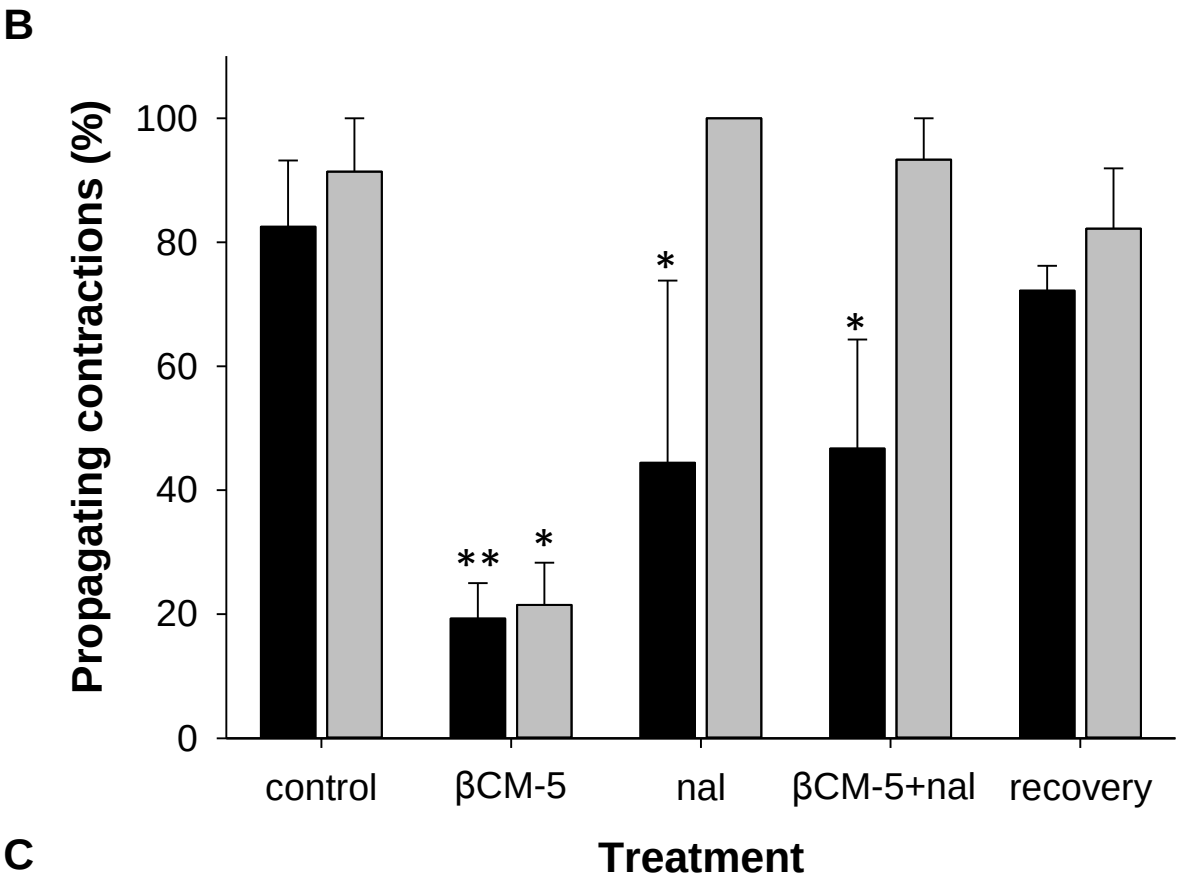


Fig. 4



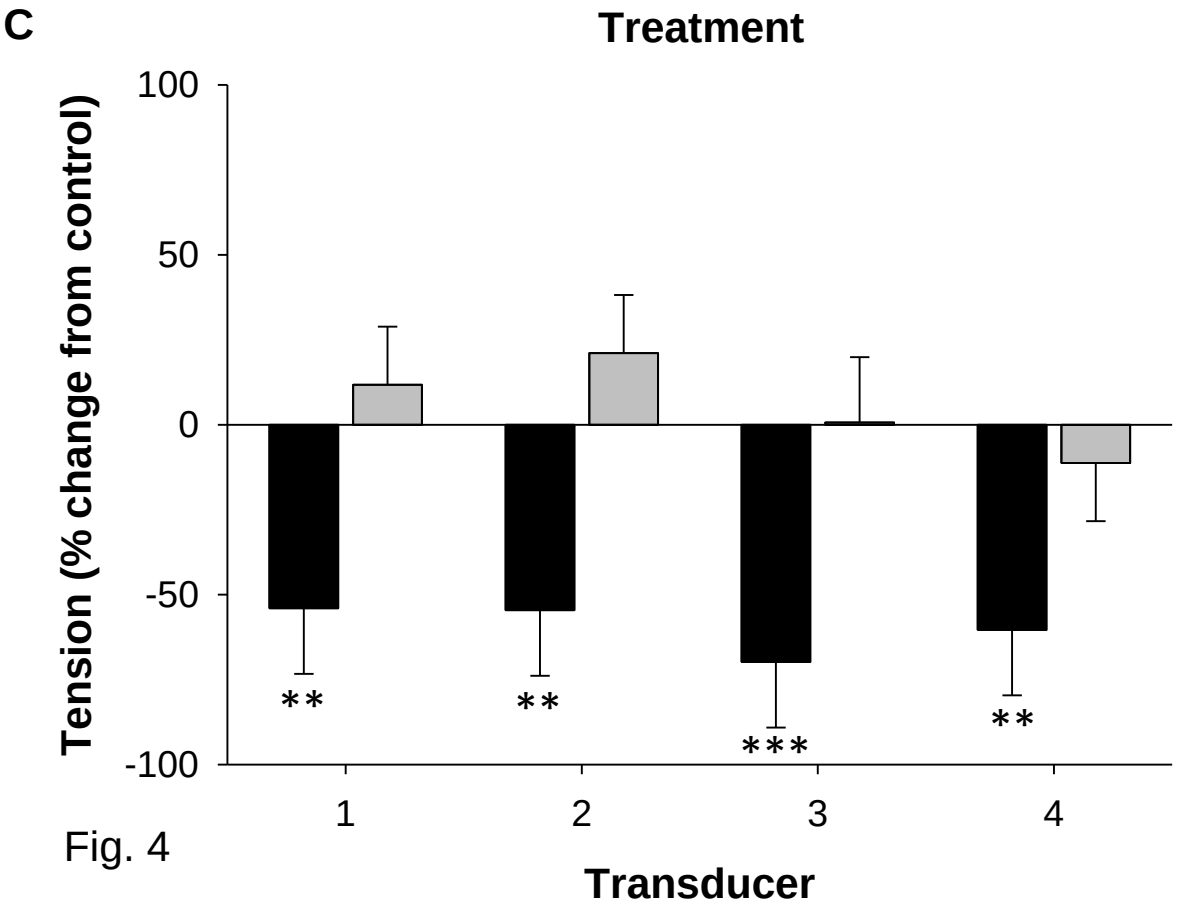


Fig. 4