Title: Copeptin, a surrogate marker of arginine[8] vasopressin, has no ability to modulate human and mouse gastric motility.

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Abstract

Copeptin, a glycosylated peptide fragment derived from the C-terminal region of the precursor of arginine^{8} vasopressin (AVP), is co-secreted with AVP in equimolar amounts. Elevated plasma AVP modulates gastric motility so we investigated whether copeptin had a similar effect.

Copeptin (10^{-9}-10^{-7}M), and AVP (10^{-12}-10^{-5}M), were evaluated for their ability to modulate spontaneous and electrically-evoked (EFS) contractions of human proximal and distal gastric circular muscle *in vitro*. Similar experiments were performed on the mouse stomach and we re-examined the published effect of copeptin on the mouse aorta.

In the presence of tetrodotoxin (10^{-6}M), atropine (10^{-6}M) and L-NAME ($3x10^{-4}$M), human proximal and distal stomach muscle contracted spontaneously and rhythmically as did mouse distal stomach.

Copeptin (10^{-9}-10^{-7}M), had no effect on baseline muscle tone or myogenic spontaneous contractions of either human or mouse stomach. However, AVP concentration-dependently increased tone, amplitude and frequency of contractions in both regions of human stomach with similar potency (pEC_{50} 9.0-9.5; n=4) and threshold concentration (10^{-11}-10^{-10}M). AVP was similarly active in the mouse stomach. EFS-evoked cholinergic contractions (human and mouse) were unaffected by both peptides EFS-evoked relaxations of mouse stomach were unaffected by copeptin.

In sub-maximally contracted mouse aorta the elevated tone was unaffected by copeptin (10^{-7}M) (cf. previously published study) but was reduced by carbachol (10^{-6}M) and sodium nitroprusside (10^{-3}M).

We conclude that in contrast to AVP, copeptin over a concentration range reported in the plasma has no direct ability to modulate the motility of the human and mouse stomach.

Key words: Copeptin, Antidiuretic hormone, Arginine^{8} vasopressin, Gastric motility Human stomach, Nausea.

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1. Introduction

Copeptin is a 39-amino acid fragment derived from the C-terminal region of the precursor of arginine^{8} vasopressin (AVP; anti-diuretic hormone, ADH), pre-pro-AVP (Holwerda, 1972). It is secreted from the posterior pituitary with AVP in equimolar amounts in response to plasma hyperosmolality, low blood volume or pressure (Christ-Crain, 2019; Dobša & Edozien, 2013; Morgenthaler et al., 2006).

In humans, >90% of the released AVP is bound to platelets (Preibisz et al., 1983) and inactivated in the liver and kidney (Baumann & Dingman, 1976; Dobša & Edozien, 2013). This renders AVP with a short half-life (4-20 min) (Christ-Crain, 2019; Treschan & Peters, 2006) *in vivo*. In comparison, copeptin is metabolically stable with a longer half-life (~90 min) (Beglinger et al., 2017) and mirrors the released concentration of AVP (Dobša & Edozien, 2013; Morgenthaler et al., 2006). Consequently, copeptin has been used as a prognostic and diagnostic biomarker of diseases and disorders associated with imbalances in AVP (Christ-Crain, 2019; Dobša & Edozien, 2013). Its use has also been extended to investigations into the heightened release of AVP in response to stressful stimuli such as strenuous exercise (Hew-Butler et al., 2011; Popovic et al., 2019), pregnancy (Evers & Wellmann, 2016) and surgery (Mastropietro et al., 2012). These studies have confirmed a positive correlation between the plasma concentration of both peptides, and a parallel 2-10 fold rise in their values above physiological levels (~1-10 pg/ml). Notably, additional stressful stimuli such as heat (Pittman & Wilkinson, 1992), numerous medications (Andrews & Hawthorn, 1988; Belton & Thomas, 1999; Chan, 1997; Edwards et al., 1989, Feldman et al., 1988; Fisher et al., 1982; Raskind et al., 1987; Shepshelovich et al., 2017), sensory irritants (Bester-Meredith et al., 2015; Wacker & Ludwig, 2019), vection (Farmer et al., 2015; Kim et al.,1997; Koch et al., 1990; Xu et al., 1993) are associated with a 2-200 fold rise in the basal plasma level

of AVP. Whilst copeptin has not been measured under the same circumstances, its concentration is likely to have been similarly increased in these studies raising the question of whether copeptin could be responsible for biological effects ascribed to the elevated AVP.

Remarkably, despite the discovery of copeptin ~50 years ago (Holwerda, 1972), the physiological function and consequence of elevated copeptin is unknown. One suggestion is that it may be involved in the structural formation of pre-pro-AVP (Morgenthaler et al., 2008). However, it seems reasonable to hypothesise that copeptin might also have activity among those functions in which AVP is known to play a role. For example, there is strong association between raised plasma AVP, the sensation of nausea and disruptions in the myoelectrical “slow wave” rhythm of the stomach (Koch, 1997; Stern et al., 2011). Further, nausea is reported by patients receiving AVP or its analogs (e.g. terlipressin) for treatment of gastric bleeding and diabetic insipidus (Stump & Hardin, 1990), and when AVP is administered experimentally in healthy volunteers (Caras et al., 1997). It is hypothesised that one way in which AVP could induce nausea is by disruption of gastric motility (Makwana et al., 2018; Stern et al., 2011). However, copeptin could be equally responsible. It is not known whether copeptin can modulate gastric motility in a similar manner but acute exposure of the mouse aorta to copeptin is reported to relax the smooth muscle (Rasor et al., 2006) raising the possibility that it may modulate gastric smooth muscle function.

We have now investigated whether copeptin could modulate human gastric motility at concentrations comparable to AVP, tested as a positive control. As a further control we also performed similar experiments on the mouse isolated stomach and in an attempt to replicate previously reported findings with copeptin we also studied the mouse aorta (Rasor et al., 2006).

2. Materials and methods

2.1. Tissues

2.1.1 Human stomach

Human stomach tissue was obtained from obese patients (1 male and 7 females, median age 43 years, mean body mass index (BMI) 47.7 $\pm$ 2.8 kg/m^2) undergoing vertical sleeve gastrectomy. Two of the 8 donors were diagnosed with Type 2 diabetes and there was no evidence of additional co-morbidities. Written, informed consent was obtained from all donors in accordance with the requirements of the local ethics committee (QMUL REC 15/LO/2127). The resected specimen was cut ~2cm from the gastro-oesophageal junction and up to ~8 cm from the pylorus and approximately two-thirds of width between the curvatures. Sections of ~5 cm^2 were cut 5 cm from both ends of the specimen, excluding the areas clearly damaged during surgery. These sections represented the fundus-proximal corpus and antrum-distal corpus, respectively, and for concision are referred to as the proximal and distal stomach hereafter.

Stomach sections were transported to the laboratory within 1 hr after resection in Krebs-Henseleit solution (x10^{-3}M: NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, D-glucose 11.1, $CaCl_2$ 2.5) pre-gassed with 95% O_2 and 5% CO_2. The mucosa, muscularis mucosa and submucosal plexus were removed by blunt dissection and discarded. A maximum of four strips (5 x 20 mm) were cut perpendicular to the greater curvature, approximately parallel to circular muscle fibres from each region. Detailed methods for collecting and minimising variation when using human gastrointestinal tissue have been previously described (Sanger et al., 2013).

Tissues were studied on the following day after overnight (15-18 h) storage in Krebs-Henseleit at 4ºC in pre-oxygenated Krebs-Henseleit solution. Previously, electrically-evoked and agonist-evoked contractions have been found not to be affected by overnight storage (Broad et al., 2011).

2.1.2 Mouse stomach and aorta

Animal care and welfare complied with the UK Animals Scientific Procedures (ASPA) Act 1986 Code of Practice and the European Directive 2010/63 provisions as transposed into the UK. Male and female virgin CD1 mice (25-30g, 7 weeks old) were purchased from Charles River Laboratories (Margate, UK) and housed in a room with a controlled temperature (22±1°C), humidity (55±10%) and 12-h light-dark cycle. Both sexes were segregated in separate cages with food (LabDiet® EURodent diet 14%, International Product Supplies Ltd, London, UK) and water provided *ad libitum* until sacrifice, and were used after a minimum of 6 days acclimatisation. The collection of cadaveric tissue did not require ASPA authority, and all humane killing by ASPA Schedule 1 methods was conducted within Queen Mary University of London, a UK Home Office licenced establishment (Ref XEDA0E7B1). Mice were killed by a Schedule 1 method that consisted of cervical dislocation followed by exsanguination to ensure death. The protocol was approved by the Animal welfare ethical review board for Queen Mary University of London and those involved were trained and signed off as competent by the Named Training and Competency Officer and Named Animal Care and Welfare Officer.

The stomach and thoracic aorta were excised and immersed in Krebs-Henseleit solution at room temperature (~20^{O}C). After removal of adherent connective tissue, ring preparations (3 mm wide) were cut from the proximal (forestomach) and distal (corpus-antrum) stomach along the axis of the circular muscle fibres. The aorta was

divided into two rings (5 mm in length). Tissues were used immediately for functional experiments.

2.1.3 Assay procedure

The test apparatus has been described previously (Broad et al., 2011). Briefly, tissues were suspended in 10 ml tissue baths between platinum wire electrodes (15 mm length, 10mm apart) and an isometric force transducer (MLT201/D, AD Instruments, Chalgrove, UK) using cotton thread for human stomach strips or L-shaped stainless steel wires for mouse stomach and aortic rings. The bath was filled with Krebs-Henseleit solution, gassed with 95% O_2 and 5% CO_2 and maintained at 37ºC. Changes in muscle tension was recorded in milliNewtons (mN) on a Dell personal computer (Dell, UK, www.dell.com/uk) using AcqKnowledge v3.8.1 recording software (BIOPAC Systems Inc., CA, USA). Both freshly dissected tissues at room temperature and those that had been stored overnight at $4^{O}C$ were initially suspended in the baths for 15 min without tension and allowed to equilibrate to the temperature of the bath. The human stomach strips and mouse tissues (stomach and aortic rings) were then stretched by 20 mN and 10mN, respectively, and allowed to equilibrate under tension for at least 60 mins until rhythmic uniform-sized spontaneous contractions and/or a stable baseline tone was recorded. Drug treatments were commenced after a minimum of 30 min of stable tone and/or contractions. During this period, the Krebs-Henseleit solution was renewed at 20 min intervals.

The action of copeptin (10^{-9} – 10^{-7} M) and AVP (10^{-12} – 10^{-5} M) was examined on the contractions of stomach tissues elicited by electrical field stimulation (EFS) and during blockade of neuronal activity by addition of tetrodotoxin (10^{-6} M), atropine (10^{-}

^{6} M) and L-NAME (3x10^{-4} M) to the bathing solution for inhibition of the voltage-dependent Na^{+} channel mediating action potentials, antagonism at muscarinic acetylcholine receptors and inhibition of nitric oxide synthase activity, respectively. EFS was applied by using a STG2008 stimulator (Multi Channel Systems, Reukingen, Germany), with a volley of pulses of 5 Hz frequency, 0.5 ms pulse width, for 10 s every 1 min at a voltage 10% greater than that required for EFS to elicit a maximal contraction. Test compounds were applied cumulatively in log-unit increments with a 15 min dosing interval, or when the effect of the previous concentration had peaked. Only one concentration-response curve was constructed per tissue. Copeptin could not be investigated at concentrations greater than 10^{-7} M because of its poor solubility. Nevertheless, this concentration would have been equivalent to a concentration 4000 fold greater than the maximal plasma concentration reported *in vivo (*Christ-Crain, 2019; Dobša & Edozien, 2013).

All experiments were performed in parallel with relevant vehicle-treated and time-matched controls. After completion of an experiment, stomach tissues were challenged with a supramaximal concentration of carbachol (10^{-3} M) in order to compare their integrity and rule out concerns about potential damage resulting from the surgery and dissection. The response to carbachol also served as a comparator to standardise the action of the test compounds on the stomach muscle from each species.

After the equilibration period, the maximal contractile capacity of each aortic ring was assessed in response to potassium chloride (KCl 8 x 10^{-2}M; Chataigneau et al., 1999). Following a wash with drug-free Krebs-Henseleit solution and a 15 min period recovery to the initial basal muscle tension, aortic rings were stimulated with either noradrenaline (10^{-6}M) or KCl (5 x 10^{-2}M) in order to elicit a stable elevated muscle tone representing ~65-70% of the reference contraction to KCl (8 x 10^{-2}M,). The

ability of copeptin (10^{-7}M) to relax the contracted aortic rings was then investigated, followed by an application of carbachol (10^{-3}M) to elicit an endogenous nitric oxide-mediated relaxation as a means to verify the integrity of the endothelium. At the end of the experiment, sodium nitroprusside (SNP, 10^{-3}M), a nitric oxide donor, was applied to the aortic rings to elicit maximal relaxation of the muscle.

2.2, Data analysis and Statistics

For experiments in which tissues were stimulated electrically, the effect of an agonist was quantified as a percentage change in amplitude of EFS-evoked contractions determined immediately before its first addition. Spontaneous contractions in the absence and presence of an agonist were quantified by measuring the change in basal muscle tone (mN), amplitude of spontaneous contractions (mN) and frequency of contractions per min (c/min) during a steady-state period. The increase in baseline muscle tension (tone) of the gastric muscle in response to the test molecule was also expressed as a percentage of the amplitude of contraction evoked by carbachol. Contractions of the aortic rings in response to noradrenaline (10^{-6}M) and KCl ($5x10^{-2}$M), were expressed as a percentage of the maximal contraction in response to KCl ($8x10^{-2}$M). Relaxation of the noradrenaline and KCl contracted aortic rings were expressed as a percentage of the maximal relaxation in response to SNP (10^{-3}M).

All agonist concentration-response data were quantified as mean ± standard error of the mean (mean ± S.E.M). Individual agonist concentration-response curves were fitted by non-linear regression to a four-parameter logistic Hill equation using GraphPad PRISM 8.0 for Windows (Graph-Pad Software, La Jolla, CA, USA) as described previously (Makwana et al., 2010).

Where appropriate, a paired or unpaired Student's *t*-test was performed for comparisons of individual means of data from within a given type of tissue or

between tissues from different donors, respectively, followed by a Dunnett's *post hoc* test for multiple comparisons. The probability $P < 0.05$ was taken to be statistically significant. *n* values represent the number of patients or animals. Only one muscle strip or ring was used per drug treatment from a given patient or animal. Drug and molecular target nomenclature conform to the IUPHAR / British Journal of Pharmacology Guide to Receptors and Channels (Alexander et al., 2019).

2.3. Materials

Atropine sulphate, Ascorbic acid, Carbachol (carbamylcholine chloride), Copeptin trifluoroacetate (human), L-NAME (Nω-nitro-L-arginine methyl ester hydrochloride, (-) Noradrenaline, SNP (sodium nitroprusside), Tetrodotoxin and reagents for making the Krebs-Henseleit solution were purchased from Sigma-Aldrich, Poole, UK. AVP (Arginine^{8} vasopressin) was purchased from Tocris Biosciences, Abingdon, UK. Ascorbic acid, Carbachol, Copeptin, L-NAME, AVP and tetrodotoxin were dissolved in distilled water. Noradrenaline was dissolved in ascorbic acid (10^{-3}M). The total volume of the solvents added to the tissue baths did not exceed 1% of bath volume.

3. Results

3.1 Electrical field stimulation (EFS)-evoked responses of human and mouse stomach

EFS elicited a short-lived monophasic contraction of human proximal and distal stomach strips during the period of stimulation, with a similar amplitude of 17.6 ± 1.48 mN and 18.7 ± 1.6 mN (n = 8), respectively. These represented 22.1 ± 3.1 % and 19.2 ± 4.2 % of the amplitude of the contraction elicited by carbachol (10^{-3} M, n = 8), respectively. The EFS-evoked contractions of both stomach regions were unaffected by copeptin or AVP up to the highest concentrations tested i.e. 10^{-7} M and 10^{-5} M, respectively (Fig. 1), but abolished by either TTX (10^{-6} M; proximal and distal

stomach: 98.7 ± 2.4 % and 99.4 ± 1.4 %, n = 4, respectively) or atropine (10^{-6} M; proximal and distal stomach: 96.5 ± 3.4 % and 98.54 ± 2.4 % n = 4, respectively).

In mouse proximal and distal stomach, EFS elicited a relaxation of 3.5 ± 0.3 mN and 1.8 ± 0.4 mN (n = 8) respectively during the period of stimulation, followed by a "rebound" contraction of 4.8 ± 0.4 mN and 5.9 ± 0.4 mN (n = 8), respectively, on termination of the stimulation. Carbachol (10^{-3}M) produced a comparable maximal contraction of 42.6 ± 2.8 mN and 40.4 ± 2.9 mN (n = 8 for both) of the mouse proximal and distal stomach, respectively. Thus, the rebound contractions were 9.5 ± 1.48 % and 16.9 ± 2.3 % (n = 8 for both) of the amplitude of the contraction elicited by carbachol (10^{-3} M,), respectively. The EFS-evoked relaxations and rebound contractions of both stomach regions were unaffected by copeptin (Fig. 2) but were both abolished by TTX (10^{-6} M; (proximal and distal stomach, n=2): 94.3 and 100 % and 98.4 and 100% respectively). The relaxations were also abolished by L-NAME ($3x10^{-4}$M; proximal and distal stomach: n = 2) whereas the rebound contractions were blocked by atropine (10^{-6} M; proximal and distal stomach: n = 2).

3.2 Myogenic responses of human and mouse stomach

Strips of both human proximal and distal stomach displayed spontaneous rhythmic contractions at a frequency of 2.3 ± 0.1 and 2.4 ± 0.1 per min; (n = 8), respectively. The contractions of the former were ~5 times smaller than those of the latter (0.9 ± 0.2 mN *vs* 4.6 ± 0.4 mN; n = 8). The maximal contraction of both types of muscle strips in response to carbachol (10^{-3}M) was comparable (respectively, 82.6 ± 4.2 mN and 87.8 ± 6.0 mN; n = 8). Copeptin (Fig. 3A and B) in concentrations as high as 10^{-7} M had no effect on the amplitude and frequency of the spontaneous contractions nor affected the baseline tension of muscle strips from either human stomach region. A longer (1 h) exposure to the highest concentration (10^{-7} M) did not induce any activity. In contrast, AVP (10^{-12} – 10^{-5} M) produced a concentration–related increase

in the amplitude and frequency of the spontaneous contractions and a rise in the baseline tension of the muscle strips from both human stomach regions (Fig. 3C and D). The potency of AVP (pEC_{50} 9.0 - 9.5) and the lowest effective concentration tested for each effect (between 10^{-11} and 10^{-10} M) was similar in both stomach regions (Fig. 3 and 4).

In mouse stomach, spontaneous contractions were produced by the muscle from the distal region only (Fig. 5D and D). These contractions had an amplitude of 3.1 ± 0.2 mN and occurred at a frequency of 1.2 ± 0.2 min^{-1} (n = 6 for both). The amplitude of these contractions equated to 8.0 ± 7.6 % (n = 6) of the carbachol-induced contraction. Copeptin had no effect on baseline muscle tone or the spontaneous contractions of either region of the mouse stomach (Fig. 5 A, B and 6). In contrast, AVP raised the muscle tone (pEC_{50} 7.3 ± 0.2; threshold concentration ~10^{-9}M, n=3) of the proximal stomach muscle only (Fig. 5C and 6), with the maximal rise representing 38.7 ± 4.8 % (n=3) of the carbachol-induced contraction. AVP (Fig. 5D and 6) also increased the amplitude of the spontaneous contractions of the distal stomach (pEC_{50} 6.0 ± 0.2; threshold concentration >10^{-6}M, n=3) and their frequency (pEC_{50} 7.3 ± 0.2, threshold concentration >10^{-9}M, n=3). Notably, the application of AVP (10^{-7}M and 10^{-6}M) was associated with a short-lived initial reduction in spontaneous contraction amplitude and arrhythmic contractions of varying amplitude at concentrations ≥10^{-6}M (Fig. 5D).

3.3 Mouse aorta

In mouse aorta, KCl ($8x10^{-2}$M) elicited a maximal contraction of 7.6 ± 0.4 mN (n = 6). Following a washout, noradrenaline (10^{-6}M) and KCl ($5x10^{-2}$M) generated a contraction of 4.9 ± 0.4 mN and 5.5 ± 0.3 mN, respectively (Fig. 7), which equated to ~65-70 % of the reference contraction elicited by KCl ($8x10^{-2}$M). The elevated submaximal tone was unaffected by copeptin (10^{-7}M) but reduced by carbachol (10^{-}

^{3}M) and sodium nitroprusside (10^{-3}M). Carbachol appeared more efficacious at inhibiting the noradrenaline-induced contraction than that elicited by KCl. Sodium nitroprusside caused complete relaxation of both noradrenaline and KCl-induced contractions (Fig. 7 and 8).

4. Discussion

The main finding of this study was that copeptin had no effect on neuronally-mediated contractions, basal tone or the spontaneous contractions of both the proximal and distal human isolated stomach. The lack of effect of copeptin occurred at concentrations greater than those measured as a free fraction *in vivo* in response to a range of stressful stimuli (Hew-Butler et al., 2011; Popovich et al., 2019; Evans & Wellmann, 2016; Mastropietro et al., 2012). Such high concentrations are unlikely to be released even during disorders such as the syndrome of inappropriate antidiuretic hormone (SIADH) that is characterised by an excessive and insupressible release of AVP and copeptin (Moritz,2019). As a positive control AVP was shown to have a positive ionotropic and chronotropic effect on the spontaneous contractions of both proximal and distal stomach, together with a spasmogenic action at concentrations comparable to those circulating in subjects reporting nausea and with gastric myoelectrical activity disturbances (Koch, 1997; Stern et al., 2011). We (Makwana et al., 2018) recently suggested that each action of AVP in human stomach was most likely mediated by activation of the V_{1A} receptor. By contrast AVP did not modulate the EFS-evoked, cholinergically-mediated contractions of human stomach muscle. Notably, in similar experiments with the stomach of different animal species AVP has been observed to stimulate or modulate cholinergic activity e.g. rat (Qin et al., 2009) and cat (Mirčić et al., 1998); suggesting a markedly different mechanism by which AVP can influence movements of the human stomach.

Several epidemiological studies in humans have associated elevated circulating levels of copeptin with obesity, including increased risk of type 2 diabetes mellitus and insulin resistance (Wannamethee et al., 2015; Then et al., 2015; Enhorning et al., 2010; Asferg et al., 2014), but no mechanistic link has been established. Obesity and diabetes are also associated with altered gastric motility and emptying (Marathe et al., 2016; Wisén & Hellström, 1995; Wright et al., 1983; Xing & Chen, 2004). This raises the possibility that the failure to observe a response to copeptin in stomach preparations from obese people was because any mechanism of activity may have become desensitised or downregulated during chronic exposure to endogenous copeptin *in situ*. In our study, although 2 out of the 8 donors had been diagnosed with type 2 diabetes, there was no apparent difference in the spontaneous and EFS–evoked contractions or responsiveness of the muscle to AVP between diabetic and non-diabetic stomachs. Thus, the incidence of diabetes was unlikely to be a confounding factor in the lack of effect of copeptin on the stomach. Because we did not have access to stomach tissue from non-obese people, comparative experiments were performed on the stomach from mice of normal weight. Whilst the results cannot exclude any confounding issues related to species differences, the finding that copeptin was also without effect on both neuronal and myogenic contractions and basal tone of mouse stomach provided a basis for suggesting that the inability of copeptin to exert activity in human stomach was not due to the use of tissue from obese people. As with human stomach, AVP also facilitated spontaneous contractile activity of the mouse stomach (without affecting EFS-evoked contractions) albeit less potently and exhibiting region- and concentration- dependent actions.

In light of the absence of an effect of copeptin on the gastric muscle of human and mouse, we examined the action of copeptin on the mouse aorta as a potential positive control (Rasor et al., 2006). In this tissue, the peptide (10^{-7}M) has previously

been reported to cause a partial (~45%) endothelium-dependent and nitric oxide-mediated relaxation (Rasor et al., 2006). However, we found that the peptide did not elicit a relaxation of both agonist- and receptor-dependent (noradrenaline) and independent (KCl) mechanisms in endothelium intact aortic rings.

In conclusion, the present study was unable to demonstrate an ability of copeptin to influence the motility in proximal and distal regions of the human or mouse isolated stomach at concentrations comparable to AVP and greater than copeptin concentrations measured in the plasma. An excitatory activity of AVP acted as a positive control. Further, we were unable to confirm an ability of copeptin to relax mouse aorta muscle. To date, no other studies are reported in which the functions of acutely-applied copeptin have been examined. A preliminary recent study with copeptin (10^{-7}M) at concentrations substantially greater than those recorded in human plasma, suggested an ability to increase mRNA expression of the early transcriptional activation marker c-FOS in three different cell types (HEK293, KATOIII, and ARPE19), indicating the potential for a biological action (Haddock et al., 2017). Further studies are needed to investigate the possibility that copeptin may be more than just a surrogate marker for AVP, responsible only for ensuring the correct structural formation of pre-pro-AVP (Morgenthaler et al., 2008). However, the results of the present study would appear to preclude an involvement in gastric motility modulation.

5. Conclusion

Copeptin has no direct ability to modulate motility of the human stomach.

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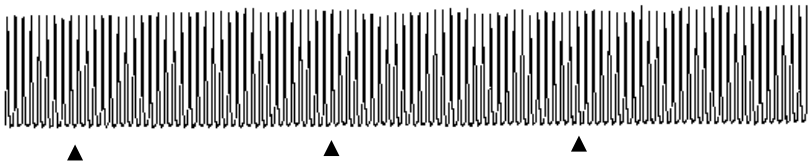
a) Proximal

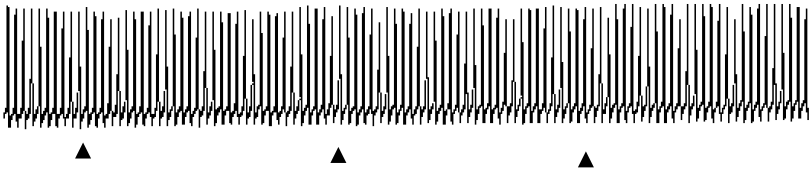


Copeptin (M): 10^{-9}M 10^{-8}M 10^{-7}M

20mN

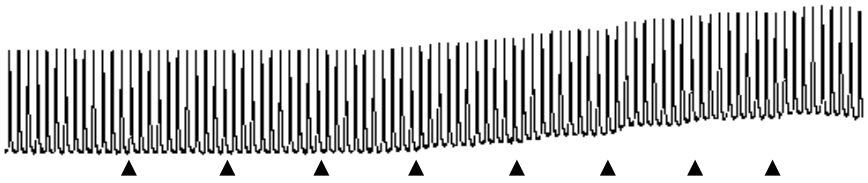
15 min

b) Distal



Copeptin (M): 10^{-9}M 10^{-8}M 10^{-7}M

c) Proximal

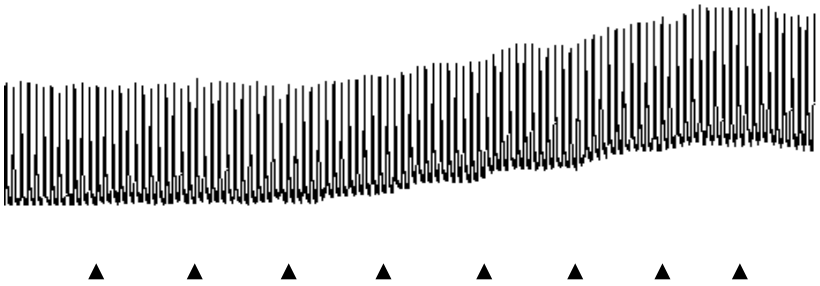


AVP (M): 10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5}

20mN

15 min

d) Distal



AVP (M): 10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5}

Figure 1: Electrically-evoked contractions of the circular muscle of the human isolated proximal (a,c) and distal (b,d) stomach in the absence and presence of copeptin (a,b) or AVP (c,d). Stimulation parameters: 5Hz frequency for 10s every 1 min, 0.5 ms duration and 110% supramaximal voltage.

a) Proximal

Copeptin (M): 10^{-9}M 10^{-8}M 10^{-7}M

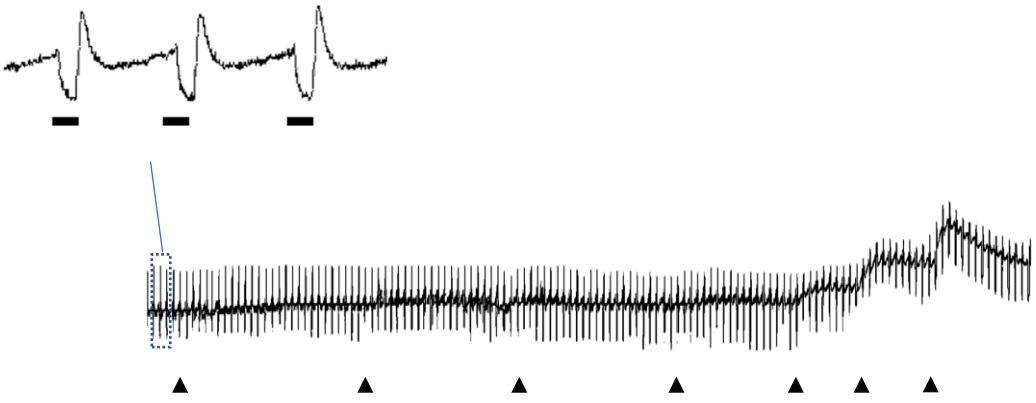
10 mN

15 min

b) Distal

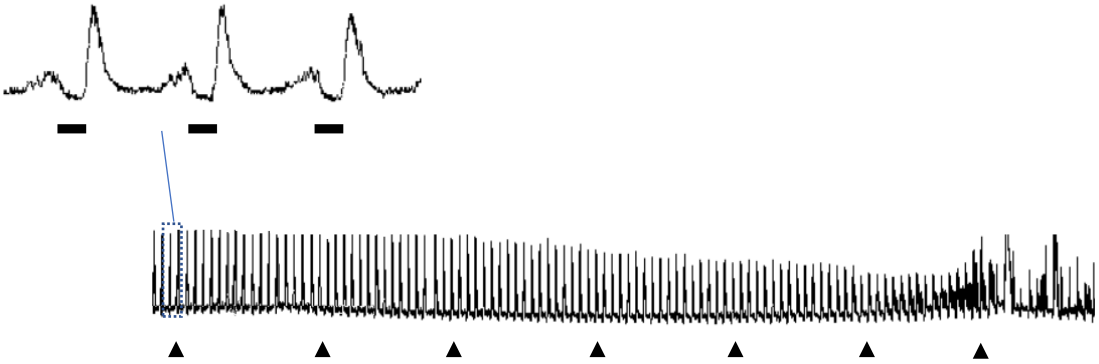
Copeptin (M): 10^{-9}M 10^{-8}M 10^{-7}M

c) Proximal



AVP (M): 10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6}

d) Distal



AVP (M): 10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6}

Figure 2: Electrically-evoked contractions (and relaxations) of the circular muscle of the mouse isolated proximal (a,c) and distal (b,d) stomach in the absence and presence of copeptin (a,b) or AVP (c,d). Stimulation parameters: 5Hz frequency for 10s every 1 min, 0.5 ms duration and 110% supramaximal voltage. In c and d the black bars under the snapshots of each EFS response indicates the period of stimulation.

a) Proximal

Copeptin (M): 10^{-9}M **10^{-8}M** **10^{-7}M**

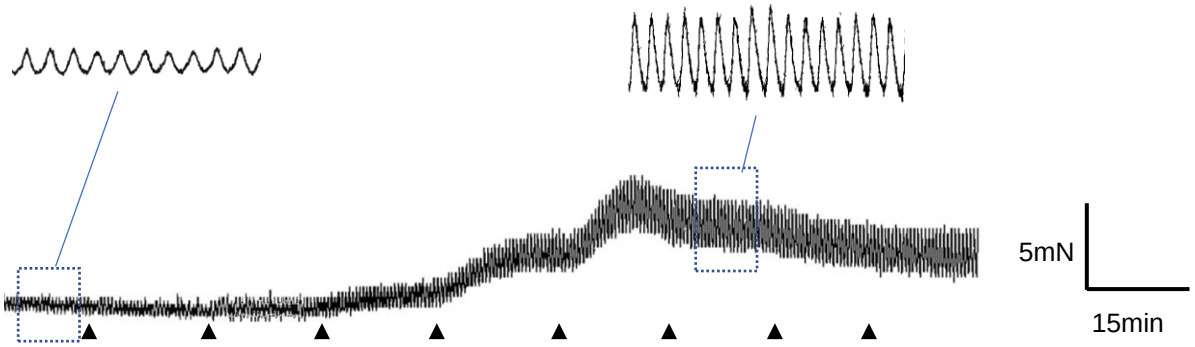
5 mN

5 min

b) Distal

Copeptin (M): 10^{-9}M 10^{-8}M 10^{-7}M

c) Proximal

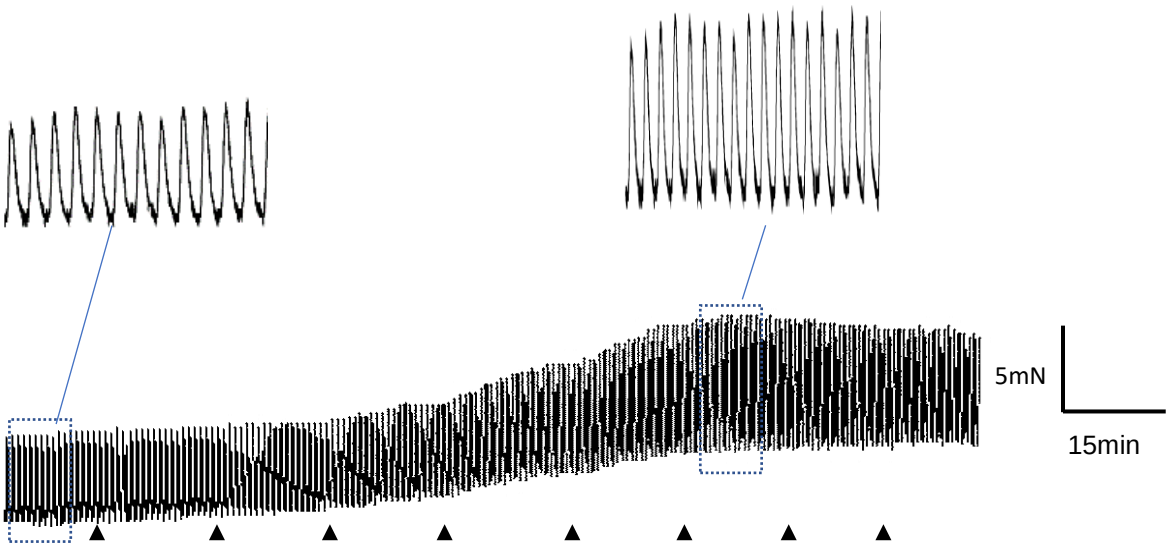


5mN

15min

AVP (M): 10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5}

d) Distal



5mN

15min

AVP (M): 10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5}

Figure 3: Recordings of the spontaneous contractions of the circular muscle of the (a, c) proximal and (b,d) distal region of the human stomach in the absence and presence of copeptin (a and b) and arginine^{8} vasopressin (AVP, c and d). The muscle strips were pre-treated with a combination of atropine (10^{-6}M), TTX (10^{-6}M) and L-NAME (3 x10^{-4}M), which themselves do not affect the rhythmic contractions.

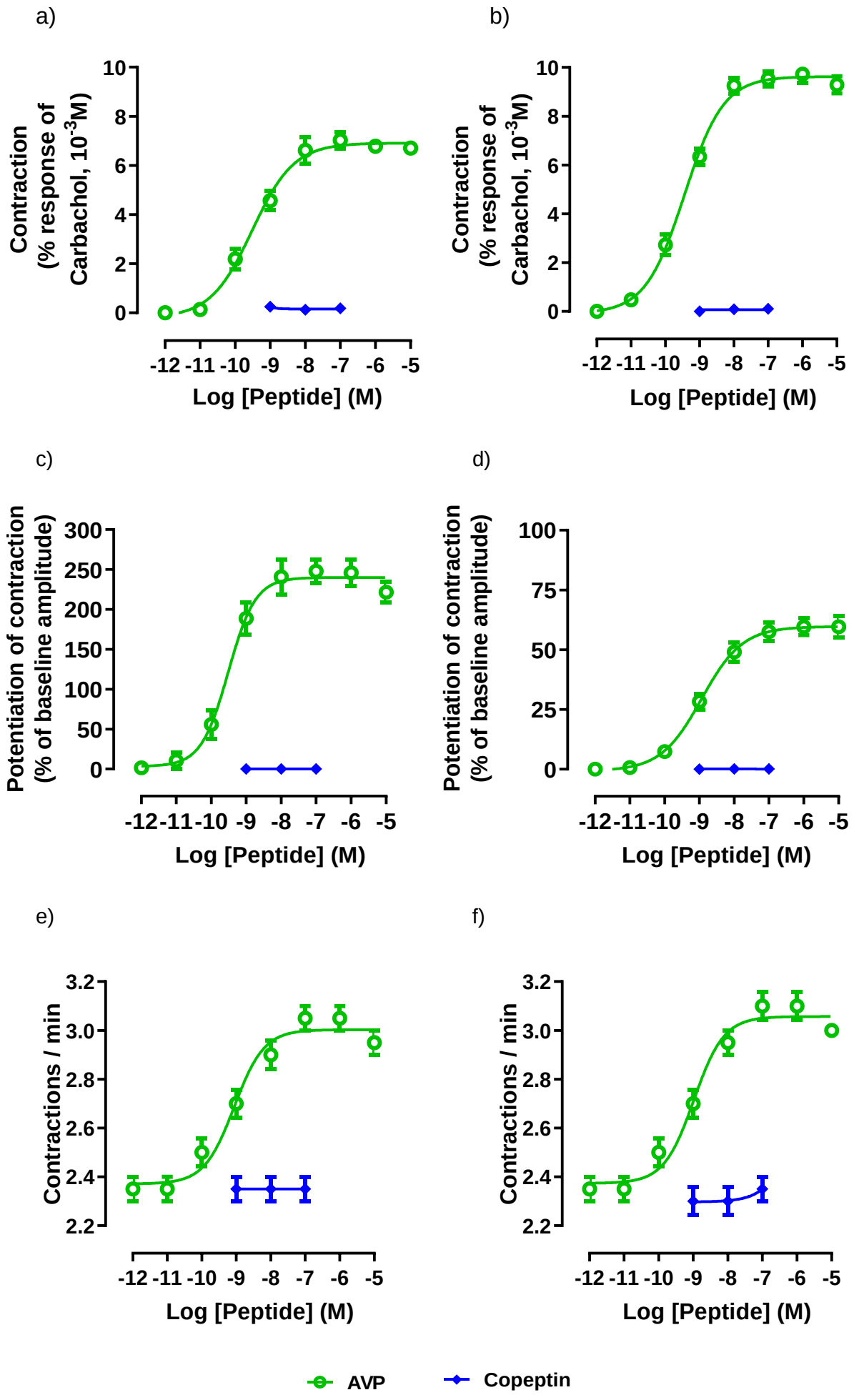

a)
b)
c)
d)
e)
f)
Contraction (% response of Carbachol, 10-3M)
Potentiation of contraction (% of baseline amplitude)
Contractions / min
Log [Peptide] (M)
-12 -11 -10 -9 -8 -7 -6 -5
AVP
Copeptin

Figure 4: Concentration response curves for the changes in baseline muscle tension (a.b), and the amplitude (c,d) and frequency (e,f) of the spontaneous contractions of the circular muscle of the proximal (graphs on the left) and distal (graphs on the right) region of the human stomach by vasopressin and copeptin. Each curve was fitted by non-linear regression analysis. Each symbol represents the mean ± s.e.m of n = 4. The change in baseline muscle tension was expressed as a percentage of the amplitude of contraction in response to carbachol (10^{-3}M) whereas the potentiation of the amplitude of contraction was expressed as a percentage increase in amplitude of the contractions before the first addition of the peptide. The change in number of contractions by a peptide is expressed relative to basal values.

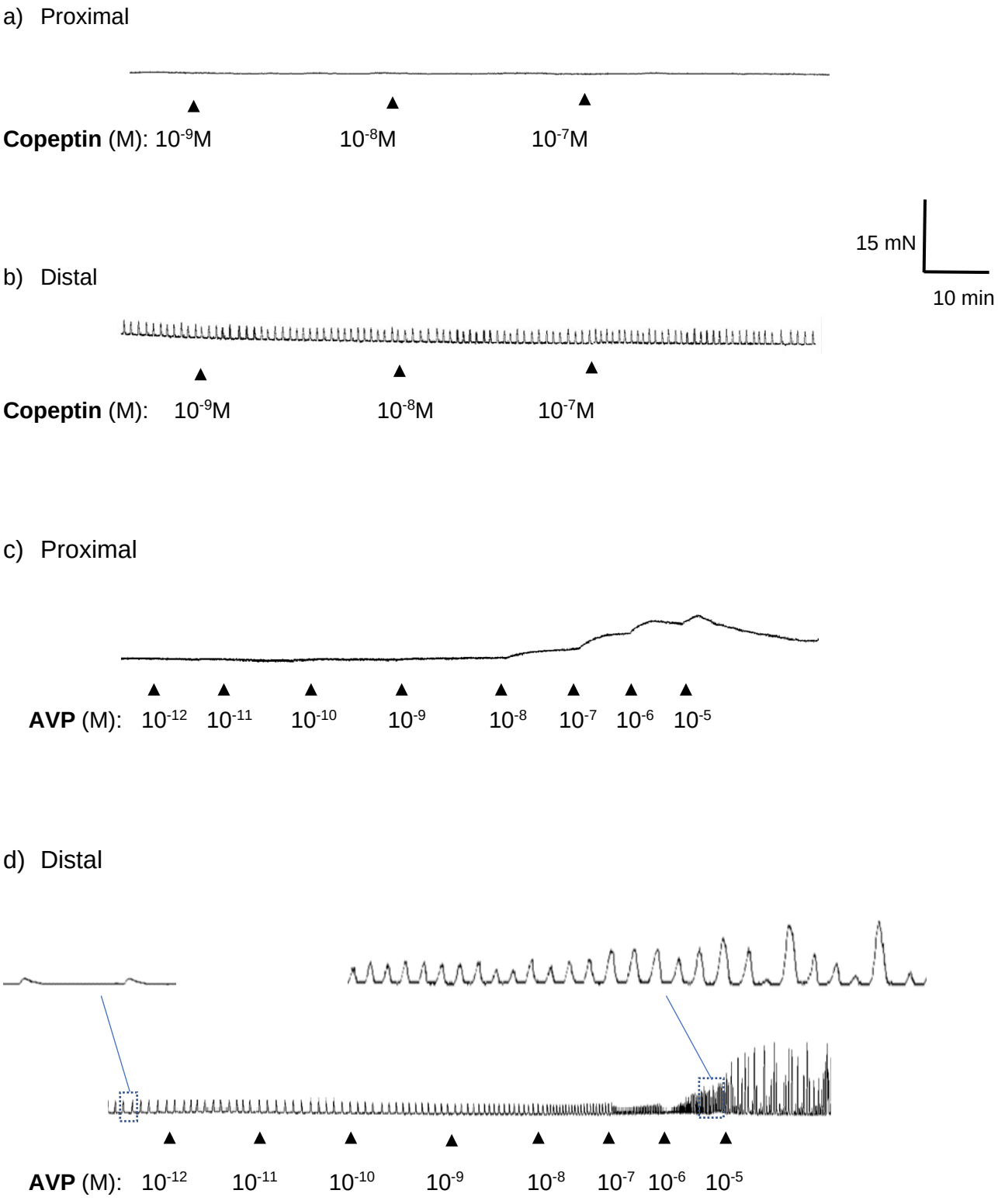


Figure 5: Recordings of the basal activity of the circular muscle of the (a, c) proximal and (b,d) distal region of the mouse stomach in the absence and presence of copeptin (a and b) and arginine^{8} vasopressin (AVP, c and d). The muscle strips were pre-treated with a combination of atropine (10^{-6}M), TTX (10^{-6}M) and L-NAME (3 x10^{-4}M), which themselves do not affect the rhythmic contractions.

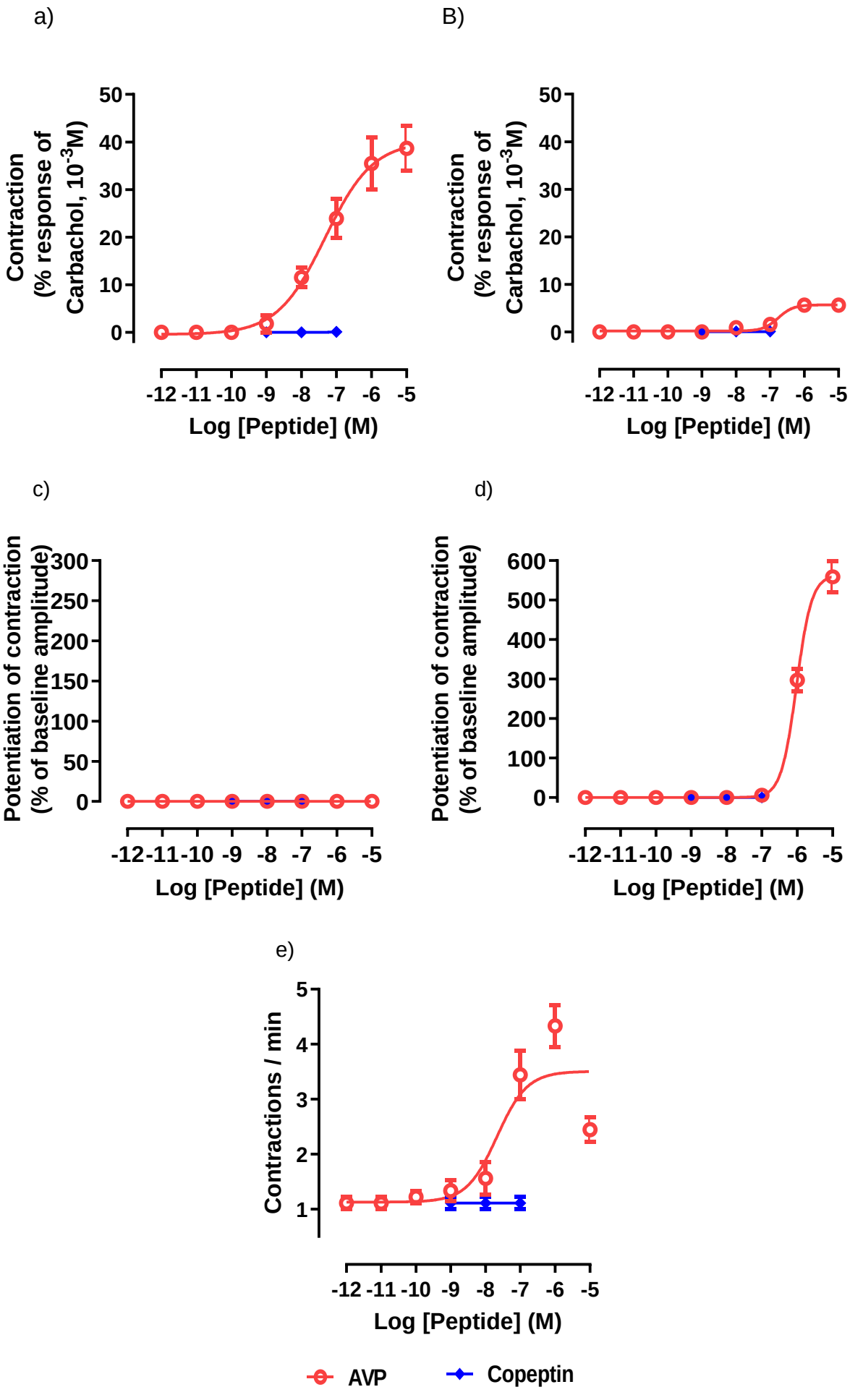
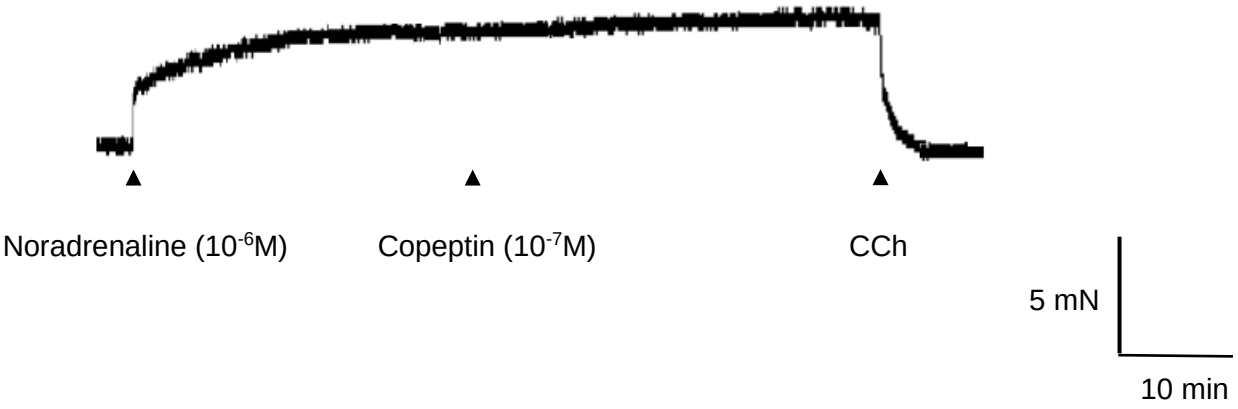

a)
B)
c)
d)
e)
Contraction (% response of Carbachol, 10-3M)
Log [Peptide] (M)
Potentiation of contraction (% of baseline amplitude)
Contractions / min
AVP
Copeptin

Figure 6: Concentration response curves for the changes in baseline muscle tension (a.b), and the amplitude (c,d) and frequency (e) of the spontaneous contractions of the circular muscle of the proximal (a,c) and distal (b,d,f) region of the mouse stomach by vasopressin and copeptin. Each curve was fitted by non-linear regression analysis. Each symbol represents the mean ± s.e.m of n = 3 mice. The change in baseline muscle tension was expressed as a percentage of the amplitude of contraction in response to carbachol (10^{-3}M) whereas the potentiation of the amplitude of contraction was expressed as a percentage increase in amplitude of the contractions before the first addition of the peptide. The change in number of contractions by a peptide is expressed relative to basal values.

a)



b)

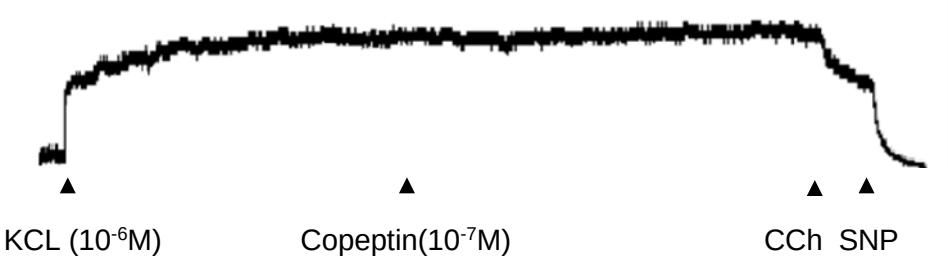


Figure 7: Contraction of mouse aortic rings by a submaximal (~65-70 %) concentration of a) noradrenaline (10^{-6}M) and b) potassium chloride (KCl, $5x10^{-2}$M) and the lack of relaxant effect of copeptin (10^{-7}M), but relaxation by carbachol (CCh, 10^{-3}M) and SNP (10^{-3}M).

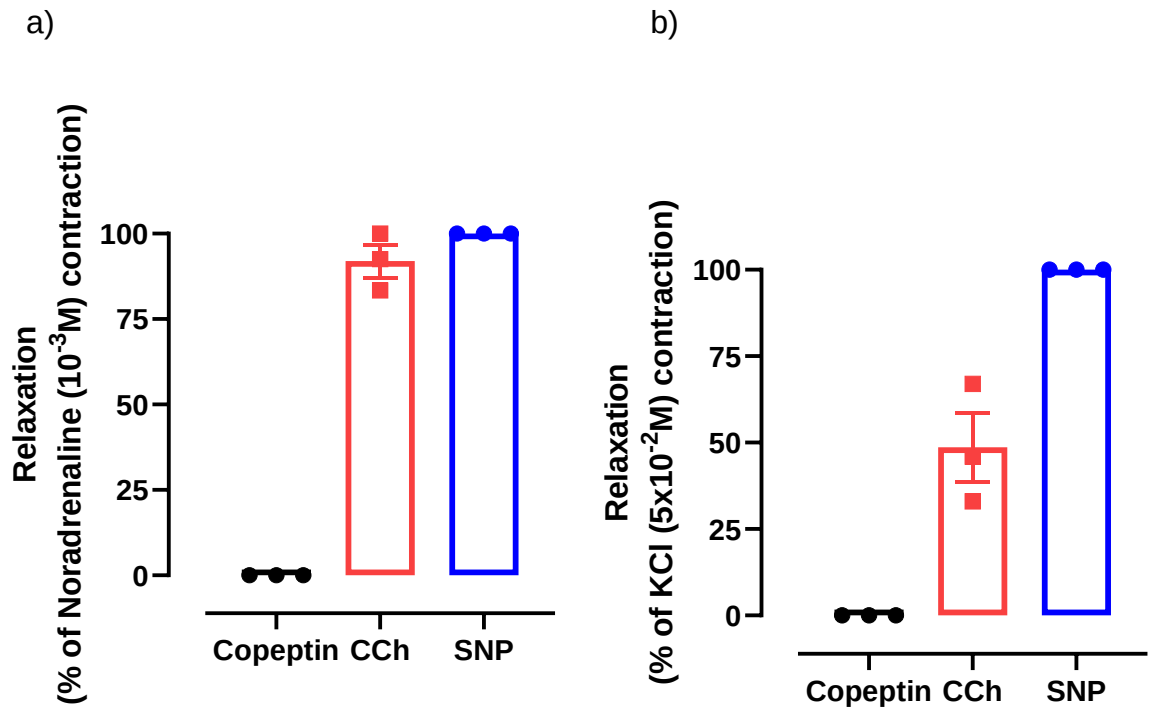
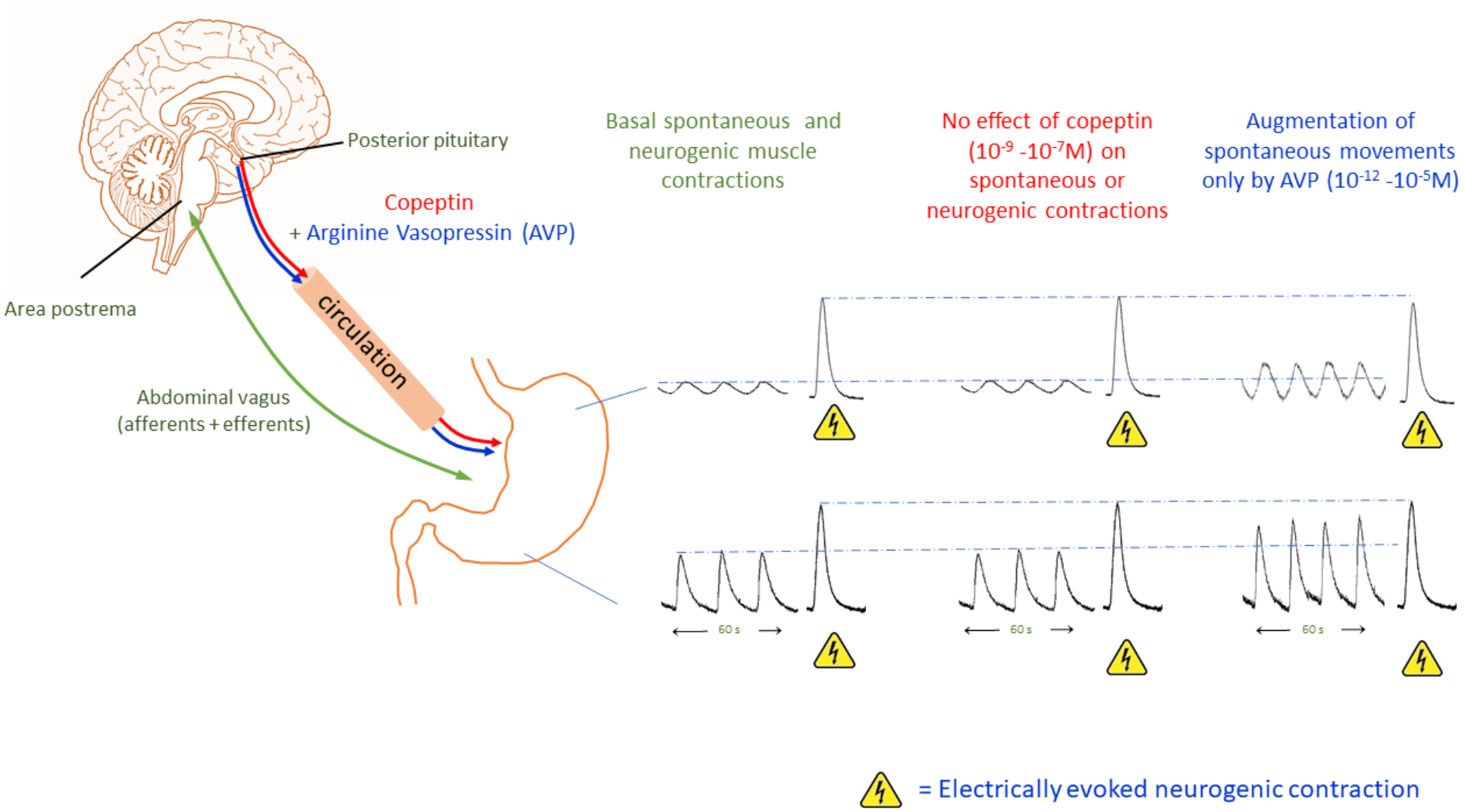


Figure 8: Relaxation of a) noradrenaline (10^{-6}M) and b) potassium chloride (KCl, $5x10^{-2}$M) induced submaximal (~65-70 %) contraction of mouse aortic rings by carbachol (CCh, 10^{-3}M) and SNP (10^{-3}M) but not copeptin. Data presented as mean ± s.e.m. n=3 for each bar.


Posterior pituitary
Copeptin
+ Arginine Vasopressin (AVP)
Area postrema
circulation
Abdominal vagus
(afferents + efferents)
Basal spontaneous and neurogenic muscle contractions
No effect of copeptin (10^{-9} -10^{-7}M) on spontaneous or neurogenic contractions
Augmentation of spontaneous movements only by AVP (10^{-12} -10^{-5}M)
60 s
60 s
60 s
= Electrically evoked neurogenic contraction

Conflict of Interest: GJS receives research funding from Takeda Pharmaceuticals. The other authors have no conflict of interest.

Highlights

- Copeptin is co-released in equimolar amounts with vasopressin (AVP) from the posterior pituitary.
- In contrast to AVP it is not known if copeptin is biologically active on the stomach.
- Copeptin at plasma concentrations was without effect on human or mouse stomach *in vitro*.
- We were unable to replicate a published effect of copeptin to relax the mouse aorta.
- Copeptin does not modulate gastric motilit in contrast to AVP implicated in genesis of nausea.

CRediT

Raj Makwana: Methodology, Investigation, Formal analysis, Data curation, Writing- original draft, Visualization, Project administration

John Loy: Resources

Miriam Adebibe: Resources

Kalpana Devalia: Resources

Paul LR Andrew: Writing-Review & Editing

Gareth J Sanger: Conceptualization, Resources, Writing original draft, Supervision, Project administration, Funding acquisition