Sanger Gareth (Orcid ID: 0000-0002-4231-1945)

Synergistic augmentation of rhythmic myogenic contractions of human stomach by [Arg^8]-vasopressin and adrenaline: Implications for the induction of nausea

Running title: Nauseagenic hormones and gastric rhythmicity

Raj Makwana[1], Ellie Crawley[1], Marilisa Straface[1], Alexandra Palmer[1,] Armen Gharibans[2], Kalpana Deavlia[3], John Loy[3], Greg O'Grady[2], Paul L.R. Andrews[4], Gareth J. Sanger[1]*

[1]Blizard Institute, Faculty of Medicine and Dentistry, Queen Mary University of London, United Kingdom. [2]Department of Surgery and Auckland Bioengineering Institute, University of Auckland, New Zealand. [3]Bariatric Surgery Department, Homerton University Hospital, United Kingdom. [4]Division of Biomedical Sciences, St George's University of London, United Kingdom.

*Corresponding author: Prof. G.J. Sanger, Blizard Institute, Faculty of Medicine and Dentistry, Queen Mary University of London, London, UK.

Email: g.sanger@qmul.ac.uk

Phone: +44 02078826027

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KEYWORDS

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ORCID

RM: 0000-0003-2175-3530

PLRA: 0000-0001-7099-6964

GJS: 0000-0002-4231-1945

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Data Availability

The data that supports the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions. SAGER Guidelines have been followed.

What is already known

- Area postrema activation by [Arg^8]-vasopressin (AVP) is considered the mechanism by which it induces vomiting
- Elevated plasma AVP and adrenaline (ADr) accompany nausea and gastric electrical dysrhythmia

What this study adds

- Spontaneous, non-neuronally-mediated human stomach muscle contractions (~2.7/minute) were larger and developed faster in the antrum
- Low concentrations of AVP and ADr acted synergistically to increase muscle tone and myogenic activity

Clinical significance

- Synergy of motor activity between low concentrations of AVP and ADr in stomach increases impact
- Signaled to the brain, such gastric motor activity may promote nausea in humans.

ABSTRACT

Background and Purpose

Nausea is associated with secretion of [Arg^8]-vasopressin (AVP) and adrenaline (ADr) but determining their role in mechanisms of nausea is limited by poor understanding of their abilities to directly affect gastric motility. We investigated their effects, interactions, and sites of action on human stomach muscle.

Experimental Approach

Muscle strips were suspended in tissue baths and neuronal-/non-neuronally-mediated contractions measured. Custom software analyzed eight motility parameters defining spontaneous phasic non-neuronally-mediated (myogenic) contractions. Receptor distributions were assessed by qPCR and immunofluorescence.

Key Results

V_{1A} receptors and α_1-adrenoceptors were located on muscle and interstitial cells of Cajal (ICC) by immunofluorescence staining. Myogenic contractions of human proximal and distal stomach (respectively, 2.6±0.1, 2.7±0.0/min; n=44) were larger in the distal area (1.1±0.1, 5.0±0.1mN), developing relatively slowly (proximal) or rapidly (distal). AVP caused tonic (proximal) or short-lived (distal) increases in muscle tone and increased myogenic contraction amplitude, frequency and rate (acting at V_{1A} receptors; thresholds 10^{-11}-10^{-10}M); separately, cholinergically-mediated contractions were unaffected. Oxytocin (OT) acted similarly but less potently (at OT receptors). ADr increased (10^{-10}-10^{-5}M; α_1-adrenoceptors) and decreased (≥10^{-6}M; β-adrenoceptors) muscle tone and enhanced/reduced myogenic contractions. Cholinergically-mediated contractions were reduced (α_2-adrenoceptors). Combined, AVP (10^{-9}M) and ADr (10^{-}

^{8}M) greatly increased muscle tone and phasic myogenic activity in a synergistic manner.

Conclusions and Inferences

AVP and ADr, at concentrations in plasma during nausea, acting via ICC and muscle cells, increased human gastric tone and myogenic contraction amplitude, rate of contraction and frequency. Combined, these actions were further increased in a synergistic manner. Such activity may promote nausea.

Abbreviations

AP: Area Postrema

ADr: Adrenaline

AVP: [Arg^{8}]-vasopressin

DMSO: Dimethyl sulfoxide

EGG: Electrogastrography

EFS: Electrical Field Stimulation

E_{max}: maximum response achieved from an applied agent

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GI: Gastrointestinal

ICC: Interstitial Cell of Cajal

L-NAME: Nω-nitro-L-arginine methyl ester hydrochloride

OT: Oxytocin

NTS: *Nucleus Tractus Solitarius*

pA_2: negative $\log_{10}$ of the molar concentration of antagonist which reduces the effect of double dose of an agonist to that of single dose.

pEC_{50}: negative $\log_{10}$ of the concentration of the agonist achieving half the maximal response

pK_B: negative $\log_{10}$ of the equilibrium dissociation constant of the antagonist-receptor complex

TTX: Tetrodotoxin.

1. INTRODUCTION

The unpleasant self-reported experience of nausea (Stern et al., 2011) often precedes vomiting and seems less effectively treated than vomiting (Sanger & Andrews 2018). This includes patients receiving anti-cancer chemotherapy (Smit et al., 2021). Drug treatments also fail to adequately alleviate nausea in gastroparesis, chronic unexplained nausea and vomiting and functional dyspepsia (Parkman et al., 2016; Camilleri et al., 2018). Thus, the pathways of nausea and vomiting can overlap, but differences exist, complicating treatments (Stern et al., 2011; Sanger & Andrews 2018).

Accompanying nausea are diverse physiological changes including heightened sympathetic and reduced parasympathetic activity (LaCount et al., 2011), pituitary secretion of [Arg^8]-vasopressin (AVP) (Koch et al., 1990), and adrenaline (ADr) and cortisol from adrenal glands (Xu et al., 1993). Additionally, dysrhythmic gastric electrical activity (Koch, 1997) and antral hypomotility (Faas et al., 2001) occur. A key challenge is to determine which of the changes contributes to the development of nausea and which represents a physiological response to the stress of nausea.

AVP is of particular interest (Stern et al., 2011). In humans, AVP enhances renal water reabsorption, increases peripheral vascular resistance (Holt & Kaspel, 2010) and is associated with nausea in several clinical paradigms (e.g., motion sickness, water intoxication, tilt-induced syncope; Rowe et al., 1979; Heyer et al., 2017) in addition to vomiting in humans (Edwards et al., 1989) and animals (Verbalis et al., 1987). Further, intravenously-administered AVP (or analogs) can induce nausea without vomiting or retching (Caras et al., 1997; Kim et al., 1997). It is assumed that systemic AVP induces vomiting by activating neurons in the area postrema (AP), also known as the ‘chemoreceptor trigger zone’. However, the evidence relies mostly on

iontophoretic application of AVP activating 50% of AP neurons tested in dogs (Carpenter et al., 1988), at a concentration (10^{-3}M) which undiluted can activate all AVP receptors, including oxytocin (OT) receptors (Bichet et al., 2019). This activity has not been causally linked to vomiting, or the cardiovascular, feeding and metabolic functions of the AP (Price et al., 2008).

Some evidence links nausea induced by systemic AVP with changed dominant frequency of gastric electrical slow waves (assessed by electrogastrography; EGG), creating tachygastria (Caras et al., 1997; Kim et al., 1997) and bradygastria (Kim et al., 1997; Lien et al., 2003). This finds some consistency with EGG studies demonstrating gastric dysrhythmia (tachygastria and/or bradygastria) before and/or during nausea induced by different stimuli (Koch et al., 1990; Koch, 1997; Heyer et al., 2017). The gastric motor correlates are not fully described but tachygastria has been linked with antral hypomotility (Ouyang et al., 2005). Reduced gastric contraction amplitude (particularly antrum; Chen et al., 1993) and delayed emptying are associated with nausea (Faas et al., 2001).

It is 25 years since the “noxious trio” of nausea, gastric dysrhythmia and AVP was described (Koch, 1997), yet remarkably AVP has only recently been shown to directly stimulate human stomach contractility (contrasting with inactivity of copeptin, a glycosylated peptide from the C-terminus of the AVP precursor, co-secreted with AVP; Makwana et al., 2021). Accordingly, the present study has several inter-linked objectives: i) Characterize the spontaneous, non-neuronally-mediated ‘myogenic’ contractions of human stomach (initiated by depolarization of interstitial cells of Cajal; ICC; Sanders et al., 2014) and compare with published measurements of gastric electrical activity recorded *in vivo*; ii) Fully characterize the effects of AVP on human stomach muscle tone, myogenic and neuronally-mediated contractions, defining the

receptor involved and clinically contextualizing by comparing active concentrations with those reported in plasma during nausea; iii) Investigate the activity of ADr, released with AVP during nausea (Hasler et al., 1995; LaCount et al., 2011; Heyer et al., 2017) and known to be involved in the accompanying disrupted gastric slow wave cycling (Chen et al., 1993); could AVP and ADr act together to disrupt gastric movements, reaching a 'tipping point' at which nausea is perceived?; iv) Determine if receptors for AVP and ADr are localized to ICCs. These objectives address the fundamental question: Could low concentrations of AVP and ADr signal nausea by directly influencing gastric motility?

2. MATERIALS AND METHODS

2.1. Human stomach

Obtained from obese donors (12:41 male:female; 23-67 [median 48] years) undergoing sleeve gastrectomy (cut ~2cm distal to gastro-esophageal junction, ~8cm proximal to pylorus, ~⅔rd of width from greater curvature). Table S1 summarizes patient demographics, including any use of current medications (these were varied and did not include GLP-1 analogues). Tissues were used as they became available, without regard to donor sex, age or body mass index, but some retrospective analysis of patient characteristics was performed. Ethical approval (REC-15/LO/2127) and written informed consent was obtained. Sections ~5cm^2 were cut ~5cm from both ends excluding areas damaged during surgery and transported 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_2/5% CO_2. The resected areas most likely represented proximal corpus and distal corpus–proximal antrum, respectively, and for

brevity, are defined as ‘proximal’ and ‘distal’ stomach. Methods for minimizing variation when using human GI tissue were followed (Sanger et al., 2013).

2.2. Functional experiments

Methods have been described (Makwana et al., 2021; Straface et al., 2022). Briefly, mucosa-free muscle strips (5x20mm) were cut (perpendicular to greater curvature, parallel to circular muscle fibres) and used immediately or after overnight storage (4ºC in Krebs-Henseleit solution). In preliminary experiments, storage did not appear to affect contractile activity (Table S2), confirming previous studies (Broad et al., 2012) and data were pooled.

Strips were mounted in tissue baths to isometric force transducers (MLT201/D, ADInstruments, UK) between platinum wire electrodes and bathed in Krebs-Henseleit solution (95% O_2/5% CO_2) maintained at 37ºC; changes in muscle tone were recorded (milliNewtons; mN) using AcqKnowledge v3.8.1 data acquisition system (BIOPAC Systems, USA) on personal computers (www.dell.com/uk). After stretching (20mN) and equilibration, strips were treated with a test substance for 30min before constructing cumulative concentration-response curves for AVP, OT or ADr. Non-cumulative AVP concentration-response curves were constructed separately.

During Electrical Field Stimulation (EFS; 5Hz frequency, 0.5ms width; 10s/min; voltage 10% greater than required to elicit maximal contractions; electrodes connected to STG2008 stimulators: Multi Channel Systems, Germany), test substances were applied for 30min before constructing cumulative concentration-response curves for AVP, OT or ADr (log_{10}-unit increments with 15min intervals or when effect of previous concentration peaked), with one curve/ tissue. Treatments were randomized between tissue baths and paired with time-matched vehicle-treated controls.

After completing an experiment, muscle strips were challenged with carbachol, 10^{-3}M to estimate maximal contractile capacity.

2.2.1. Expression of Data

Spontaneous contractions were quantified using custom software (https://github.com/agharibans/GISMCA) measuring change in baseline muscle tone (mN), amplitude of contractions (mN), number of contractions/minute (c/min) during steady-state, total contraction time (s), time for contractions to peak and decay (s), rates for contraction development and decay (mN/s) and areas under the contraction wave and during the development and decay phases (mN.s) (Figure 1C-E). Measurements were visualized as radar plots using OriginPro 2020 (OriginLab Corporation, USA).

Agonist-induced increases in baseline muscle tone were expressed as percentage of carbachol-evoked contraction. For experiments with EFS, the effects of agonists were quantified as percentage change in amplitude of EFS-evoked contractions immediately before first addition.

Individual agonist concentration-response curves were fitted by non-linear regression. Ratios for increased agonist pEC_{50} in the presence of increasing concentrations of antagonist were analyzed using Schild plots, determining the pK_B of the antagonist-receptor complex. When using single concentrations of antagonist, the potency (pA_2) was determined by Gaddum–Schild equation (see Supplementary Methods for more details about the curve fitting and data manipulation).

2.2.2. Chemicals

Ascorbic acid, atropine sulphate, carbachol, cromolyn sodium, L-NAME (Nω-nitro-L-arginine methyl ester-hydrochloride), TTX (tetrodotoxin) and salts for Krebs-Henseleit solution (Sigma-Aldrich, Gillingham, UK). AVP ([Arg^8]-vasopressin), Oxytocin (OT), SR49059 (V_{1A} receptor antagonist; Serradeil-Le Gal et al., 1993) and L371257 (oxytocin receptor antagonist; Griffante et al., 2005) (Tocris Biosciences, Abingdon, UK). ADr (L-Epinephrine), yohimbine hydrochloride (Fisher Scientific, Loughborough, UK). Propranolol hydrochloride (Insight Biotechnology, London, UK). Mepyramine maleate and prazosin hydrochloride (Cambridge Bioscience, Cambridge, UK). Ascorbic acid, AVP, carbachol, L-NAME, oxytocin and TTX dissolved in water. Stock/dilutions of ADr in ascorbic acid (10^{-1}M). Other drugs in 100% dimethyl sulphoxide (DMSO). Total volume of solvents added to tissue baths not >1% bath volume.

2.3. Receptor expression and localization

Stomach muscle ~0.5cm^2 (n=8; 4:4 male:female; 2/8 diabetic; 28-54 [median 41] years) was stored at -80ºC in RNAlater™ and receptor mRNA expression was determined by qPCR (Supplementary Methods).

Receptor localization was investigated by immunofluorescence (3:3 male:female; 3/6 diabetic, 40-59 [median 47] years); Supplementary Methods). Full-thickness distal stomach (~1cm^2) was fixed in 10% formalin and embedded in paraffin wax. Transverse sections (4µm thickness) were deparaffinized, rehydrated, and subjected to heat-mediated antigen retrieval before incubation with optimized concentrations of primary antibody overnight at 4°C. Subsequently, sections were incubated with secondary antibodies for 1h after epitope blockade with 1% BSA,

followed by an auto-fluorescence quencher (Vector® TrueVIEW® Autofluorescence Quenching Kit; Vector Laboratories) and nuclei counterstain DAPI at room temperature. Coverslips were mounted (VECTASHIELD Vibrance Antifade Mounting; Vector Laboratories, USA) before imaging (Olympus BX61 microscope; Olympus, UK), SmartCapture-3 (DSUK, UK) and analyses (Image-J; https://imagej.nih.gov/ij/).

2.4. Statistical analysis

Data were quantified as mean ± standard error of the mean (S.E.M). In functional studies paired or unpaired Student's *t*-tests compared individual means of data from a given stomach or between stomachs from different donors, respectively. Shifts of agonist concentration-response curves by test ligands were compared by one-way ANOVA followed by Dunnett's *post hoc* test for multiple comparisons. $P<0.05$ represented statistical significance. *n*-values represent number of patients. Only one muscle strip was used per drug treatment from a given patient.

Inter-rater agreement between two independent observers assessing immunofluorescence of a given receptor was investigated using Bland-Altman plots.

All authors had access to study data and reviewed and approved the final manuscript. The data and methods that support the findings are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

2.5. Nomenclature of Targets and Ligands

Key protein targets and ligands are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY, permanently archived in the Concise

Guide to PHARMACOLOGY 2020/21. Key family targets are G protein-coupled receptors (Alexander et al., 2021).

3. RESULTS

3.1. Receptor expression

Relative to GAPDH expression (similar in both stomach regions), V_{1A}, V_{1B}, V_2, OT and adrenergic α_{1A}, α_{1B}, α_{1D}, $\alpha_{2A\text{-}C}$, $\beta_{1\text{-}3}$ receptor mRNA was present in muscle of both regions (Figure S1). There was no clear difference between results from donors with or without diabetes and these were combined. Rank orders of expression for the two families were $V_{1A}>V_{1B}=V_2=OT$ and $\beta_2=\beta_3>\alpha_{1D}=\alpha_{1B}>\alpha_{2A}=\alpha_{2C}>\beta_1=\alpha_{2A}=\alpha_{1A}$.

3.2. Spontaneous contractions

Proximal and distal stomach muscle displayed spontaneous phasic contractions (respectively, 1.1±0.1mN and 5.0±0.2mN; n=44), the former x5 (P<0.001) smaller than the latter (Figures 1A,B,E). These and other measurements were independent of patients' sex or diabetic status (Figure S2); no patient was diagnosed with diabetic gastroparesis associated with damaged ICCs (Ördög, 2008) (Table S1). The duration of individual contractions was similar (respectively, 23.5±0.4s, 22.8±0.3s; P>0.09, providing matching rhythmic frequencies (2.6±0.1c.p.m., 2.7±0.1c.p.m; P>0.19). Correspondingly, contractions of proximal stomach developed and decayed with comparable rates whereas those of distal stomach peaked ~5x faster and decayed more slowly.

TTX (10^{-6}M) did not alter baseline muscle tone or spontaneous contractions (Figure 2). Atropine (10^{-6}M) alone or with TTX and L-NAME ($3x10^{-4}$M) caused small tone reductions in proximal and distal stomach (respectively, by 1.6±0.2mN,

1.4±0.1mN, n=6 both) compared to time-matched controls but had no effect on spontaneous contractions (Figure S3).

Carbachol produced similar maximal increases in muscle tone in both stomach regions (Figure 1E).

3.3. [Arg^{8}]-vasopressin (AVP) and oxytocin (OT)

3.3.1. Muscle tone and spontaneous contractions

Concentration-response curves for AVP were similar when constructed using single concentrations/ muscle strip or by cumulative application, suggesting minimal tachyphylaxis (Figures S4A-C, Table S3). Subsequent experiments used cumulative application. Notably, low single concentrations of AVP (≤10^{-9}M) evoked slow, sustained contractions and increases in spontaneous contractions, whereas higher concentrations caused shorter-lived responses (Figure S4D).

AVP concomitantly increased muscle tone, amplitude and frequency of contractions in both stomach regions (Figure 3). OT acted similarly but was less potent and effective (Figure 3). The potency and threshold concentrations of both hormones were similar in each stomach region (Table 1A). The maximum increase in baseline muscle tone by AVP and OT was greater in distal, compared with proximal stomach. Further, both caused larger increases in muscle tensions developed by the larger phasic contractions of distal stomach, relative to the smaller contractions of proximal stomach (although % increase greater in proximal stomach). AVP (10^{-7}M) increased the frequency of contractions by respectively increasing and decreasing the rate and duration of contraction development and decay. Coupled to the increased amplitude of contraction, this resulted in similar changes in 'area under the curve' for each phase of contraction (Figures 3E,F). TTX (10^{-6}M) had no effect on AVP- and OT-induced

responses (Table S4; Figure S5). Atropine (10^{-6}M) and its combination with TTX and L-NAME ($3x10^{-4}$M), previously shown to cause small reductions in baseline muscle tone, caused small decreases in potency of AVP and OT (Table S4; Figure S5). Finally, in preliminary experiments the response to AVP was unaffected by the mast cell stabilizer cromolyn (10^{-5}M) or the histamine H_1 receptor antagonist mepyramine (10^{-7}M) (Figure S6, Table S5), which alone did not appear to affect baseline muscle tone and spontaneous contractions.

The V_{1A} and OT receptor antagonists, respectively SR49059 (10^{-9}-10^{-7}M) and L371257 (10^{-9}-10^{-7}M), did not alter baseline muscle contractility of either stomach region, but antagonized AVP and OT (Figures 3C,D) with pK_B's independent of stomach region (Table 2A). SR49059 (10^{-8}M) also antagonized OT (Figure S5) with comparable pA_2 values of ~9.5 (Table 2B).

3.3.2. Electrically-evoked contractions

EFS elicited monophasic contractions (Figure 4A,B) of similar amplitude in both stomach regions, prevented by TTX (10^{-6}M) or atropine (10^{-6}M) (Table S6).

Neither AVP nor OT affected EFS-evoked contractions in either stomach region but increased baseline muscle tone (Figures 4A-D). SR49059 (10^{-8}M) and L371257 (10^{-9}M) were without effect on the contractions, but antagonized (Figure 4C,D), respectively, AVP and oxytocin (pA_2 values consistent with pK_B's estimated on muscle strips not subjected to EFS; Table 2A,B).

3.4. Adrenaline (ADr)

In both stomach regions, ADr produced sustained, graded increases in muscle tone up to 10^{-7}M followed by relaxation (≥10^{-6}M), accompanied by enhanced (10^{-10}-10^{-5}M)

or reduced (10^{-4}M) amplitude and frequency of spontaneous contractions (Figure 5). The rank order for pEC_{50}'s (Table 1B) was: Augmenting Muscle tone >(2-$\log_{10}$ units) Contraction amplitude ≥Contraction frequency. ADr generated a larger maximal increase in muscle tone in distal stomach but at higher concentrations, a larger, complete relaxation in proximal stomach. Prazosin (10^{-7}M) antagonized the excitatory actions of ADr, weakly reducing the muscle relaxation (Figure 5B, Table 2C). Propranolol (10^{-5}M) caused a small increase in the ability of ADr to increase muscle tone without affecting spontaneous contractions, almost abolishing the relaxations induced by high concentrations (Figure 5B). Neither prazosin nor propranolol affected spontaneous contraction activity.

ADr concentration-dependently inhibited EFS-evoked contractions (Figure 4A,B,F), approximately equipotently in both stomach regions (Table 1B) but ~15% more efficacious in distal stomach. Yohimbine (10^{-7}M) had no effects alone but antagonized ADr-induced inhibition of contractions with similar pA_2's (Table S3B). Together, prazosin, propranolol and yohimbine (10^{-7}M each) abolished all actions of ADr.

3.5. [Arg^8]-vasopressin and adrenaline

To investigate whether AVP and ADr could interact functionally, they were applied at concentrations equivalent to that producing 75% of their maximum: 10^{-9}M and 10^{-8}M, respectively. Co-administration caused synergistic augmentation of muscle tone and amplitude and frequency of spontaneous contractions, compared to when applied individually (Figure 5C,D). There was no difference in pattern of synergy between AVP and ADr in the two stomach regions.

3.6. Receptor expression

In preliminary studies ICCs (DAPI-stained nucleus with c-Kit-positive irregular processes emanating from cell bodies) were not stained by mast cell tryptase, further distinguishing these cells from the rounder mast cells, both c-Kit-positive (Figure S7). V_{1A} receptor and α_1-adrenoceptor immunofluorescence was detected on respectively, 69.4±12.5% and 61.1±3.5% (mean of three ICCs in circular muscle from each of 6 donors; Figure 6). These data were not influenced by diagnosis of diabetes or inter-observer differences (Figure S8). Smooth muscle cells were identified by a DAPI-stained nucleus and αSMA-positive staining. Each of three smooth muscle cells from each of 6 donors showed positive immunofluorescence for V_{1A} receptors, with 44.5.1±18.1% immunofluorescent for α_1-adrenoceptors.

4. DISCUSSION

Proximal and distal human stomach spontaneously and rhythmically contracted at ~3c.p.m., independent of donor sex or diabetes. The contractions were 'myogenic' (unaffected by blocking neuronal functions) and likely initiated by spontaneous ICC depolarization (Rhee et al., 2011). Others, measuring electrical and sometimes mechanical activity of human stomach, reported higher frequencies (fundus, corpus, antrum: 4.2, 3.9, 4.9 c.p.m.; fundus, corpus: 5-6; antrum: 4-7; El-Sharkawy et al., 1978; Sinn et al., 2010; Rhee et al., 2011). The reasons for the differences are unclear. Perhaps pinning/impalement with microelectrodes caused tissue damage (O'Grady et al., 2012). A less likely possibility is biological variation between patients with different diagnoses, since others found similar gastric electrical activity (~3c.p.m.) in different GI disorders (Hinder & Kelly, 1977).

Myogenic contraction frequency was within the ranges recorded by EGG in healthy volunteers (2-4 c.p.m., Yin & Chen, 2013), showed no region-dependence and appeared similar to slow wave electrical frequencies in the same areas of anesthetized, fasted humans (O'Grady et al., 2010). Thus, it seems unlikely that the present data are greatly skewed by studying tissue from obese individuals. Others have suggested the existence of a frequency gradient of electrical activity from corpus to antrum (Hinder & Kelly, 1977), but robust measurements of muscle contraction frequency have yet to be obtained *in vivo*. Further, it remains possible that gastric electrical or myogenic activity *in vivo* can be influenced by substances released from the mucosa (e.g., Cai et al., 2022), removed in the present experiments. Notably, the myogenic contractions *in vitro* were 5x larger in distal, compared with proximal stomach, reaching maximum tension more quickly and returning to baseline more slowly. Since smooth muscle cells and ICCs (together with platelet-derived growth factor receptor-α^+ cells) exist as interactive electrically-coupled syncytia (Sanders et al., 2014), these contraction parameters might be expected to reflect the rates of rise and amplitude of electrical slow waves. Indeed, similar characteristics were recorded by high-resolution mapping in stomach of patients undergoing surgery (O'Grady et al., 2010). Region-dependent differences could reflect different resting membrane potentials (Rhee et al., 2011) and the need for distal stomach to generate stronger contractions following meals (Pal et al., 2004).

Low concentrations of AVP caused muscle contraction and stimulated myogenic contractions. This was independent of neuronal activity or mast cell functions (stimulated by AVP activating enteric neurons in mice with TNBS-induced colitis; Dou et al., 2019) and AVP did not modulate cholinergically-mediated contractions. In proximal stomach the AVP-induced increase in tone was maintained

and appeared unrelated to increases in spontaneous contractions. The likely consequences *in vivo* would be reduced proximal stomach storage capacity, 'pushing' contents to the antrum, particularly liquids (Indireshkumar et al., 2000). In distal stomach, the smaller increases in muscle tone appeared to track the increase in spontaneous contraction activity. In both regions, AVP increased amplitude, rate and frequency of myogenic contractions, especially in distal stomach. These actions were mediated through V_{1A}, not OT receptors, independently of neuronal activity. This finds consistency with V_{1A} receptor mRNA in human stomach neuromuscular tissue (extending previous findings for full-thickness human stomach biopsies; Monstein et al., 2008).

The effects of AVP occurred at concentrations measured in human blood plasma during nausea (Table S7). For example, AVP concentrations were $8.2x10^{-10}$M following apomorphine (near EC_{50} in present study). In other investigations, infusing AVP induced nausea and EGG changes at blood levels of $3.9\text{-}6.4x10^{-10}$M, concentrations causing stomach contraction. Notably, OT was less effective than AVP, although both OT and V_{1A} receptors were activated by OT. These observations, combined with the absence of significant increases in plasma OT during nausea in humans (see Introduction) is consistent with the view that OT does not induce nausea. In healthy volunteers, OT did not affect gastric emptying although satiety scores were reduced (Borg et al., 2011).

The use of single concentrations of AVP showed that the effects on gastric motility plateaued in a concentration-dependent manner over 2-10min., consistent with the onset of nausea within 5min of AVP infusion (Caras et al., 1997).

To address the question '*could AVP activity combine with other substances released during nausea*?', the actions of ADr were studied. Immediate, sustained

increases in plasma ADr occur during motion sickness (~5-15x10^{-11}g/ml) and tilt-induced syncope, when ADr and AVP are released (Table S7). Further, ADr infusions disrupt human gastric slow waves (Lien et al., 2003). In the present study, low ADr concentrations caused muscle contraction and increased amplitude and rate of myogenic contractions in both stomach regions (via α_1-adrenoceptor activation), whereas higher concentrations relaxed the muscle (β-adrenoceptor activation). ADr reduced EFS-evoked, cholinergically-mediated contractions via α_2-adrenoceptor activation. Surprisingly, these data represent the first complete analysis for human stomach. Others showed ADr evoked muscle relaxation in human corpus/antrum (Bennett & Whitney, 1966), contractions in some stomach and pylorus muscle strips and biphasic responses or inhibition in others (Hafner et al., 1969). In human internal anal sphincter α_{1A}-adrenoceptor activation caused muscle contraction (Owaki et al., 2015).

Co-administration of submaximally-effective concentrations of AVP and ADr augmented muscle tone, contraction amplitude, rate and frequency of spontaneous contractions in a synergistic manner, compared to individual applications. Notably, the increased antrum contraction frequency caused by AVP and ADr together, falls within the EGG tachygastria range recorded in subjects reporting nausea during AVP infusion (Kim et al., 1997) and vection (Koch et al., 1990; Xu et al., 1993; Lien et al., 2003). A pathway by which AVP and ADr might interact is suggested by localization of V_{1A} receptor and α_1-adrenoceptor immunofluorescence on the ICC and muscle cells. These experiments were challenging because of tissue autofluorescence, possible loss of epitopes with degradation during tissue processing, and use of paraffin-embedded sections, which sometimes made it difficult to see the full extent of ICC extensions with nuclei. Nevertheless, co-expression of the receptors with muscle

was consistent with the functional data and co-expression with both muscle and ICCs suggests mechanisms of synergy that involve intracellular pathways. These are unknown, although vascular studies demonstrated functional synergy between AVP and noradrenaline involving Ca^{2+} channel activation (Noguera et al., 1997) or prostaglandin release (Karmazyn et al., 1978). Interestingly, only an insignificant expression of V_{1A} and α_1-adrenoceptor receptor mRNA occurred in ICCs from mouse intestine (Lee et al., 2017), suggesting species differences in receptor distribution, consistent with release of OT instead of AVP by rodents following emetogenic stimuli (Horn et al., 2013).

The ability of AVP and ADr (especially together) to increase myogenic contractions are consistent with AVP-induced EGG tachygastria (Caras et al., 1997), a role for catecholamines in inducing tachygastria during motion sickness (Chen et al., 1993) and with electrical tachygastria during nausea (Koch, 1997). However, they differ from bradygastria reported in response to AVP (Kim et al., 1997) (perhaps because of different recording methods and concentrations of AVP; Koch, 1997), with antral hypomotility and reduced gastric contraction amplitude associated with the electrical tachygastria/dysrhythmia (Faas et al., 2001; Lien et al., 2003; Ouyang et al.,2005), and with delayed emptying during nausea (Faas et al., 2001; Koch, 2014). This mismatches between the different data are difficult to dismiss, given the robustness of the present results *in vitro*. Perhaps the large increase in rate, frequency and amplitude of contractions of distal stomach by AVP and ADr, stimulates vagal nerve mechanoreceptors, resulting in vago-vagal reflex inhibition of gastric motility. Additionally, the use of muscle strips limits functional interpretation. For example, we do not know: i) if the large, high frequency contractions *in vitro* are propulsive; ii) if the larger increase in contraction parameters in distal stomach causes dysrhythmia by

destroying entrainment with the more slowly-developing smaller contractions originating in the corpus (as in mice; Kim et al., 2003); iii) if the changes occur evenly across the distal stomach (in dogs, intravenous AVP caused ectopic dysrhythmia; Du et al., 2016); iv) if the contractions operate against a closed pyloric sphincter (which itself may be affected by AVP/ADr) resulting in delayed gastric emptying.

5. Conclusions

The present experiments demonstrate region-dependent differences in human stomach rhythmic spontaneous myogenic contractions, generating greater maximum muscle tension and occurring at a faster rate in distal stomach. Marked stimulation by AVP occurred at low concentrations, within the range reported in human plasma during nausea and vomiting. Pathophysiological relevance was re-enforced by synergistic activity between AVP and ADr, reflecting co-release during nausea. ADr also decreased cholinergic transmission to the muscle. These observations support the suggestion that multiple stimuli act together, reaching a 'tipping point' at which central pathways generate nausea (Stern et al., 2011; LaCount et al., 2011; Napadow et al., 2013; Farmer et al., 2015). Interestingly, this could mean that blocking the gastric actions of either AVP or ADr alone may not fully abolish nausea. Thus, following illusory-self motion, the α_1-adrenoceptor antagonist phentolamine delayed the time to reach maximum tachygastria and reduced its magnitude and reduced but did not abolish nausea (Hasler et al., 1995).

Studies are now needed to understand exactly how the combined activity of AVP and ADr delays gastric emptying and causes nausea. Nevertheless, the proposed pathway challenges the long-held assumption that 'nauseagenic hormones' necessarily induce nausea solely via the AP (Borison, 1989).

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AUTHOR CONTRIBUTION

RM & EC performed the studies and initial data analysis, planned by RM, EC, AP and GJS. MS was involved in the data collection, JL, KD and EC enabled access to the human tissue and patient demographics, AG wrote the software enabling analysis of the parameters of contraction, visualized by RM. RM & GJS wrote the first draft of the paper, critiqued by GO'G and PLRA with the final draft agreed by all authors, funding was obtained by GJS.

CONFLICT OF INTEREST

GJS was in receipt of research funding from Takeda Pharmaceuticals and acts as scientific advisor for BYOMass. GO'G and AG are affiliated with Alimetry Ltd. Other authors do not have competing interests.

DATA AVAILABILITY STATEMENT

All data pertinent to this work are contained within the manuscript.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design & Analysis and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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Table 1: Threshold concentration, potency (pEC_{50}) and maximal effect (E_{max}) of (A) AVP and OT, for increasing muscle tone, amplitude and frequency of spontaneous contractions of human proximal and distal stomach circular muscle. (B) Data for effect of ADr on spontaneous and EFS-evoked contractions of human proximal and distal stomach.

A

	Proximal						Distal					
	AVP			OT			AVP			OT		
Effect on	**Threshold (M)**	**pEC_{50}**	**E_{max} (%)**	**Threshold (M)**	**pEC_{50}**	**E_{max} (%)**	**Threshold (M)**	**pEC_{50}**	**E_{max} (%)**	**Threshold (M)**	**pEC_{50}**	**E_{max} (%)**
Tone	10^{-11}-10^{-10}	9.5± 0.2	7.1 ± 0.2	10^{-9}-10^{-8}	7.5 ± 0.0	4.4 ±0.1	10^{-11}-10^{-10}	9.5 ± 0.1	9.5 ± 0.2	10^{-9}-10^{-8}	7.1 ± 0.1	6.3 ± 0.12
Amplitude	10^{-11}-10^{-10}	9.5 ± 0.1	244.8 ± 3.9	10^{-8}	7.2 ± 0.1	145.6 ±2.3	10^{-10}	9.0 ± 0.1	55.0 ± 2.0	10^{-9}-10^{-8}	7.4 ± 0.1	51.5 ± 1.5
Frequency	10^{-11}-10^{-10}	8.9 ± 0.1	30.4 ± 2.6	10^{-9}-10^{-8}	7.4 ± 0.1	30.0 ± 2.6	10^{-11}-10^{-10}	9.0 ± 0.1	33.6 ± 1.0	10^{-9}-10^{-8}	7.2 ± 0.2	30.5 ± 2.5

Data are mean±S.E.M. n=5.

B

Experimental condition	Effect	Proximal		Distal	
		pEC_{50}	E_{max} (%)	pEC_{50}	E_{max} (%)
Baseline conditions	Tone (contraction)	9.5 ± 0.2	6.3 ± 0.5	9.3 ± 0.1	7.6 ± 0.5
	Tone (relaxation)	5.1 ± 0.2	100	5.1 ± 0.2	100
	Amplitude	8.6 ± 0.1	244.3 ± 10.2	8.7 ± 0.1	56.4 ± 3.8
	Frequency	7.6 ± 0.4	23.4 ± 1.9	8.1 ± 0.4	25.2 ± 1.2
EFS conditions	Tone (contraction)	8.9 ± 0.1	5.1 ± 0.3	9.0 ± 0.1	6.4 ± 0.4
	Tone (relaxation)	5.4 ± 0.2	100	4.8 ± 0.1	100
	EFS-evoked contraction inhibition	5.9 ± 0.2	58.8 ± 4.9	6.2 ± 0.1	73.4 ± 4.0

Data are mean±S.E.M. n=4.

Table 2: Potency (pEC_{50}) and tissue maximal response (E_{max}) of (A) AVP and (B) OT for the increase in muscle tone, amplitude and frequency of the spontaneous contractions of human proximal and distal stomach circular muscle in the absence and presence of blockade of neurogenic, cholinergic and nitrergic activity and (A) OT receptor and (B) AVP V_{1A} receptor.

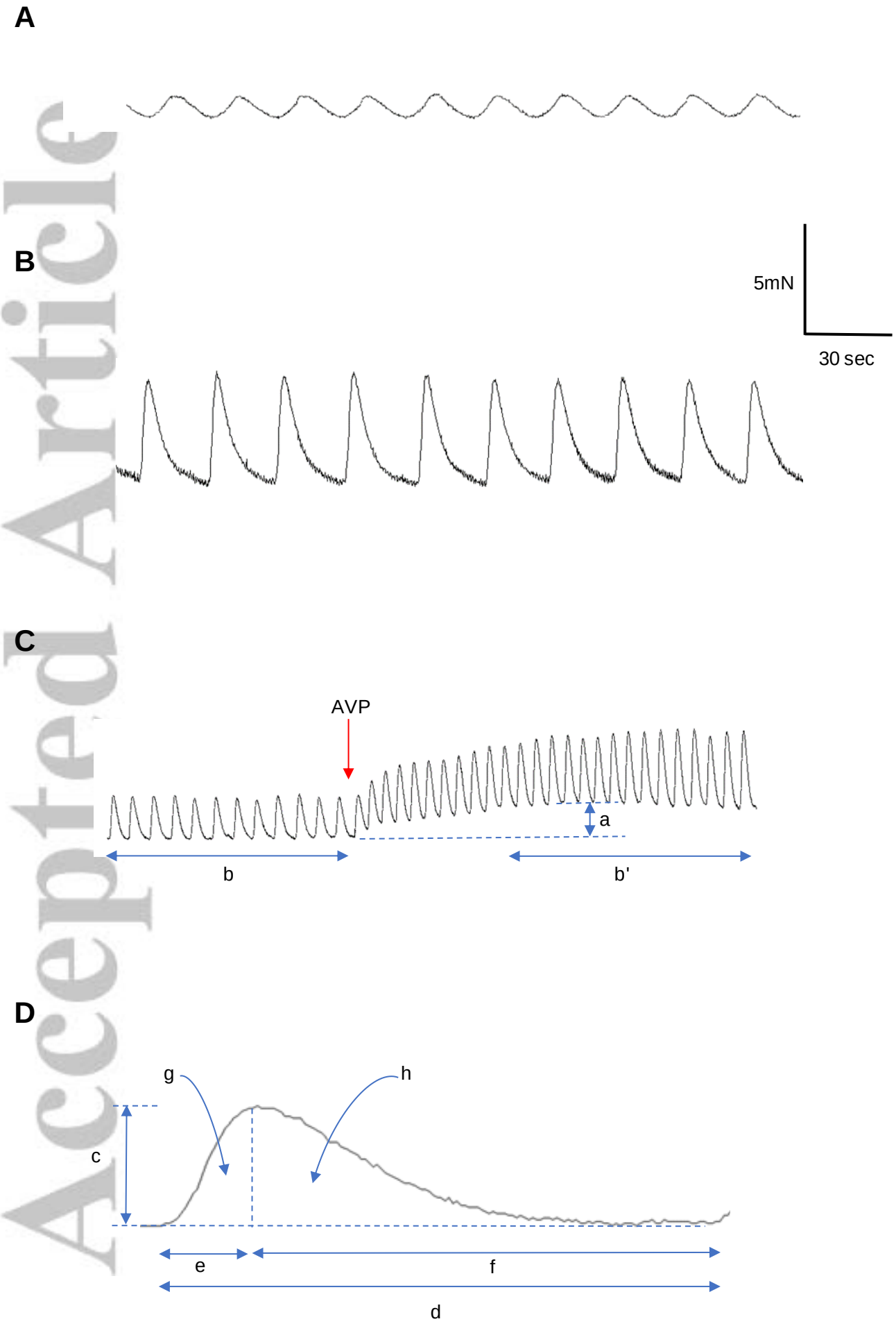
A

	Proximal						Distal					
	Control		+ TTX + Atropine + L-NAME		+ L371257		Control		+ TTX + Atropine + L-NAME		+ L371257	
Effect on	***p*EC$_{50}$**	**Emax (%)**	***p*EC$_{50}$**	**Emax (%)**	***p*EC$_{50}$**	**Emax (%)**	***p*EC$_{50}$**	**Emax (%)**	***p*EC$_{50}$**	**Emax (%)**	***p*EC$_{50}$**	**Emax (%)**
Tone	9.4 ± 0.1	6.9 ± 0.2	9.1 ± 0.2	6.4 ± 0.4	9.2 ± 0.1	6.7 ± 0.2	9.4 ± 0.1	9.3 ± 0.35	8.9 ± 0.1	9.0 ± 0.3	9.2 ± 0.1	8.1 ± 0.3
Amplitude	9.7 ± 0.8	246.6 ± 2.8	9.1 ± 0.1	246.8 ± 2.8	9.3 ± 0.1	246.8± 2.8	8.5 ± 0.1	59.0 ± 1.0	8.9 ± 0.1	59.0 ± 0.9	8.9 ± 0.1	59.0 ± 0.9
Frequency	9.0 ± 0.2	3.0 ± 0.1	9.2 ± 0.2	3.0 ± 0.1	9.1 ± 0.1	3.0 ± 0.1	8.9 ± 0.2	3.0 ± 0.1	9.1 ± 0.1	3.2 ± 0.1	9.0 ± 0.2	3.1 ± 0.1

B

	Proximal						Distal					
	Control		+ TTX + Atropine + L-NAME		+ SR49059		Control		+ TTX + Atropine + L-NAME		+ SR49059	
Effect on	***p*EC$_{50}$**	**Emax (%)**	***p*EC$_{50}$**	**Emax (%)**	***p*EC$_{50}$**	**Emax (%)**	***p*EC$_{50}$**	**Emax (%)**	***p*EC$_{50}$**	**Emax (%)**	***p*EC$_{50}$**	**Emax (%)**
Tone	7.2 ± 0.1	4.1 ± 0.2	6.9 ± 0.8	3.9 ± 0.2	5.8 ± 0.3	3.5 ± 0.8	8.3 ± 0.1	6.1 ± 0.2	6.9 ± 0.1	6.1 ± 0.2	5.8 ± 0.1	6.1 ± 0.2
Amplitude	7.2 ± 0.1	141.1 ± 3.3	6.8 ± 0.1	141.1 ± 3.3	5.7 ± 0.1	141.1 ± 3.3	7.3 ± 0.1	48.6 ± 2.5	6.8 ± 2.5	48.6 ± 2.5	5.8 ± 2.5	48.6 ± 2.5
Frequency	7.4 ± 0.3	2.9 ± 0.1	7.2 ± 0.1	2.9 ± 0.1	5.8 ± 0.1	2.9 ± 0.1	7.7 ± 0.2	2.9 ± 0.1	7.5 ± 0.2	2.9 ± 0.1	6.1 ± 0.1	2.9 ± 0.1

Blockade of neurogenic, cholinergic (muscarinic) and nitrergic transmission was achieved by simultaneous incubation with TTX (10^{-6}M), atropine (10^{-6}M) and L-NAME ($3x10^{-4}$M), respectively, 30 min before examining (A) AVP and (B) OT. In separate experiments, (A) AVP and (B) OT were also investigated after blockade of OT and V_{1A} receptors, respectively, using L371257 (10^{-7}M) and SR49059 (10^{-7}M), respectively. Data are mean ±S.E.M. n=5.



E

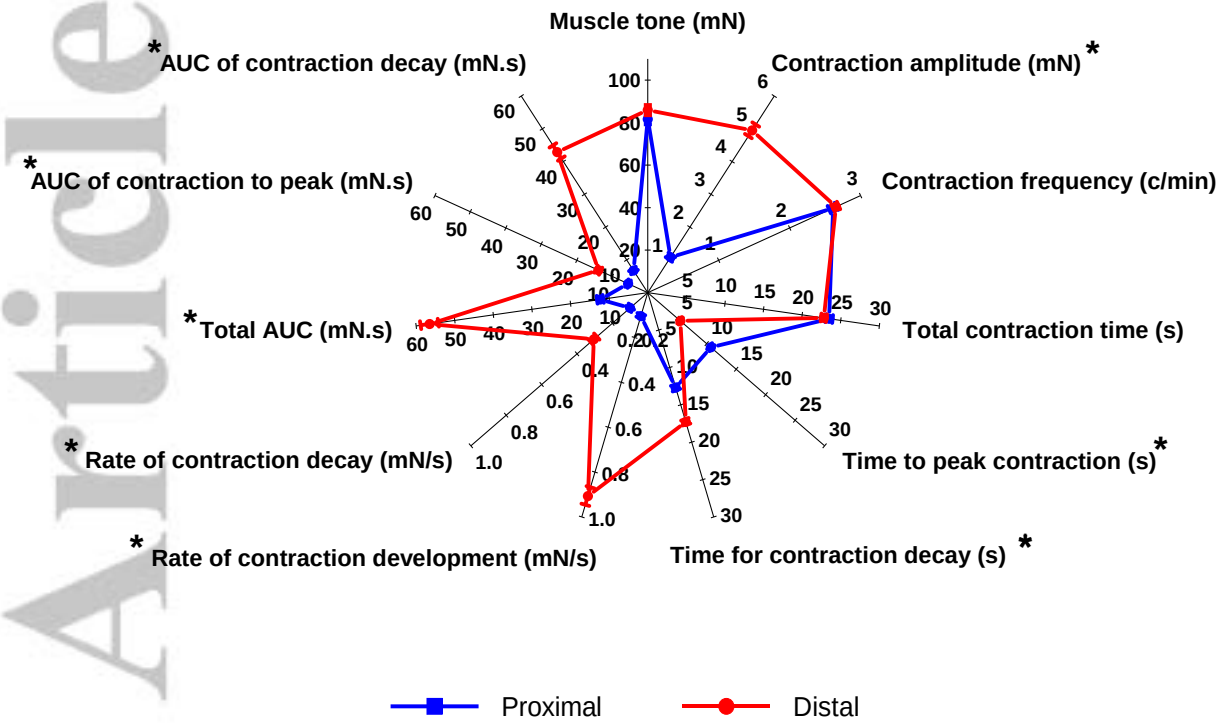
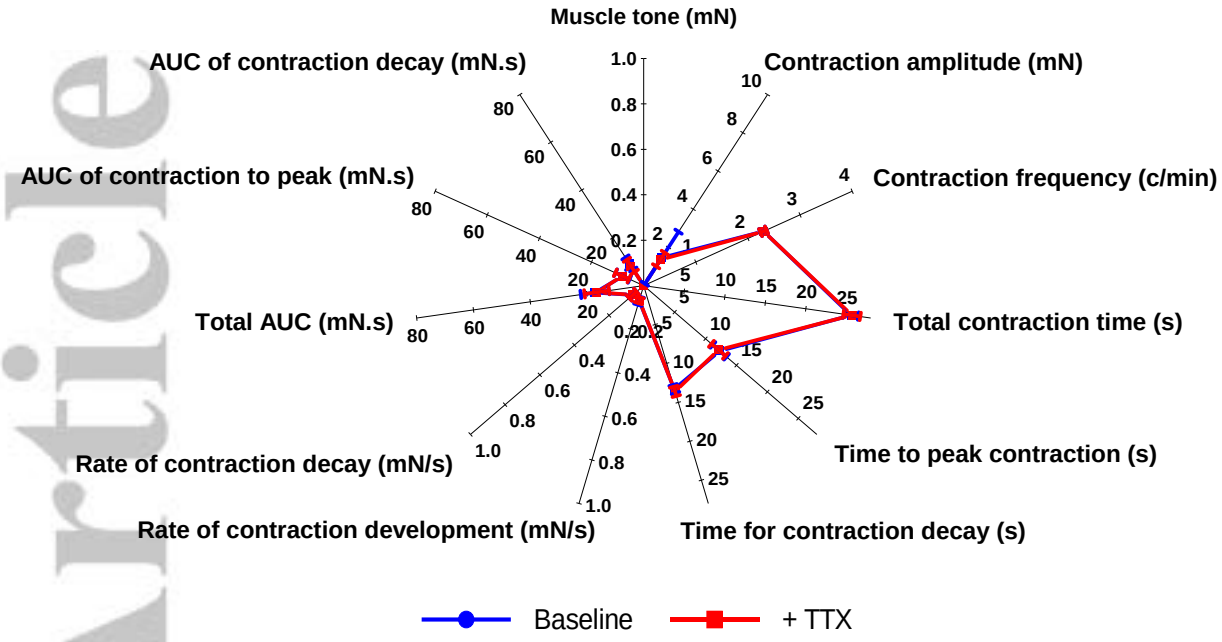


Figure 1: Spontaneous contractions of human (A) proximal and (B) distal stomach circular muscle. (C): Spontaneous contractions of distal stomach and change in a= muscle tone (mN) and frequency of contractions (number of contractions/minute; c.p.m.), usually measured over 5min b= before and b'= after application of an agonist (e.g AVP). (D): Features measured of a given contraction wave, c= Contraction amplitude (mN), d= Total contraction time (s), e and f= Time for contraction to peak and decay (s), g and h= Area under the curve for contraction to peak and decay (mN.s). g and h= Total area under curve (mN.s), mean change in c over e and, c over f= Rates of contraction development and decay (mN/s). (E): Radar plot comparing features of the baseline contraction waveform of proximal and distal stomach. Also shown is the carbachol (10^{-3}M)-induced maximal increase in muscle tone. *P<0.05 between data sets. Data are mean±S.E.M. n=44.

A



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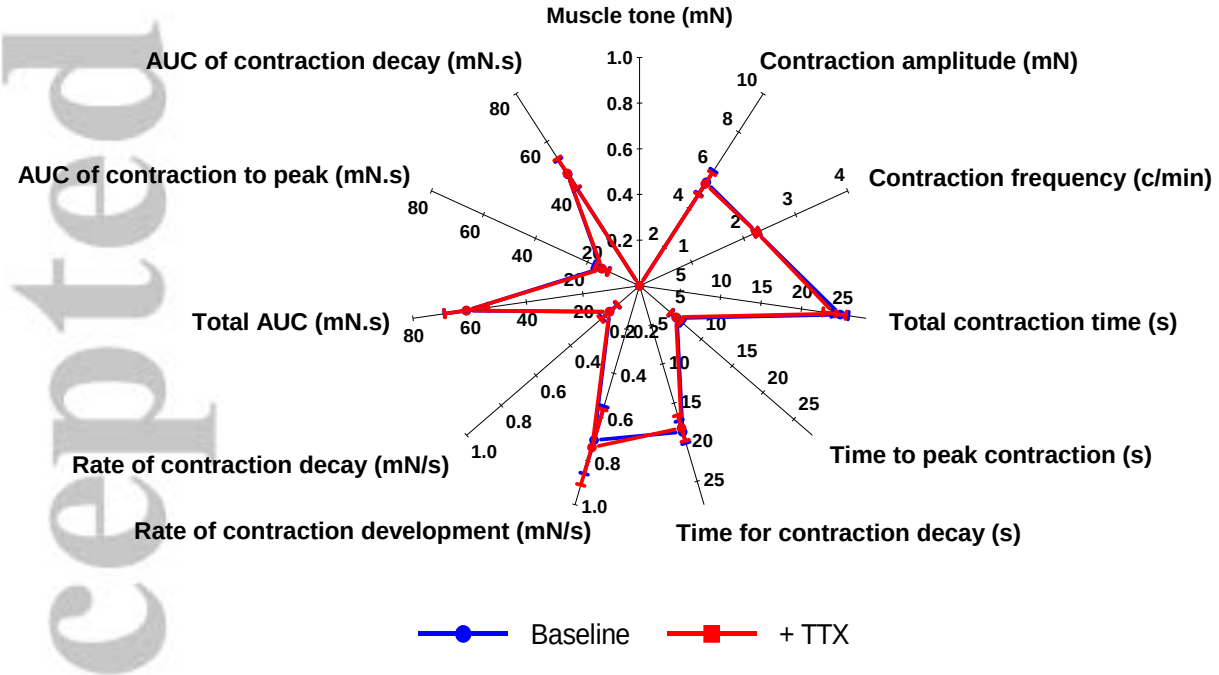
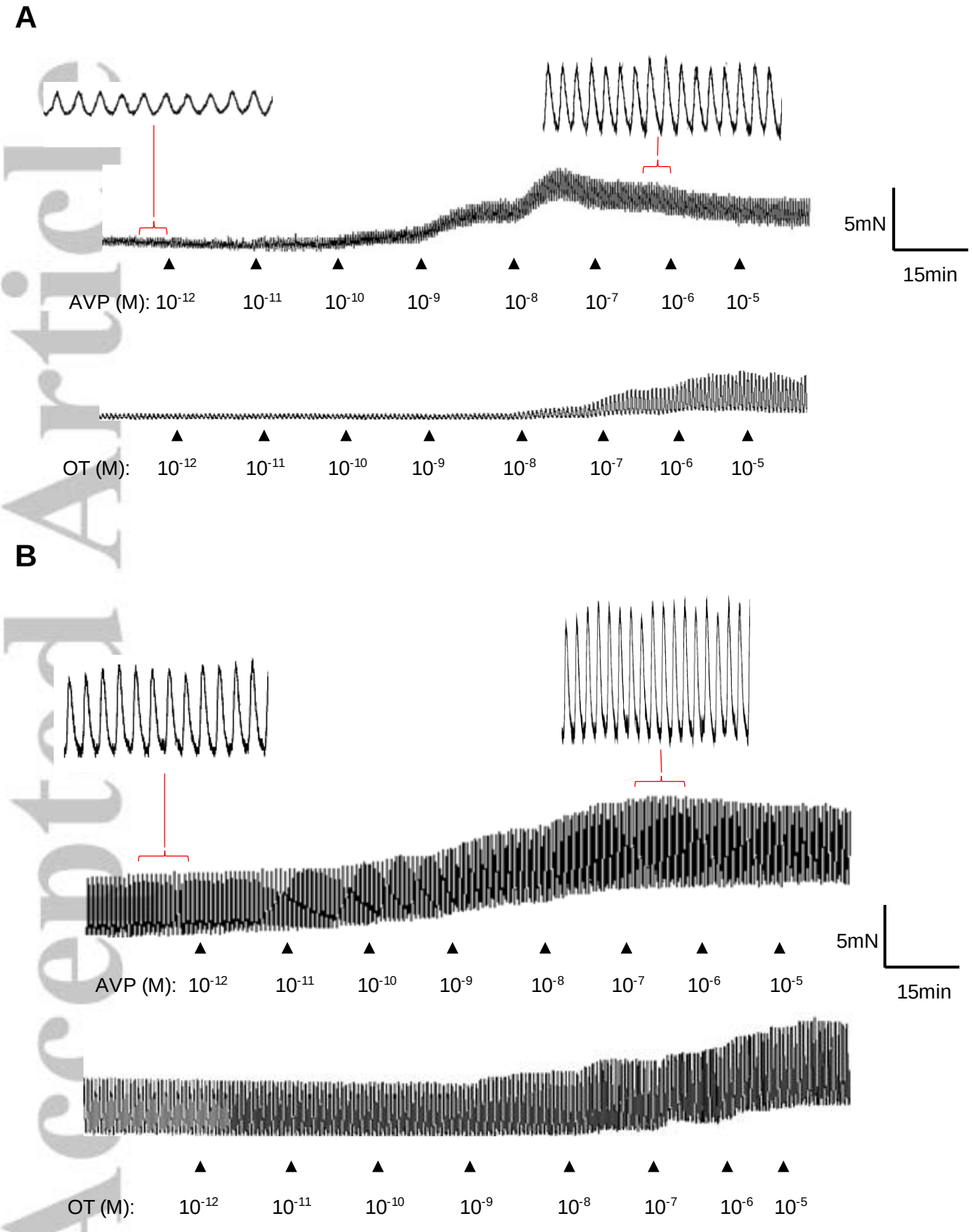
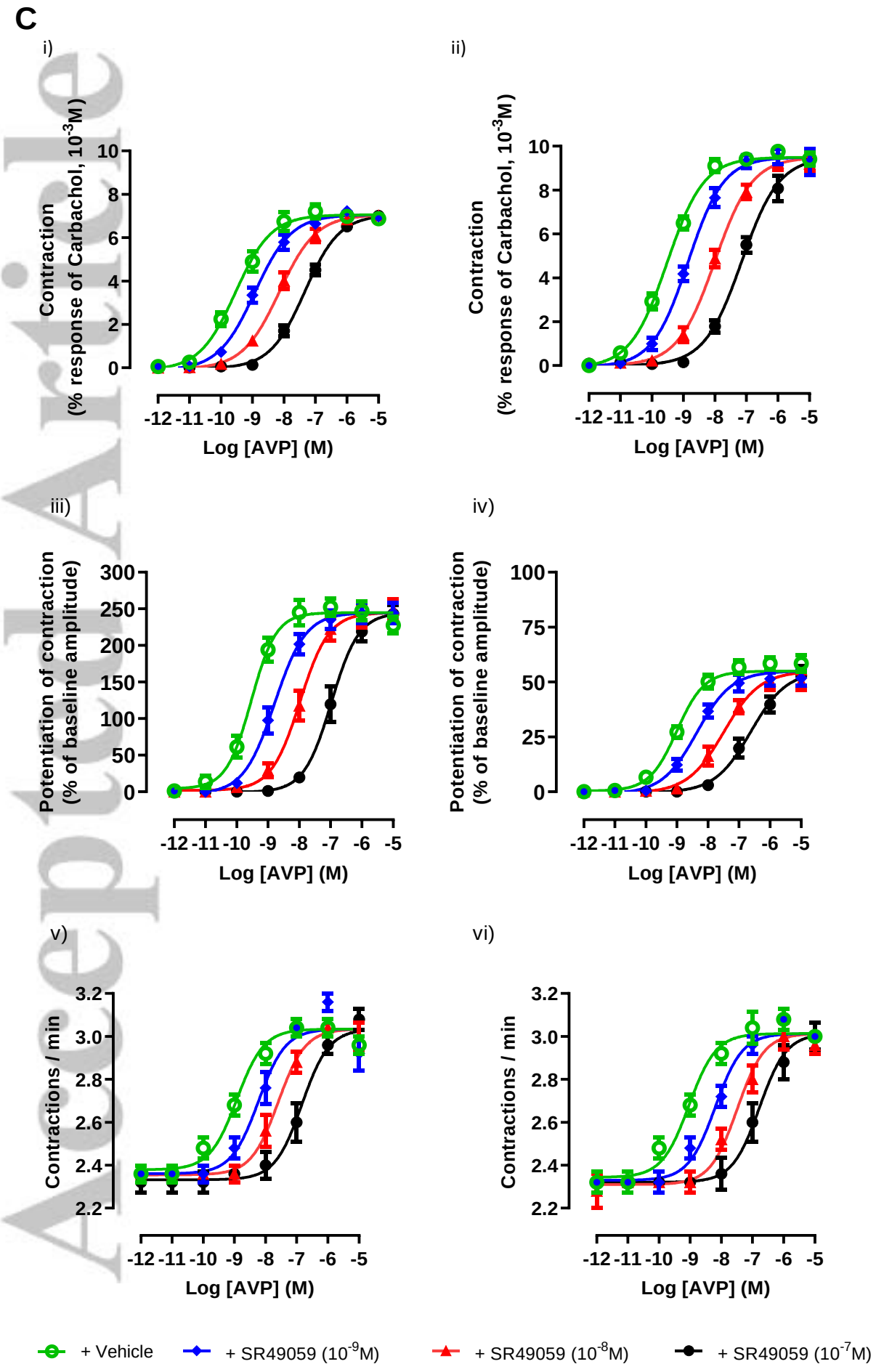
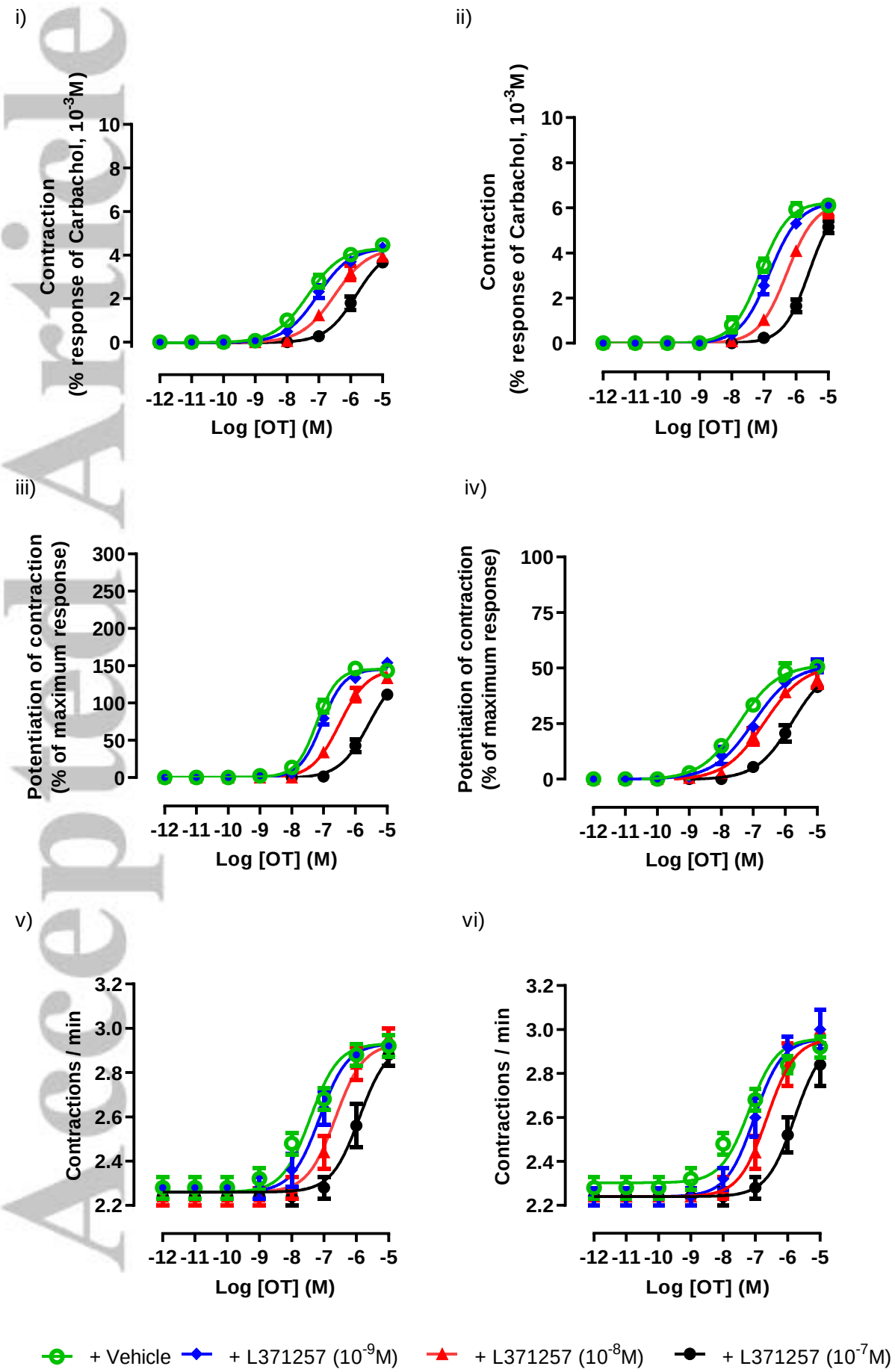


Figure 2: Comparison between the various features of the spontaneous contraction waveform of human (A) proximal and (B) distal stomach circular muscle before (Baseline) and after 30 min incubation with tetrodotoxin (TTX, 10^{-6}M). Each value represents the mean±S.E.M. n=4.



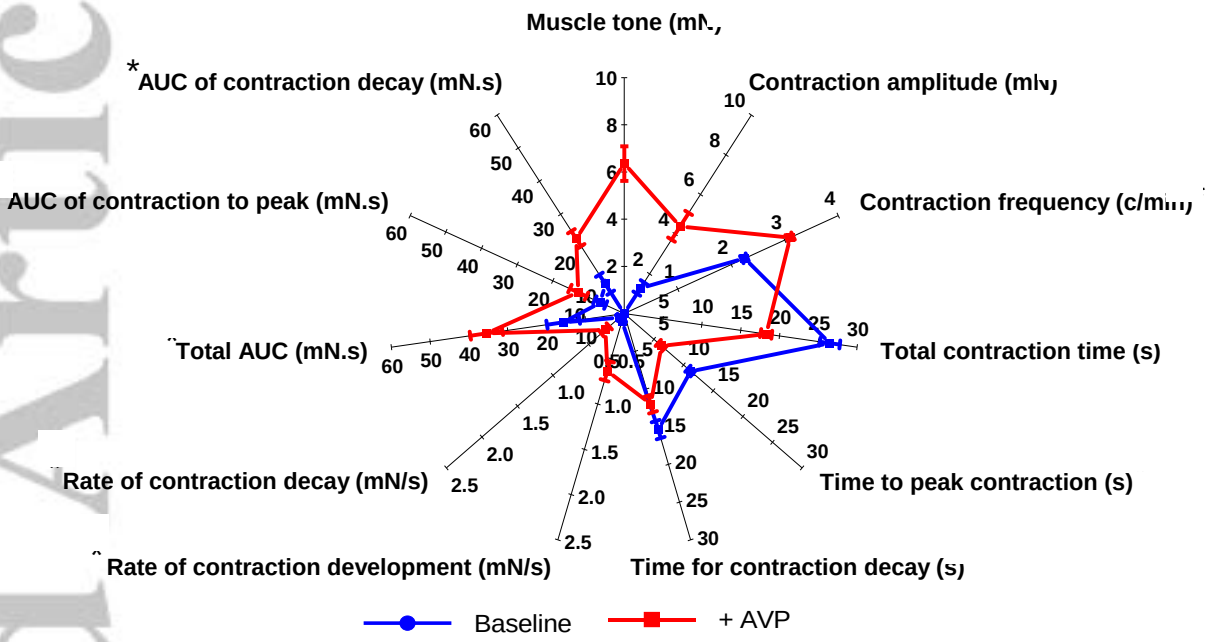


D



E

Baseline + AVP



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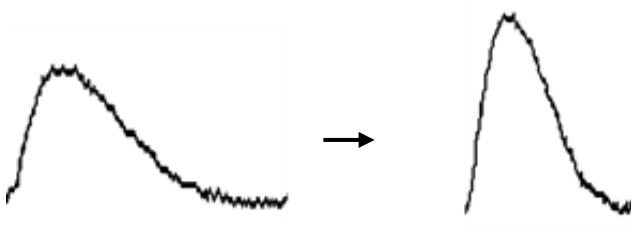


Baseline + AVP

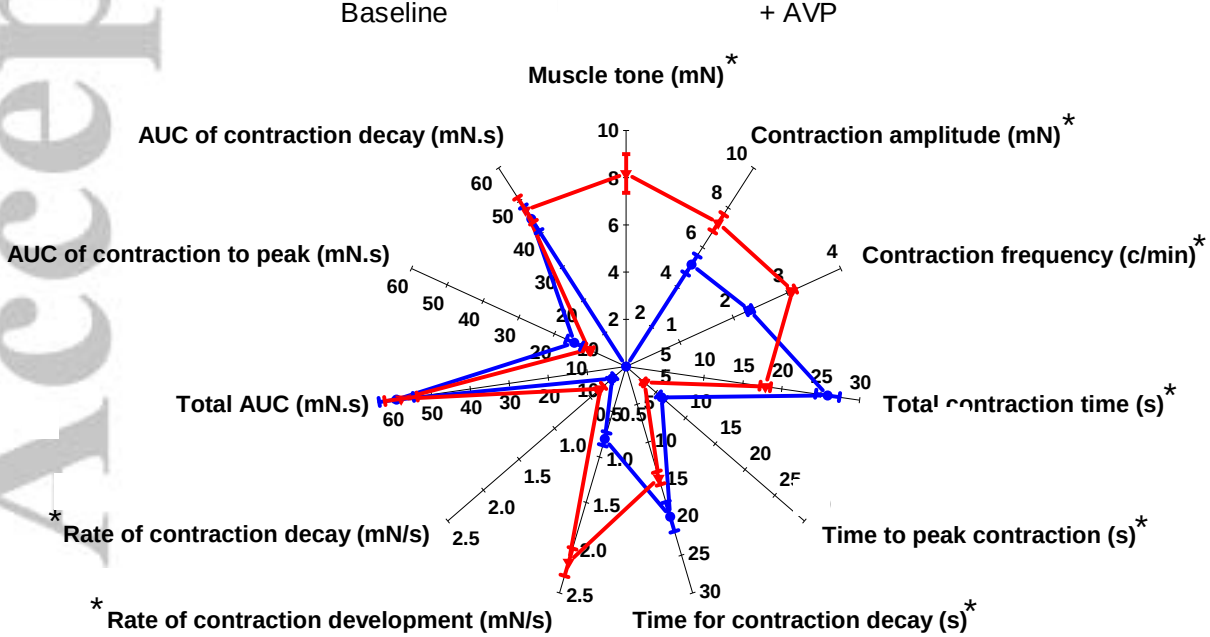
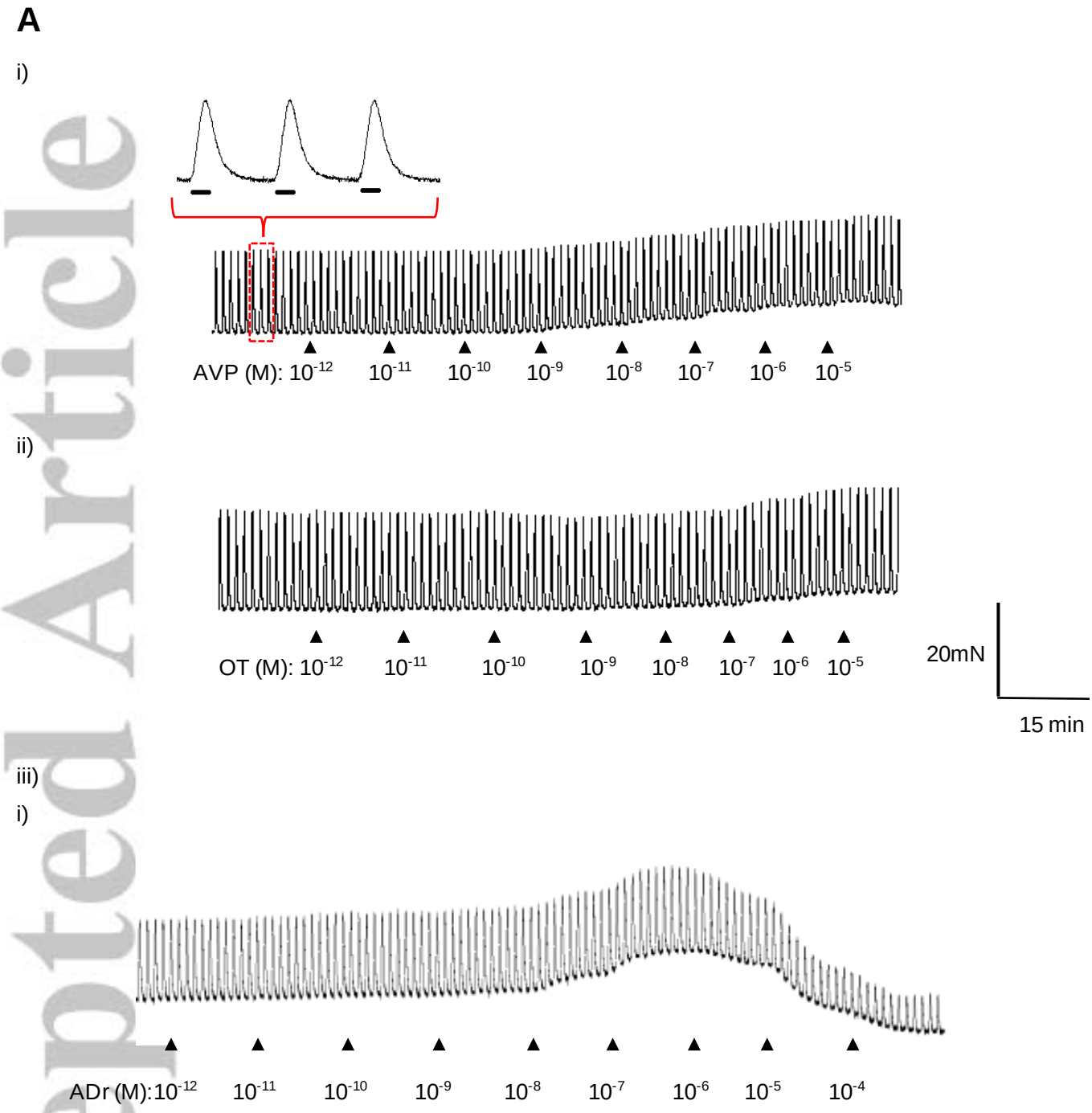


Figure 3: Effects of AVP and OT on spontaneous contractions of human (A) proximal and (B) distal stomach circular muscle. (C) AVP- and (D) OT-induced increase in muscle tone (i, ii) and amplitude (iii, iv) and frequency (v, vi) of spontaneous contractions of human proximal (graphs on left) and distal (right) stomach, after 30min incubation with SR49059 and L371257, respectively or vehicle (DMSO 0.1%v/v). n=5. (E) and (F) compare a spontaneous contraction of human (A) proximal and (B) distal stomach before (Baseline) and after a single concentration of AVP (10^{-7}M), together with the AVP-induced increase in muscle tone. n=4. Data are mean±S.E.M.



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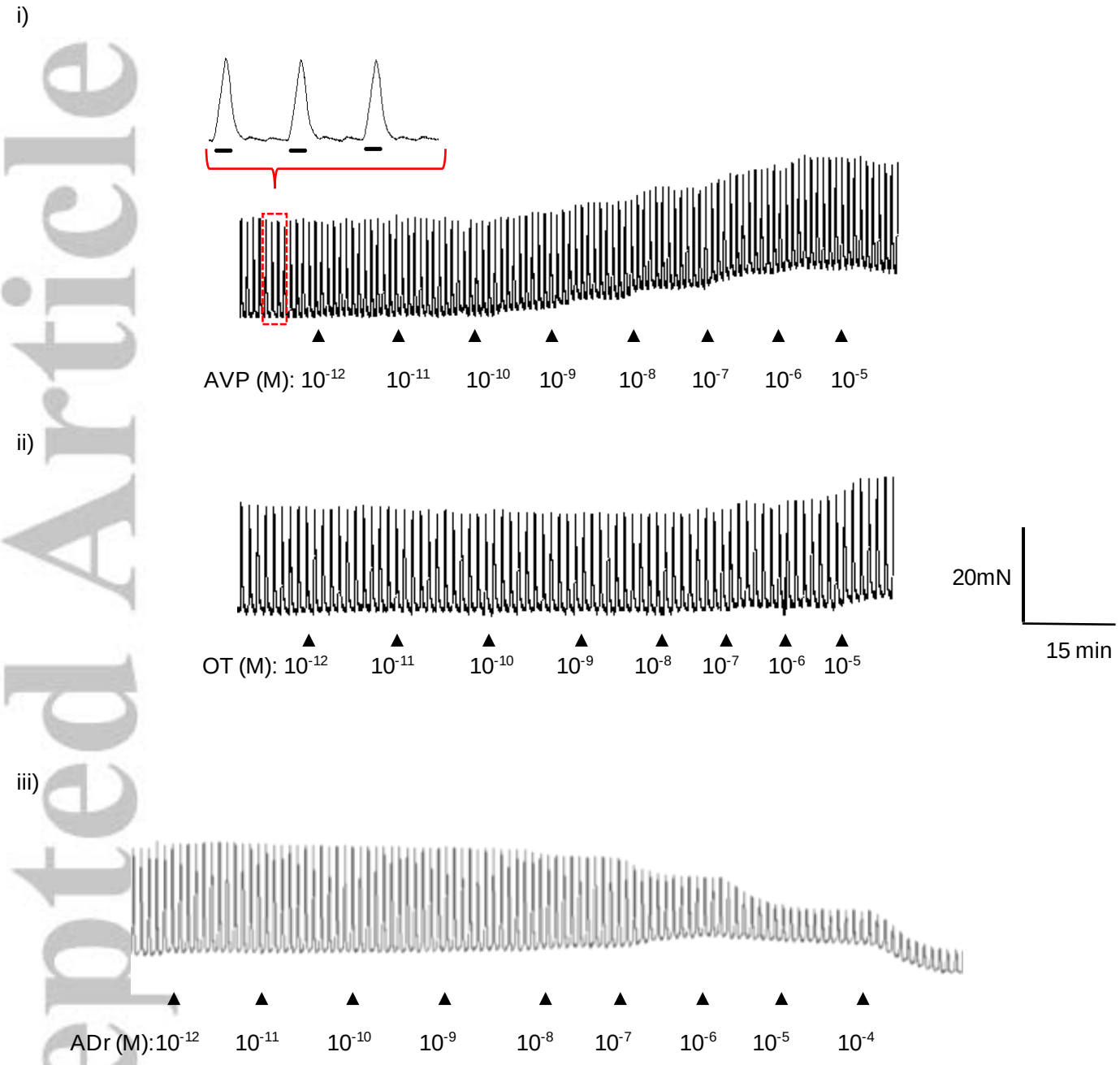
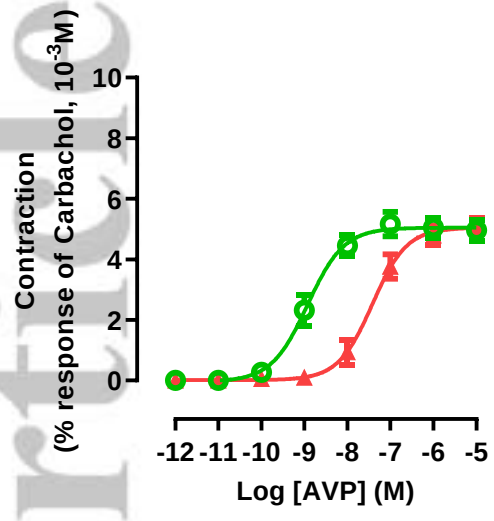


C

i)



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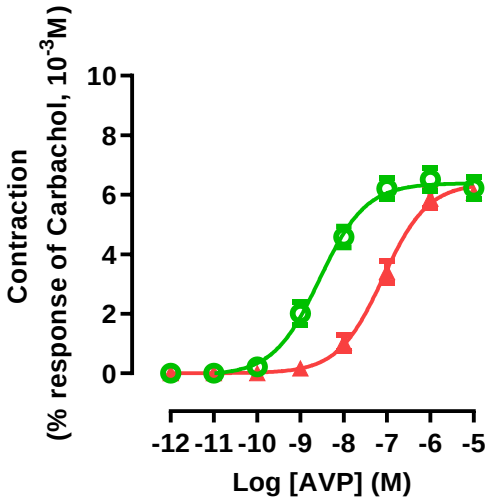
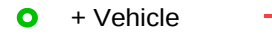
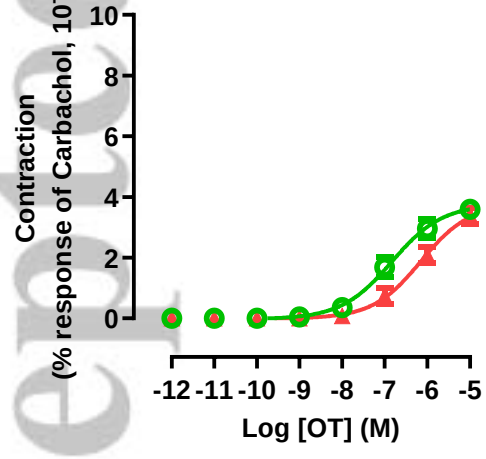


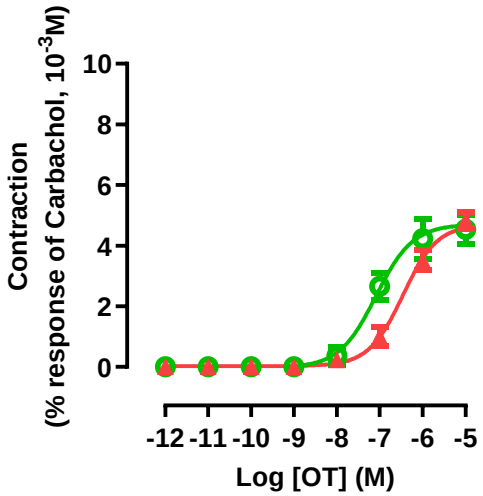


D

i)



ii)



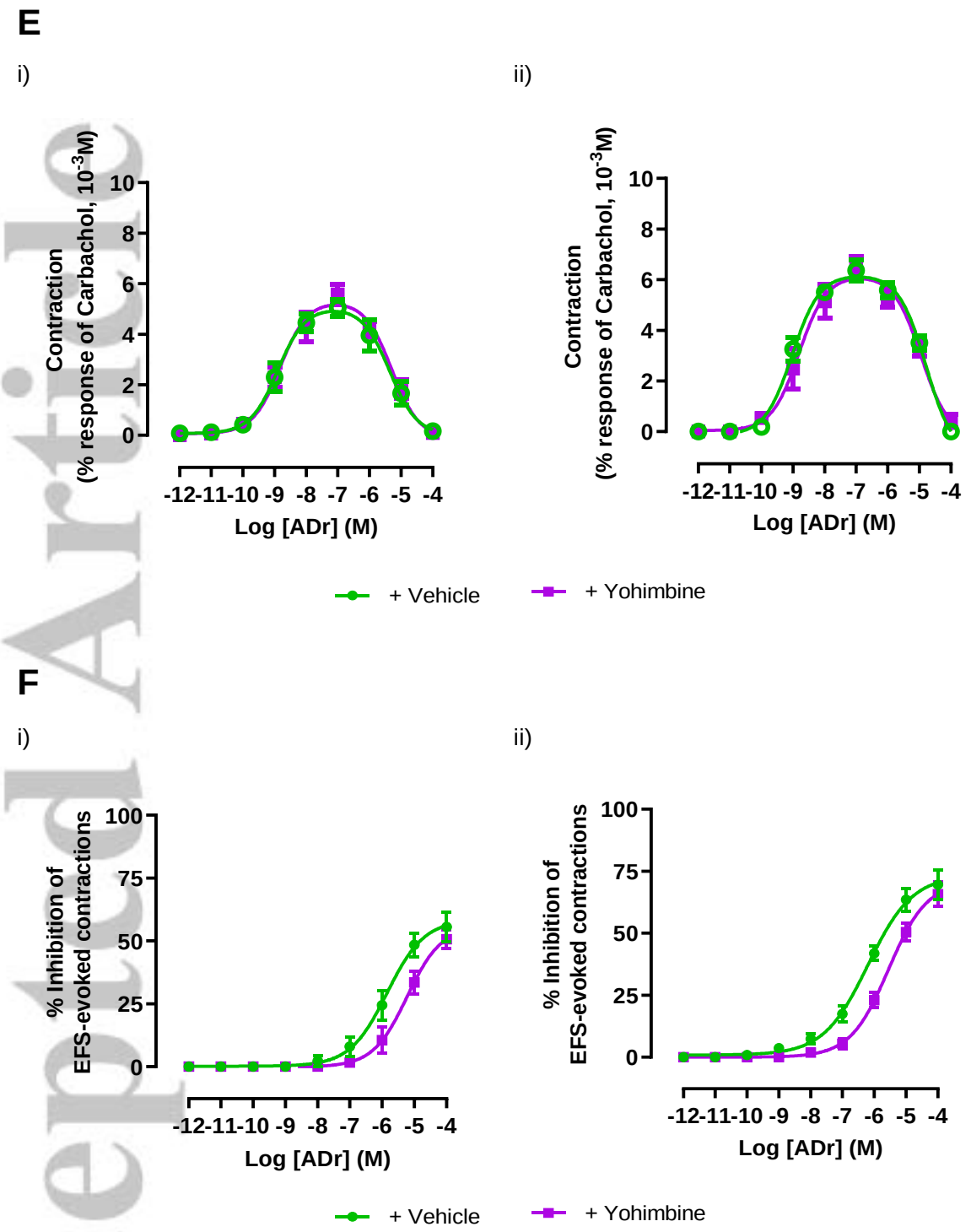
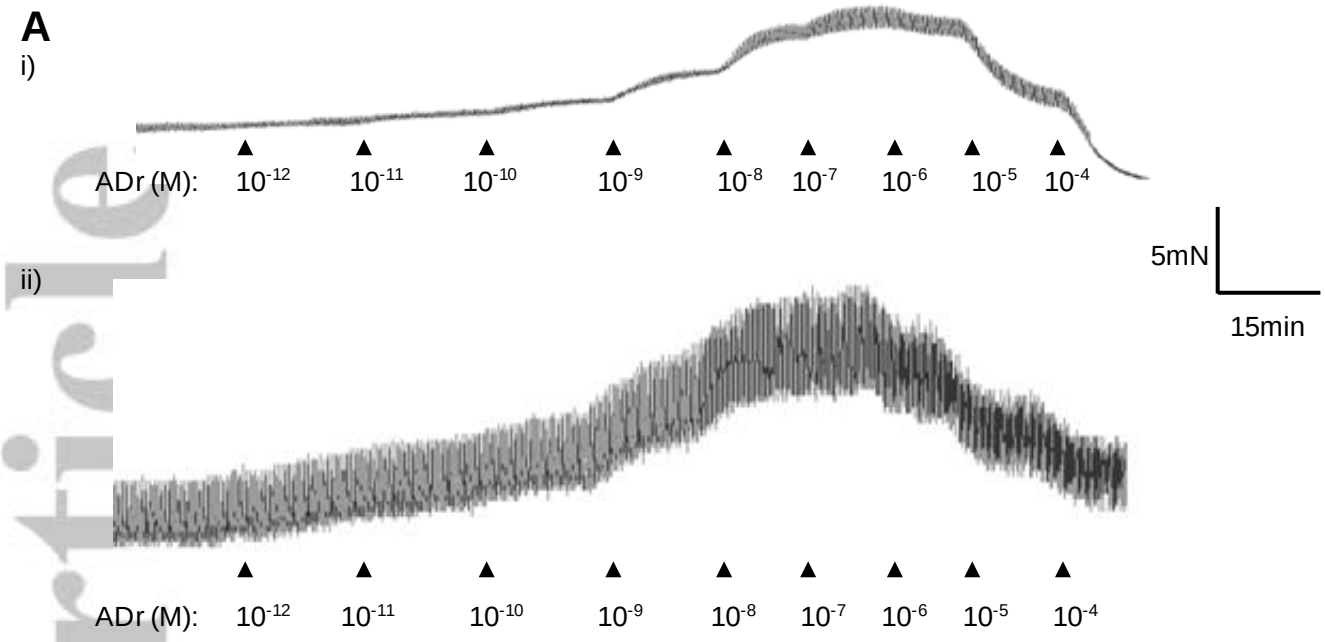
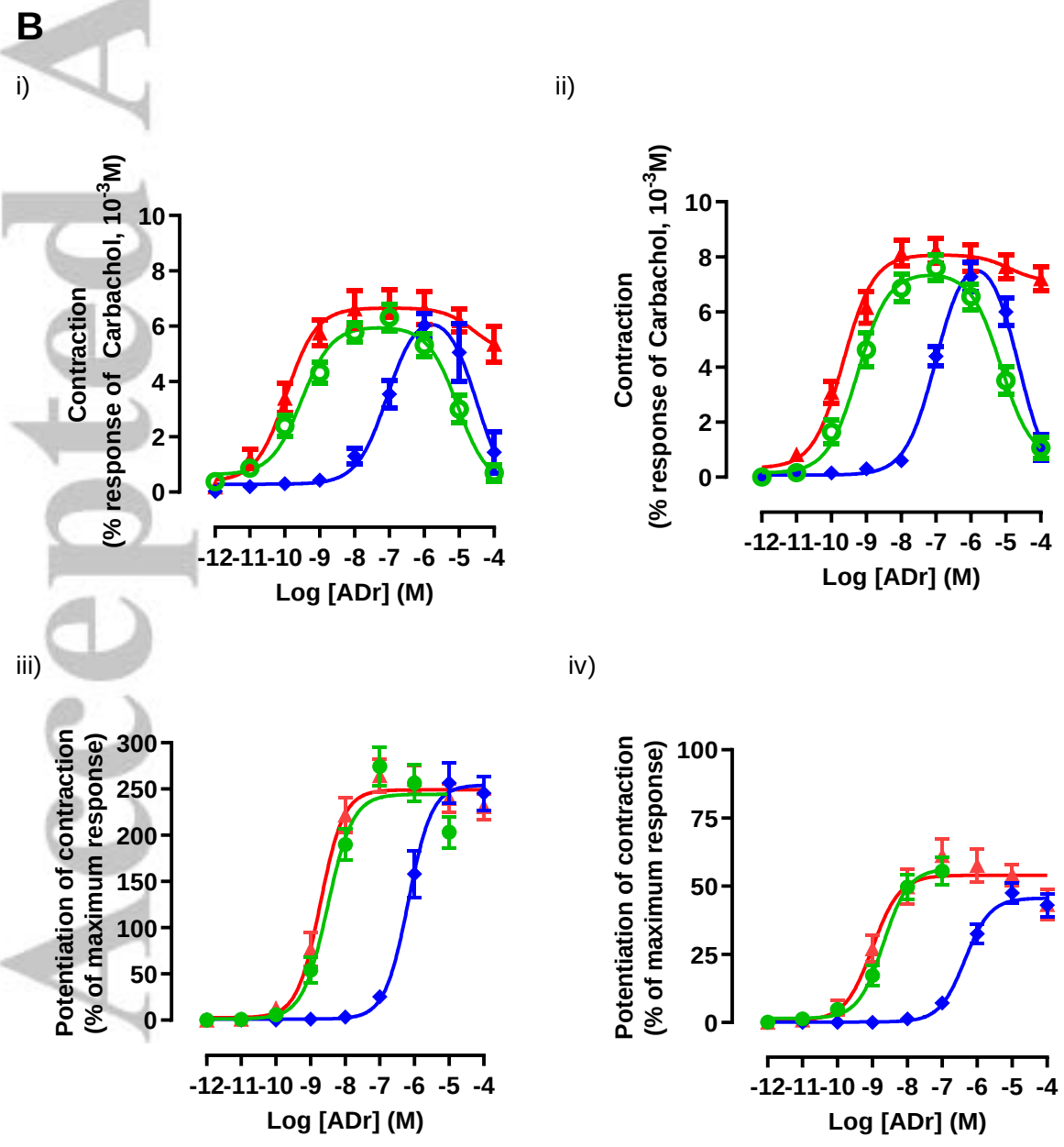


Figure 4: Effects of AVP, OT and ADr on EFS-evoked contractions of human (A) proximal and (B) distal stomach. Bars under magnified contractions indicate period of EFS. Concentration-response curves for increase in muscle tone by (C) AVP, (D) OT and (E) ADr on proximal (graphs on left) and distal (right) stomach subjected to EFS. (F) ADr-induced inhibition of EFS-evoked contractions of proximal (left) and distal (right) stomach. AVP, OT and ADr were applied after 30min incubation with, respectively, SR49059 (10^{-8}M), L371257 (10^{-9}M), Yohimbine (10^{-7}M) or vehicle (DMSO/ water, 0.1% v/v). Data are mean±S.E.M, n=4.





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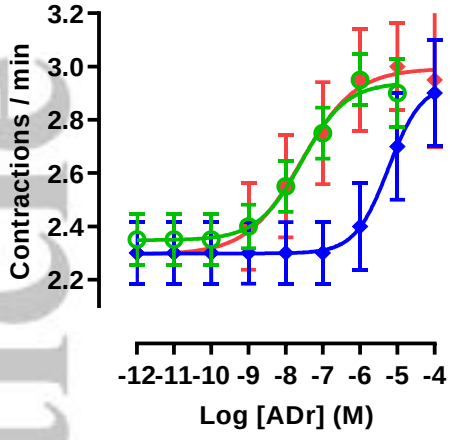

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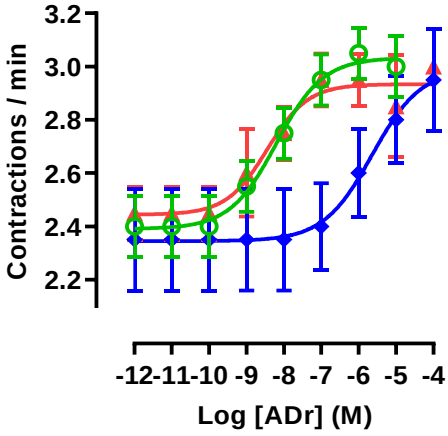

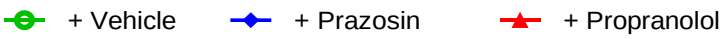

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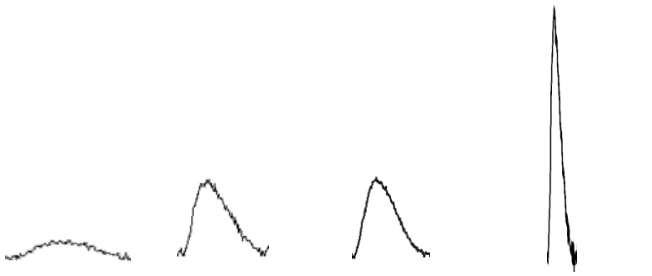

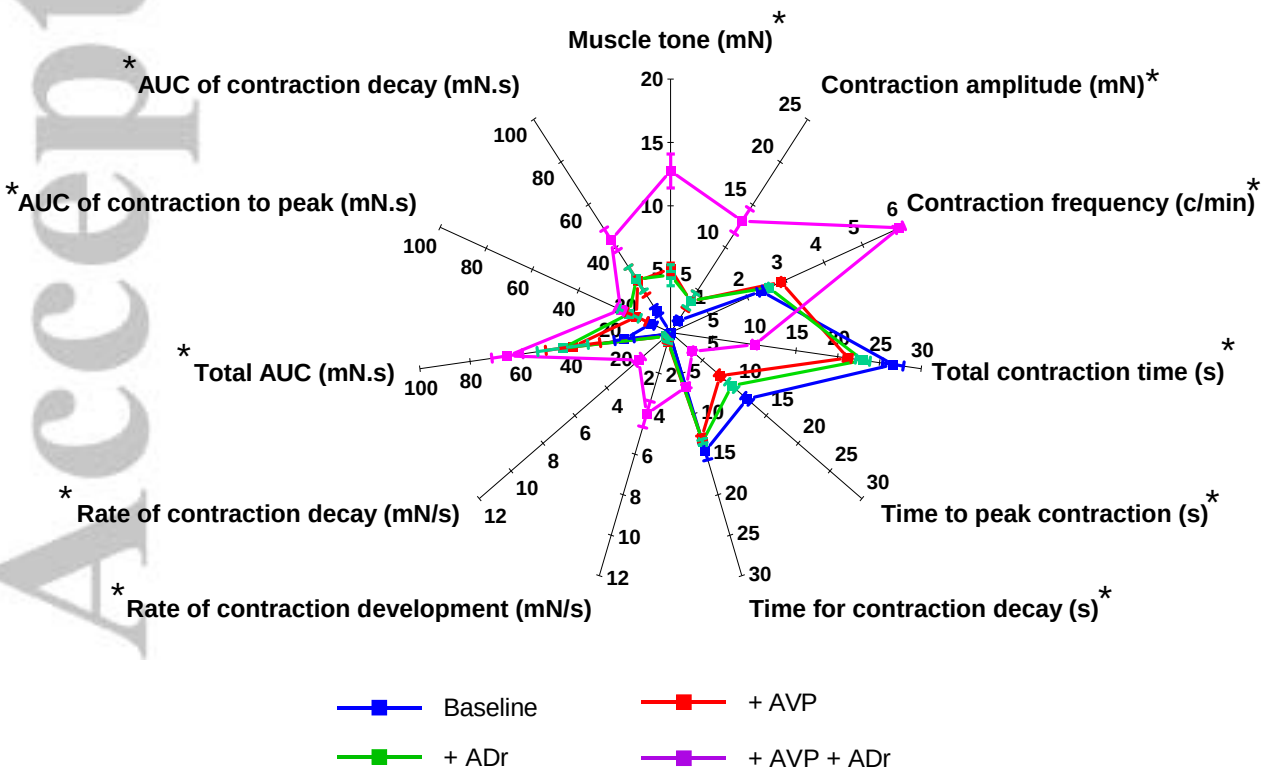

D

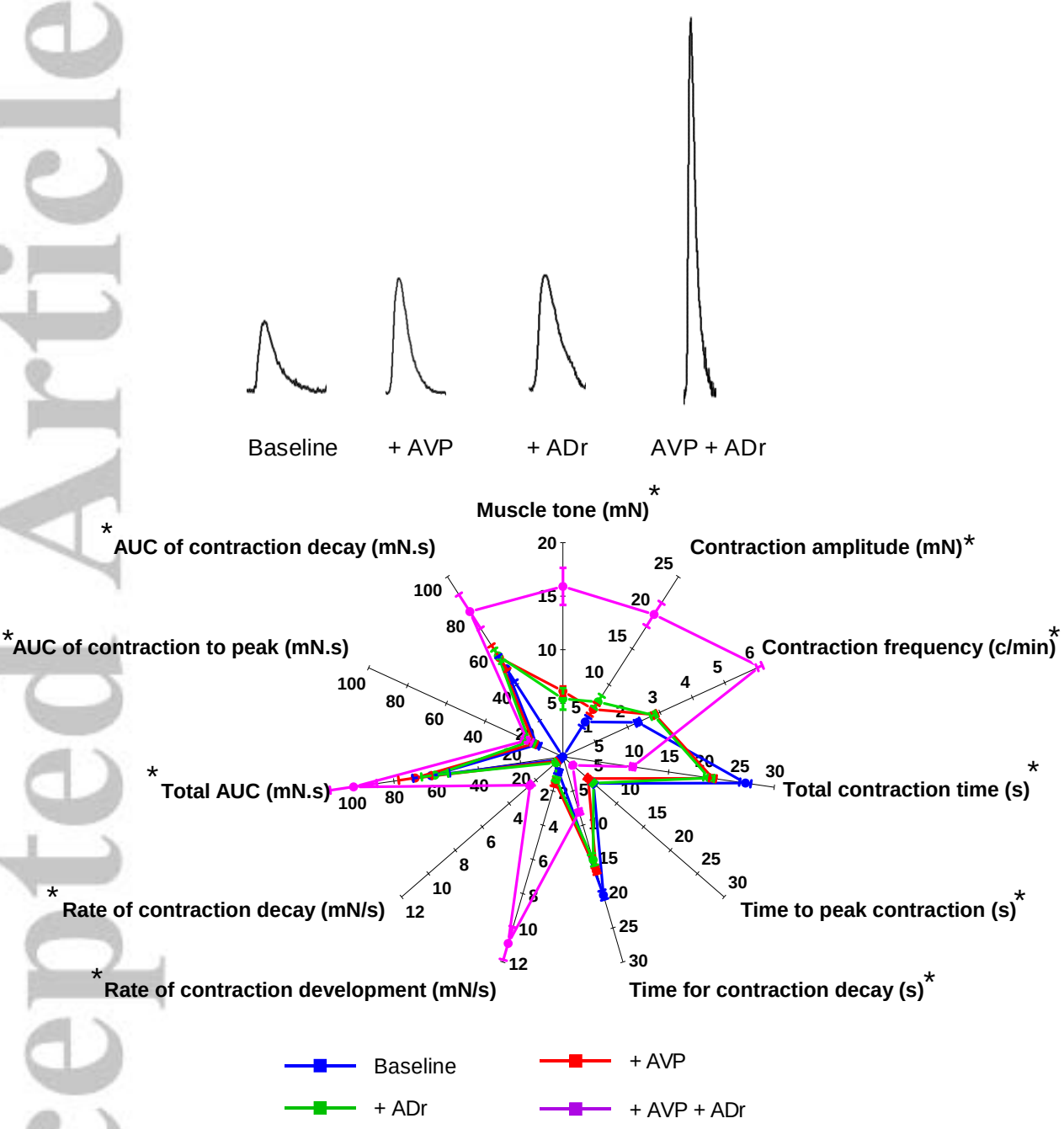

Figure 5: Effect of ADr on (A) spontaneous contractions of human (i) proximal and (ii) distal stomach. (B) ADr-induced increase in baseline muscle tone (i,ii), amplitude (iii,iv) and frequency (v,vi) of spontaneous contractions of proximal (graphs on left) and distal (right) stomach after 30min incubation with prazosin (10^{-7}M), propranolol (10^{-5}M) or vehicle (DMSO/water, 0.1% v/v). (C) Comparison of spontaneous contractions and their features in absence (Baseline) and presence of AVP (10^{-9}M), ADr (10^{-8}M) or co-application of AVP (10^{-9}M) and ADr (10^{-8}M). Concentrations of both hormones approximately equivalent to their EC_{75}. *P<0.05 between data sets for AVP and ADr applied together vs sum of corresponding data for AVP and ADr applied individually. Data are mean±S.E.M. n=5.

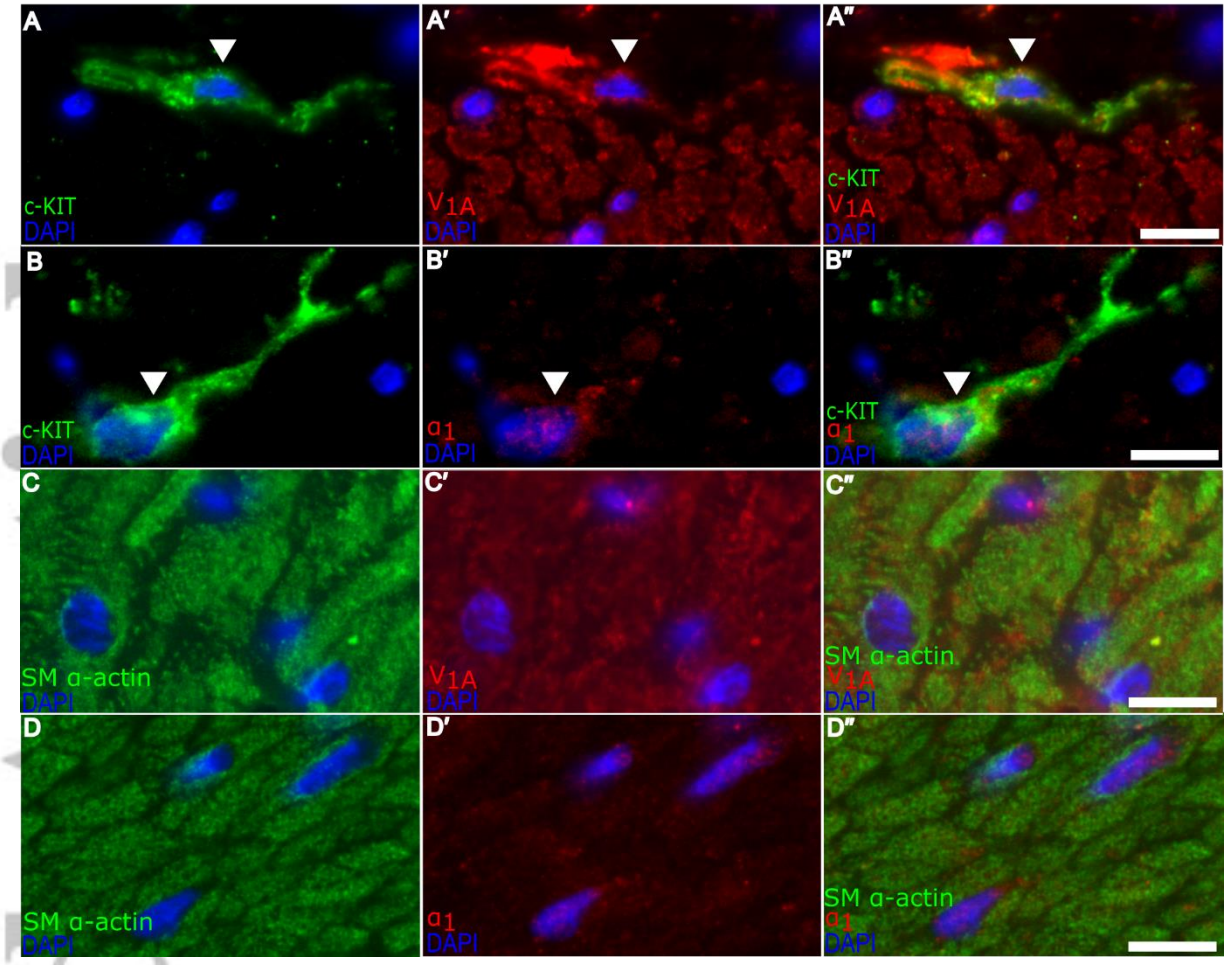

Figure 6: Fluorescence staining of c-KIT and V_{1A} or α_1 in human distal stomach circular muscle. White arrows across the panels indicate the same cell. (A) Shows a cell with DAPI-stained nucleus and emanating c-KIT-positive extensions, indictive of an ICC (white arrow). The same cell is shown (A') to stain for V_{1A}, and again in the merged images (A''). (B) Shows a DAPI-stained cell (white arrow) with c-KIT-positive extensions, (B') shows the same cell staining for α_1, while (B'') merges these images. Panel C shows a representative area of muscle with SM α-actin-positive muscle cells (C') shows these same cells staining for V_{1A} and (C'") merges these images. Panel D is arranged similarly and shows muscle cells staining for α_1. All images were captured at 40x magnification using Olympus BX61 microscope and SmartCapture3 software. The white scale bar represents 10µm.