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RESEARCH ARTICLE

Evidence for tetrodotoxin-resistant spontaneous myogenic contractions of mouse isolated stomach that are dependent on acetylcholine

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Background and Purpose: Gastric pacemaker cells, interstitial cells of Cajal (ICC), are believed to initiate myogenic (non-neuronal) contractions. These become damaged in gastroparesis, associated with dysrhythmic electrical activity and nausea. We utilised mouse isolated stomach to model myogenic contractions and investigate their origin and actions of interstitial cells of Cajal modulators.

Experimental Approach: Intraluminal pressure was recorded following distension with a physiological volume; tone, contraction amplitude and frequency were quantified. Compounds were bath applied.

Key Results: The stomach exhibited regular large amplitude contractions (median amplitude 9.0 [4.7–14.8] cmH_2O, frequency 2.9 [2.5–3.4] c.p.m; $n = 20$), appearing to progress aborally. Tetrodotoxin (TTX, 10^{-6} M) had no effect on tone, frequency or amplitude but blocked responses to nerve stimulation. ω-conotoxin GVIA (10^{-7} M) ± TTX was without effect on baseline motility. In the presence of TTX, (1) atropine (10^{-10}–10^{-6} M) reduced contraction amplitude and frequency in a concentration-related manner (pIC_{50} 7.5 ± 0.3 M for amplitude), (2) CaCC channel (previously ANO1) inhibitors MONNA and CaCCinh-A01 reduced contraction amplitude (significant at 10^{-5}, 10^{-4} M respectively) and frequency (significant at 10^{-5} M), and (3), neostigmine (10^{-5} M) evoked a large, variable, increase in contraction amplitude, reduced by atropine (10^{-8}–10^{-6} M) but unaffected (exploratory study) by the H1 receptor antagonist mepyramine (10^{-6} M).

Conclusions and Implications: The distended mouse stomach exhibited myogenic contractions, resistant to blockade of neural activity by TTX. In the presence of TTX, these contractions were prevented or reduced by compounds blocking interstitial

Abbreviations: CaCC, calcium-activated chloride channel; CTX, ω-conotoxin GVIA; GISMCA, Gastrointestinal Smooth Muscle Contraction Analysis; ICC, interstitial cells of Cajal; LALF, large amplitude, low frequency; MONNA, *N*-((4-methoxy)-2-naphthyl)-5-nitroanthranilic acid; pIC_{50}, negative $logarithm_{10}$ of the half-maximal inhibitory concentration; SAHF, small amplitude, high frequency; TTX, tetrodotoxin.

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cells of Cajal activity or by atropine and enhanced by neostigmine (antagonised by atropine), suggesting involvement of non-neuronal ACh in their regulation.

KEYWORDS
ACh, ANO1, CaCC, interstitial cells of Cajal, myogenic contractions, nausea, stomach

1 | INTRODUCTION

Gastric contractions depend on functional interactions between the vagus nerve, enteric nerves and interstitial cells of Cajal (ICC) (Goyal et al., 2019). Within the gastric body and antrum, the ICC act as pacemakers, spontaneously depolarising to generate regular, slow waves of electrical activity, conducted to the smooth muscle to depolarise and thereby initiate or facilitate rhythmic myogenic contractions (Sanders, 2020). However, whilst the electrical properties of the ICC and smooth muscle have been extensively studied, the relationships between ICC activity and the resultant myogenic contractions are less well understood. Where this has been investigated, muscle strips or sheets (usually with mucosa removed) from laboratory animals were used, to record electrical activity directly from the ICC in addition to the electrical and mechanical activity (tension) of the smooth muscle. These studies included the mouse (Forrest et al., 2006; Hwang et al., 2016, 2019; Worth et al., 2015) and the guinea pig stomach (Edwards & Hirst, 2006; Hashitani et al., 2005; Hirst & Edwards, 2006). However, the actions of compounds modulating electrical activity within the ICC have not always correlated with anticipated changes in mechanical or myogenic contractile activity of muscle strips (Boddy et al., 2004). An additional complication in understanding the relationships between ICC and muscle functions is illustrated by recent recordings of gastric myoelectric activity from the entire mouse and house musk shrew stomach *in vitro* (Liu et al., 2019). Such techniques require suppression of contractile activity by calcium channel antagonists as does recording of transmembrane potentials in the ICC and smooth muscle using microelectrodes. Together, these difficulties suggest a need to obtain a greater understanding of myogenic contractile activity in intact stomach preparations, both to establish a baseline of activity for comparison with electrical activity of the ICC and to facilitate the study of drugs and disease that modulate gastric movements.

We utilised an *in vitro* mouse whole stomach preparation to investigate and characterise rhythmic spontaneous contractions by recording intragastric pressure. The mouse was chosen as although its gastric morphology differs markedly from humans (e.g. location of the entry of the oesophagus and the aglandular proximal stomach) and is unable to vomit (associated with different brainstem connectivity and the absence of key endocrine and signalling pathways) (Horn et al., 2013; Sanger et al., 2011), it is nevertheless the species, together with the guinea pig, in which ICC functions have been studied in most detail (see Section 4 for references). Additionally, the mouse is readily amenable to molecular modification to study gastric motility (e.g. Mule & Serio, 2002). Intraluminal pressure was recorded via a cannula inserted into the stomach through the duodenum, preventing gastric emptying.

What is already known

- Depolarisation of interstitial cells of Cajal (ICC) expressing calcium-activated chloride channels (CaCC) initiates gastric contractions.
- In whole stomach, mechanisms regulating phasic 'myogenic' contractions (not neurally mediated) are poorly characterised.

What this study adds

- Large amplitude, low frequency myogenic contractions of the stomach are attenuated by CaCC channel block.
- Atropine blocks myogenic contractions, implicating tonic release of ACh from non-neuronal sources in their regulation.

What is the clinical significance

- Cooperativity between gastric ICC activity and non-neuronal ACh is a novel mechanism.
- Muscarinic receptor antagonism may influence gastric dysmotility associated with damaged ICCs (e.g. gastroparesis).

The aims were to (1), record and characterise rhythmic gastric muscle contractions following distension with a physiological volume of fluid, (2) pharmacologically isolate a myogenic (non-neuronal) component of the gastric contractions (following administration of the **Na_v channel** blocker **tetrodotoxin [TTX]**) and (3), investigate the involvement of ICC activity in generating myogenic contractions in the intact stomach. As the **calcium-activated chloride channel (CaCC)**, previously known as anoctamin-1 (Alexander et al., 2021), is a specific marker for ICCs in the mouse and human digestive tract (Gomez-Pinilla et al., 2009), we investigated compounds that block this channel, namely, **$CaCC_{inh}$-A01** (Namkung et al., 2011) and *N*-((4-methoxy)-2-naphthyl)-5-nitroanthranilic acid (MONNA; Oh et al., 2013, 2008). The results unexpectedly identified a control mechanism within the whole stomach involving non-neuronal ACh, not previously reported when using muscle strips. The latter has implications in understanding mechanisms by which gastric motility is controlled and disrupted during disorders in which nausea and dysrhythmic gastric electrical activity are reported (O'Grady et al., 2014).

2 | METHODS

2.1 | Animals

The study used 20 male and 20 female CD1 mice (25–35 g; 6–7 weeks old; randomly assigned to experimental groups of equal size except where exploratory studies were undertaken) obtained from Charles River UK Ltd. and housed in single sex groups (five animals per cage, cage size 194 mm × 181 mm × 398 mm; Allentown Inc, UK) with lignocel bedding (shredded nesting and envirotube), *ad libitum* access to pelleted food (5R58 irradiated diet; Labdiet®, IPS Products Supplies Ltd, UK) and water, in a light (07.00–19.00), temperature (21 ± 2°C) and humidity (55% ± 5.5%) controlled room. The mice were killed by cervical dislocation following the guidelines of our institutional Animal Ethics Committee in compliance with Annexe IV of EU 2010/63 and the UK, Animals (Scientific Procedures) Act 1986. Animal studies are reported in compliance with parts of the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020) where these relate to in vitro studies using animal tissue.

2.2 | Measurement of intragastric pressure

After opening the abdomen, the stomach was removed with the distal oesophagus and proximal small intestine (∼5 cm) attached and placed in a Sylgard 184 (Dow Corning Corp, USA)-lined chamber (50 ml) containing modified Krebs' solution (see below) gassed with 95% O_2/5% CO_2 and maintained at 37°C (TC120, Grant Instruments). A small, longitudinal incision (3–5 mm) was made in the proximal forestomach along the greater curve to facilitate removal of gastric contents by flushing with modified Krebs' solution. The oesophagus was ligated at the gastro-oesophageal junction and the forestomach incision closed with a ligature. A cannula (1.3-mm external diameter) filled with Krebs' solution was inserted into the stomach via the remnant duodenum (<1 cm) and secured with a ligature at the gastro-duodenal junction; the cannula tip was located in the antrum ∼5 mm from the ligature. Intragastric pressure was monitored following distension with 0.3-ml modified Krebs' solution (a volume within the range measured post-prandially in pilot studies) using a pressure transducer (TSD104A, BIOPAC System Inc., UK) connected to a DA 100C amplifier (BIOPAC System Inc) and data recorder (MP100A, BIOPAC System Inc), with the output displayed on a computer (Dell OptiPlex 7460). Pressure recording began ∼30 min after removing the stomach from the animal. The transducer was mounted at the same level as the Sylgard-lined base of the tissue bath and 'zero pressure' defined as the pressure measured by the transducer open to atmosphere. The effects of different compounds on the pressure changes were measured following their application to the solution bathing the serosa. No further injections of Krebs' solution were made into the stomach lumen throughout the experiment.

2.3 | Motility analysis

Two methods were used for analysis of pressure recordings. Firstly, a manual method (see below) measured baseline parameters (tone, contraction amplitudes and frequency; see below). These data defined preparation stability and were used to determine the effect of TTX. Secondly, an automated analysis (Gastrointestinal Smooth Muscle Contraction Analysis, see below) was used to average contraction waveforms and for comparison of changes in amplitude and frequency of the 'large amplitude, low frequency' (LALF) myogenic contractions (see Section 3) following administration of drugs in the presence of TTX.

2.3.1 | Manual method

This was undertaken by direct measurement (using a cursor) from the original recordings of the different parameters. The raw data (tone, contraction amplitude and frequency) before and after application of TTX, and in stomachs without TTX treatment over a comparable time period, were analysed based upon the average of measurements at specific time points and periods. The parameters are defined below.

- *Tone*: The prevailing pressure difference in cmH_2O between atmospheric zero and the level upon which rhythmic contractions were superimposed, measured at three time points (10, 20 and 30 min) before and after (40, 50 and 60 min) TTX.
- *Contraction amplitude*: This is the difference in pressure between the tone and the peak pressure achieved during a contraction. The amplitudes of the last 10 consecutive LALF contractions (see Section 3.1) were measured between 10 and 20 min and 20–30 min pre-TTX, with another 10 contractions measured post-TTX between 40–50 and 50–60 min. These were averaged for each stomach to represent the amplitude of contractions pre- and post-TTX application. Rhythmic contractions occurring in the presence of TTX are defined as 'myogenic' (Costa et al., 2013; Dinning et al., 2012).
- *Contraction frequency*: The number of LALF contractions (see Section 3.1) during specified 10-min periods expressed as contractions per minute (c.p.m.).

2.3.2 | Automated analysis

Contractile activity was analysed using Gastrointestinal Smooth Muscle Contraction Analysis (GISMCA, Version 0.4.1; https://github.com/agharibans/GISMCA/tree/master/GISMCA) software.

The motility parameters measured were defined exactly as in the manual analysis described above, using the GISMCA programme default setting (in which contractions were automatically identified and analysed only if the detected peaks were above the minimum amplitude of 0.05 cmH_2O and interpeak interval was 10 s). Time periods for analysis were defined by drug administration protocols

(see below) with the data on LALF contraction amplitude and frequency, and tone used to define the effects of drugs in the presence of TTX. The average value of the contraction amplitude and tone from the last five consecutive LALF contractions during each selected period of analysis (post-TTX [myogenic], post-each drug concentration) was used to quantify the effect of drug treatments on myogenic activity (i.e., in the presence of TTX). The automated analysis averaged contractions in the selected periods to produce a single contraction profile with a shadow showing the SEM. Frequency was measured over 10-min periods, as above.

The effects of drugs are expressed as a percentage of the measurements made in the presence of TTX alone (10^{-6} M) or TTX + **neostigmine** (10^{-5} M) (assigned a value of 100%), prior to their addition (see below). The effects of **atropine** displayed a sigmoidal concentration–response curve, so a pIC_{50} (the negative logarithm$_{10}$ of the half-maximal inhibitory concentration) was calculated for all parameters. For other drugs, the relationship was not sigmoidal so data are presented as histograms.

2.4 | Nerve stimulation

A limited number of experiments investigated the effect of TTX (10^{-6} M) and **ω-conotoxin GVIA (CTX**; 10^{-7}–1.3×10^{-6} M) on the gastric motility response to stimulation of electrodes placed around the distal oesophagus (bipolar platinum wire ring electrodes, likely to stimulate vagal nerve fibres and possibly, enteric neurones; $n = 6$) or across the wall of the stomach (transmural stimulation, using 10-mm-long bipolar platinum prong electrodes; one pole inside the stomach via the fundic incision and the other adjacent to the serosa; $n = 5$), each at 5- and 10-Hz frequency, 0.5-ms pulse width, up to 25 V (to elicit a maximal response at the frequencies used) for 60 s using a S88 stimulator, Grass Instrument Co., USA. These parameters were comparable with those used in similar mouse stomach preparations (Black & Shankley, 1985; Welsh et al., 1993).

2.5 | Protocol

Group size ($n = 5$) for investigating each potential modulator of myogenic contractions was selected based on the minimum number of mice, which Curtis et al. (2018) consider can be used to obtain a valid statistical analysis and is consistent with the minimal number of four to six mice used in comparable published studies (e.g. Mule & Serio, 2002; Worth et al., 2015).

Tissue manipulation was performed in Krebs' solution ($\times 10^{-3}$M: NaCl, 118.3; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; glucose, 11.1; $NaHCO_3$, 25; $CaCl_2$, 2.5) modified by addition of the H^+/K^+ ATPase inhibitor **omeprazole** (10^{-6} M) to reduce gastric acid secretion, volume change and possible mucosal damage (Coruzzi et al., 1986). A pilot study showed that when added to Krebs' solution separately, this concentration was the maximum possible without affecting motility. The Krebs' solution was gassed with 95% O_2/5% CO_2 and maintained at 37°C. Although pilot studies showed that preparations were viable for ~3.5 h, most studies were confined to ~2 h to ensure stability of baseline parameters.

The pharmacology of the contractions was investigated during the presence of TTX (30 min after application of TTX 10^{-6} M) by adding increasing concentrations of atropine to the bathing solution ($n = 5$; 10^{-10}–10^{-6} M) or the CaCC antagonists $CaCC_{inh}$-A01 ($n = 5$; 10^{-10}–10^{-5} M) and MONNA ($n = 5$; 10^{-9}–10^{-4} M), with 15 min between each increase in concentration. After pilot experiments establishing the duration of the response to neostigmine (10^{-5} M), it was added to the bathing solution 30 min after TTX (10^{-6} M) and left to equilibrate for 15–30 min prior to adding incremental concentrations of atropine ($n = 5$; 10^{-9}–10^{-6} M) with 15 min between each concentration. We investigated the potential role of **histamine (H_1) receptors** in the response to neostigmine using **mepyramine** ($n = 3$; 10^{-6}–10^{-4} M), added to the bathing solution with 15 min between each concentration. However, because effects were observed only at concentrations considered non-selective, we limited the study to $n = 3$ (precluding statistical analysis) but show all data points and an example of an original record. The effects of CTX (10^{-7} M) were investigated at least 30 min after application as prolonged contact times are required to achieve maximal activity in rat and guinea pig tissues (Sanger et al., 2000).

2.6 | Materials

$CaCC_{inh}$-A01 and MONNA were obtained from Tocris Bioscience, UK. Atropine sulphate, neostigmine bromide, omeprazole, TTX and CTX were from Sigma, Dorset, UK. Mepyramine maleate was from Cambridge Bioscience, UK. MONNA and $CaCC_{inh}$-A01 were dissolved in DMSO and atropine in 50% ethanol; the maximum final concentrations of DMSO and ethanol in the bathing solution were 0.1% and 0.02%, respectively, and were without effect at these concentrations. Neostigmine bromide, TTX and CTX were dissolved in double distilled water. Omeprazole was dissolved in Krebs' solution. DMSO and all other regents such as those for preparing Krebs' solution were from Fisher Scientific, UK.

2.7 | Data analysis and statistics

Analysis of recordings was undertaken in the knowledge of the drug treatment applied to each stomach *in vitro*. Although the analysis was not blinded (i.e. the drug applied was identified in the recording file), a large proportion of data analysis used an automated method independent of the operator and all analysed data were checked by another researcher. No data were excluded, and all data points are presented in the figures. For each drug, an example of an original recording is shown to support the validity of the numerical data.

Baseline motility data (contraction amplitude, frequency and tone) pre- and post-TTX (Figure 1) are presented (figures or text) in the original units (cmH_2O and c.p.m.). To illustrate the

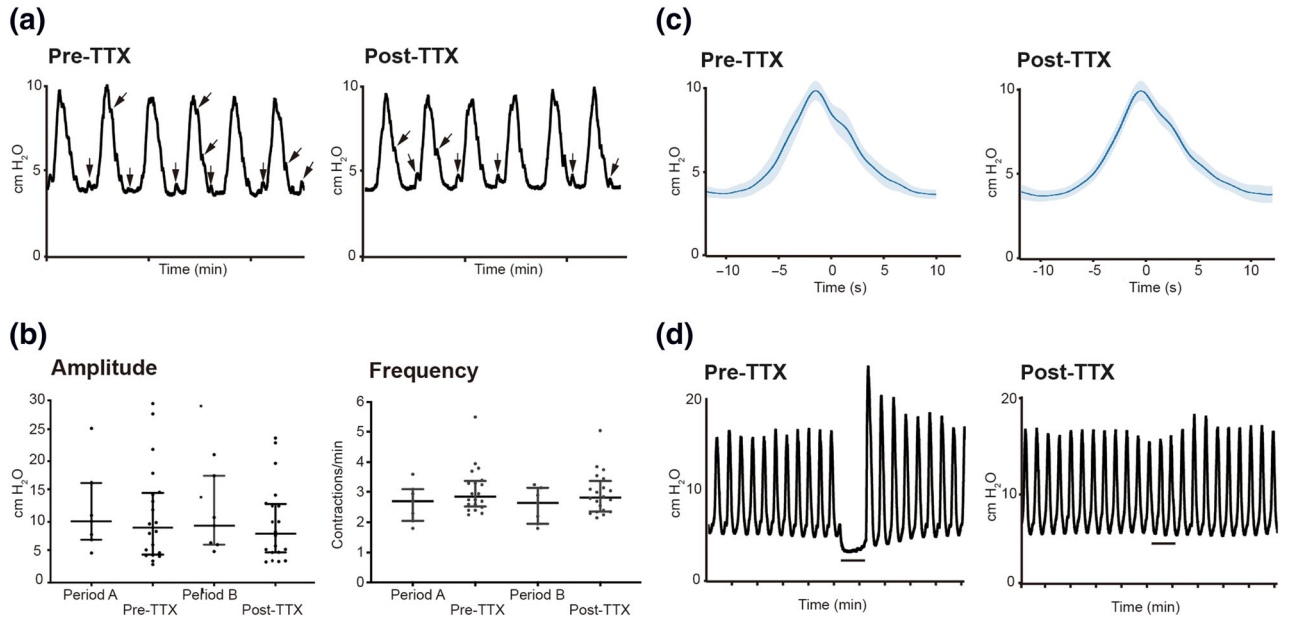

FIGURE 1 Baseline analysis of the mouse isolated stomach spontaneous contractions pre- and post-tetrodotoxin (TTX). (a) The spontaneous contractions pre- and post-TTX (10^{-6} M) administration. Black arrows show the small amplitude, high frequency (SAHF) contractions, between and superimposed upon the high amplitude, low frequency (LALF) contractions. (b) Analysis (see Section 2.3.1) of the spontaneous contraction amplitude (LALF) and frequency from 20 preparations pre-TTX and post-TTX (10^{-6} M) administration (see Section 2 for details) and 7 time-matched (periods A and B) preparations not treated with TTX. The median value and interquartile ranges are shown together with the individual data points. (c) An example of the pressure record averaged (see Section 2.3.2) over 10 min before TTX and between 20 and 30 min after TTX (10^{-6} M). The solid line and the shadow represent the average and the SEM value, respectively. Time 0 corresponds to the peak of each contraction. (d) Representative recording showing block of electrical stimulation (circumoesophageal electrodes 10 Hz, 0.5 ms, 20 V, 1 min, horizontal bar) induced reduction in tone and abolition of contractions by TTX (10^{-6} M)

concentration-related activity of drugs, the magnitude of which may depend upon baseline values, we normalised the effects of atropine, MONNA and $CaCC_{inh}$-A01 (which were inhibitory) as a percentage of the post-TTX baseline values for each stomach in that group, using these values for statistical analysis (Figures 2–4). Similarly, the inhibitory effects of increasing concentrations of atropine on the excitatory response to neostigmine (in the presence of TTX) were normalised relative to the neostigmine (% neostigmine + TTX; Figure 5).

The declared group size is the number of independent values; statistical analysis was done using these independent values and was only undertaken where group size was ≥5. Data in the text are reported as median with interquartile range in parentheses (n = number of stomachs) and in histograms as medians with all individual data points shown, and in Figure 1 as medians with interquartile range and all data points. Calculations were made using Microsoft Excel (Microsoft Excel, RRID:SCR_016137) and GraphPad Prism Version 8 or 9 (GraphPad Prism, RRID:SCR_002798; GraphPad Software, USA) also used for plotting histograms. Statistical comparisons of the effects of increasing concentrations of drug on each parameter (amplitude, frequency and tone) were undertaken by comparing the value normalised to the baseline value in the presence of TTX (100%) using the non-parametric Friedman test (matched samples where assumption of equal variances is not applicable) with Dunn's multiple comparison test. For specific data sets, a Mann–Whitney test was used for comparison. Statistical significance throughout was $P < 0.05$.

For the cumulative concentration–response study of the effects of atropine following TTX (Section 2.5), a sigmoidal $\log_{10}$ (inhibitor) concentration–response curve was fitted using a four-parameter logistic equation with automatic outlier elimination using GraphPad Prism Version 8.0 (GraphPad Prism, RRID:SCR_002798; GraphPad Software). pIC_{50} values are reported as mean ± SEM (n = number of stomachs).

The manuscript complies with the *British Journal of Pharmacology*'s recommendations and requirements on experimental design and analysis (Curtis et al., 2018).

2.8 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander et al., 2021).

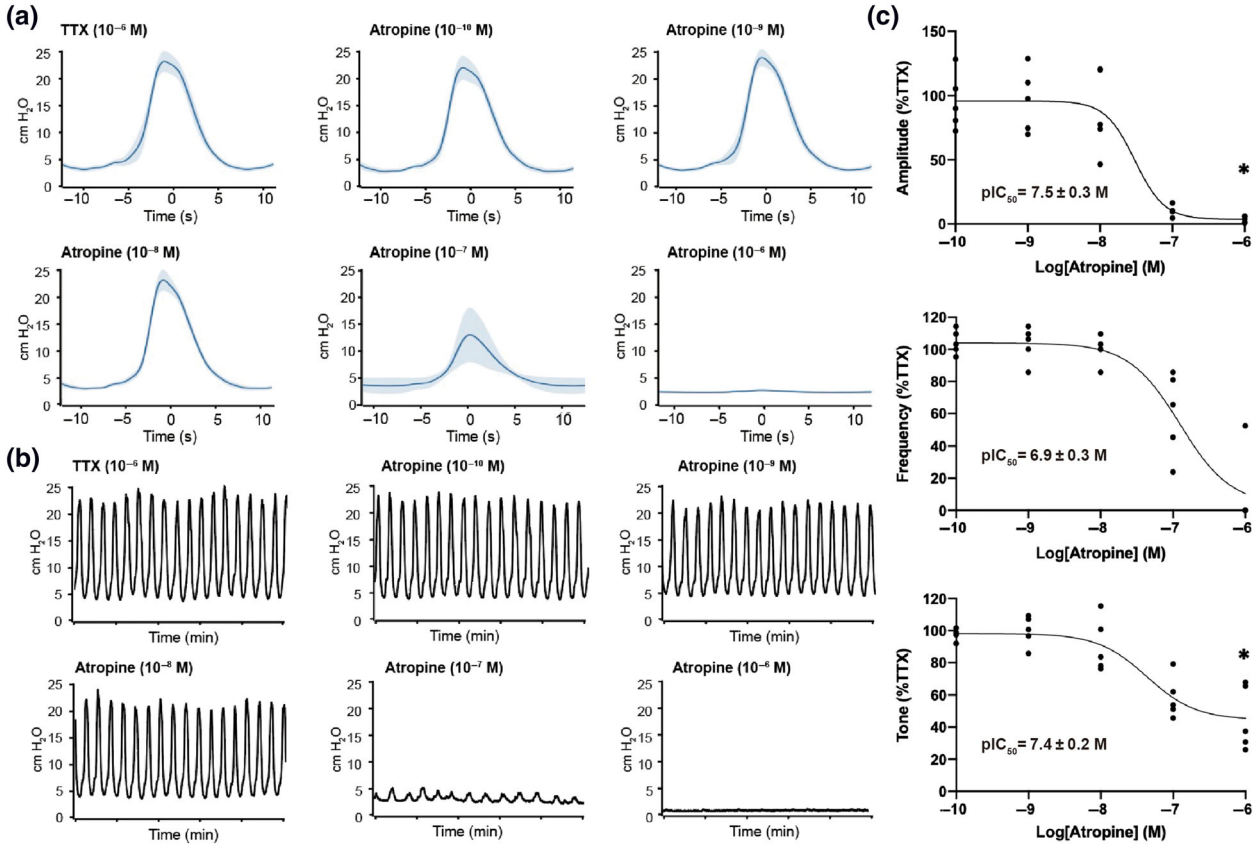

FIGURE 2 Antagonism of mouse stomach myogenic contractions by atropine. (a) Examples of the pressure record averaged following incremental concentrations of atropine in the presence of TTX (10^{-6} M; see Section 2.3.2 for analysis). The solid line represents the average, and the shadow illustrates the SEM. Time 0 corresponds to the peak of each contraction. (b) Examples of 5-min segments of original pressure recordings showing the effect of incremental concentrations of atropine in the presence of tetrodotoxin (TTX; 10^{-6} M). (c) Concentration–response curves for the effect of atropine on the contraction amplitude, frequency and tone relative to the baseline values in the presence of TTX (10^{-6} M). Individual values are plotted together with the best fit curve ($n = 5$). Friedman's test/Dunn's comparison of each atropine concentration with TTX; *$P < 0.05$

3 | RESULTS

3.1 | Characterisation of baseline motility

Immediately on placing the stomach in the tissue bath, rhythmic contractile activity, passing as a deepening ring wavefront from the proximal to the distal stomach, became visible and continued following distension with a physiological volume (0.3 ml; see Section 2.2) of Krebs' solution. Inspection of the pressure recordings following distension revealed two types of contractile activity with differing amplitudes and frequencies. Based on analysis of baseline recordings from 20 stomachs prior to application of TTX (see below), the most prominent contractions were LALF (Figure 1a). These had an amplitude of 9.0 (4.7–14.8) cmH_2O and frequency of 2.9 (2.5–3.4) c.p.m. ($n = 20$).

Smaller (<1 cmH_2O) contractions were also visible with a higher frequency (>5 c.p.m). These 'small amplitude, high frequency' (SAHF) contractions were manifest in the pressure record as oscillations visible between the LALF contractions and also superimposed on the larger contractions giving some a serrated appearance (Figure 1a). Close inspection of 10 large amplitude contractions from each of 20 stomachs (in the 10 min immediately prior to TTX) revealed that in all stomachs the 'large' contractions were recorded but 'small' contractions were apparent in 75% of stomachs. Of these, 63% (126/200) of LALF contractions had SAHF contractions visible as a serration on the contraction and/or present between the larger contractions. Analysis of the SAHF contractions over a 5-min period from 10 animals in which these contractions were clearly recorded (between or superimposed upon the larger contractions) identified a frequency for the large contractions of 3.0 (2.4–3.7) c.p.m., but when all contractions (small and large) were counted, the overall frequency was 8.8 (6.8–9.3) c.p.m. When determining the effects of TTX and other drugs, this study confined itself to the prominent, consistently recorded LALF contractions for quantitative analysis.

3.2 | Effect of tetrodotoxin (TTX) and ω-conotoxin GVIA (CTX) alone and in combination

Comparison of baseline motility data with that in the presence of TTX (10^{-6} M) using 20 stomachs revealed that TTX had no effect on

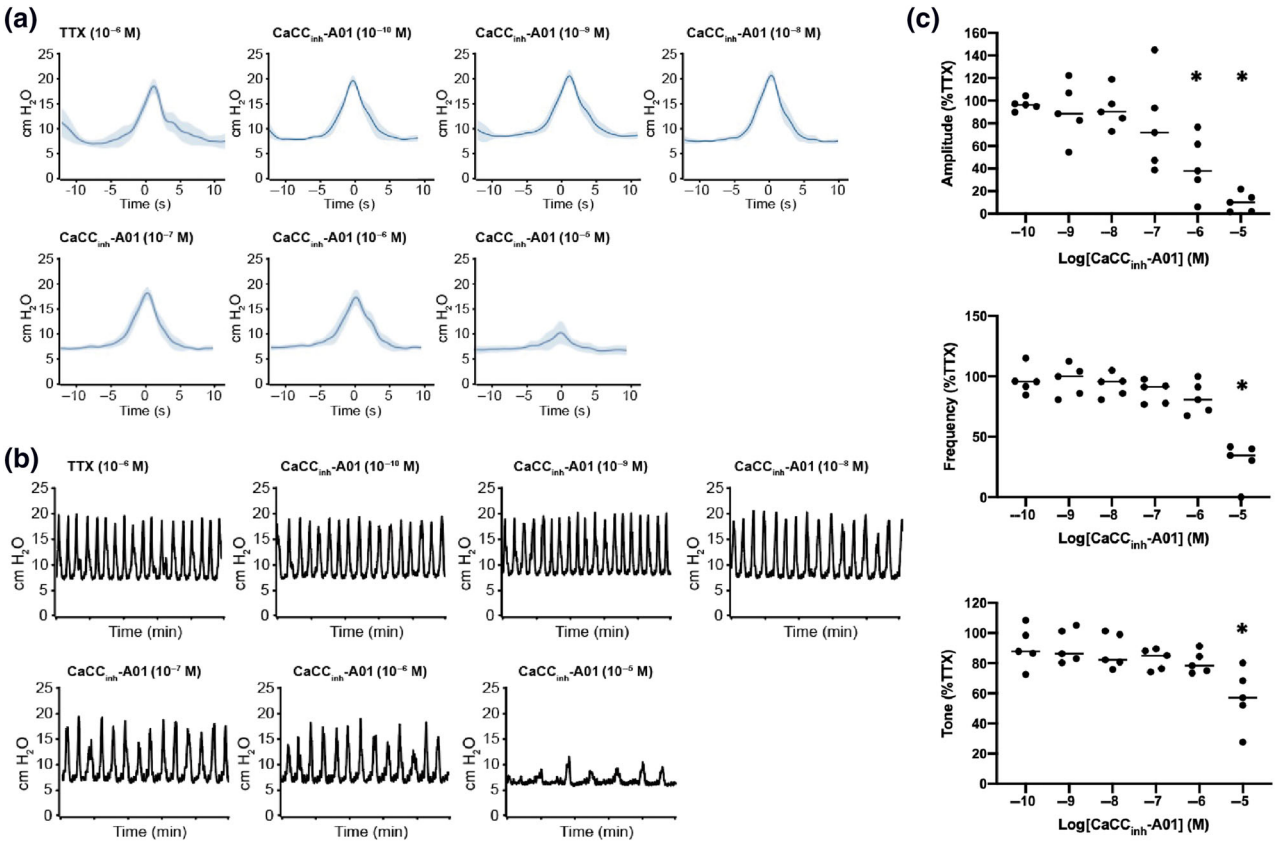

FIGURE 3 Antagonism of mouse stomach myogenic contractions by the calcium activated chloride channel (CaCC) inhibitor ($CaCC_{inh}$-A01). (a) Examples of the pressure record averaged following incremental concentrations of $CaCC_{inh}$-A01 in the presence of tetrodotoxin (TTX; 10^{-6} M; see Section 2.3.2 for analysis). The solid line represents the average, and the shadow illustrates the SEM. Time 0 corresponds to the peak of each contraction. (b) Examples of 5-min segments of original pressure recordings showing the effect of incremental concentrations of $CaCC_{inh}$-A01 in the presence of TTX (10^{-6} M). (c) Concentration–response curves for the effect of $CaCC_{inh}$-A01 on the contraction amplitude, frequency and tone relative to the baseline values in the presence of TTX (10^{-6} M). Values are median with all individual values plotted ($n = 5$). Friedman's test/Dunn's comparison of all concentrations of $CaCC_{inh}$-A01 with TTX (see Section 2.7); *$P < 0.05$

contraction amplitude or frequency when compared with time-matched controls (Figure 1a,b). Further, the profile of averaged contractions (Figure 1c) measured using automated analysis revealed no change in the shape of the LALF contractions with TTX. Inspection of the recordings showed that SAHF contractions were also present following TTX (Figure 1a), but as noted above, quantification of these contractions was not undertaken.

Electrical nerve stimulation (electrodes around oesophagus [$n = 6$] and positioned transmurally [$n = 5$], stimulated at 5 and 10 Hz for 60 s) was used to evaluate the efficacy of TTX (10^{-6} M), in blocking nerve-mediated activity. Both stimulation methods reduced gastric tone and markedly reduced the amplitude of ongoing rhythmic contractions, and these responses were abolished by TTX (see Figure 1d for example using circumoesophageal electrodes).

The effects of TTX do not preclude the spontaneous release of ACh from nerve terminals, so the effects of CTX (10^{-7}–1.3×10^{-6} M) were investigated. Addition of CTX (10^{-7} M; $n = 7$) was without significant effect on the averaged spontaneous contraction waveform or motility parameters when given alone or in the presence of subsequently administered TTX (10^{-6} M) (Figure S1). CTX (10^{-7}–1.3×10^{-6} M) also failed to prevent the inhibitory response to electrical nerve stimulation (Figures S2 and S3). TTX (10^{-6} M) administered after application of CTX blocked the inhibitory responses to electrical nerve stimulation (Figure S3).

3.3 | Effect of atropine in the presence of TTX

Addition of atropine (10^{-10}–10^{-6} M) to stomachs treated with TTX (10^{-6} M) reduced the amplitude of spontaneous contractions with the effect particularly marked at ≥10^{-7} M (~90% inhibition), when tone was also reduced (~40%); at 10^{-6} M, contractions were virtually undetectable (Figure 2a,b). The $\log_{10}$ concentration–response curves for all parameters were approximately sigmoidal, with pIC_{50} values of 7.5 ± 0.3, 6.9 ± 0.3 and 7.4 ± 0.2 (not significantly different; $n = 5$) for the effect of atropine on contraction amplitude, frequency and tone, respectively (Figure 2c).

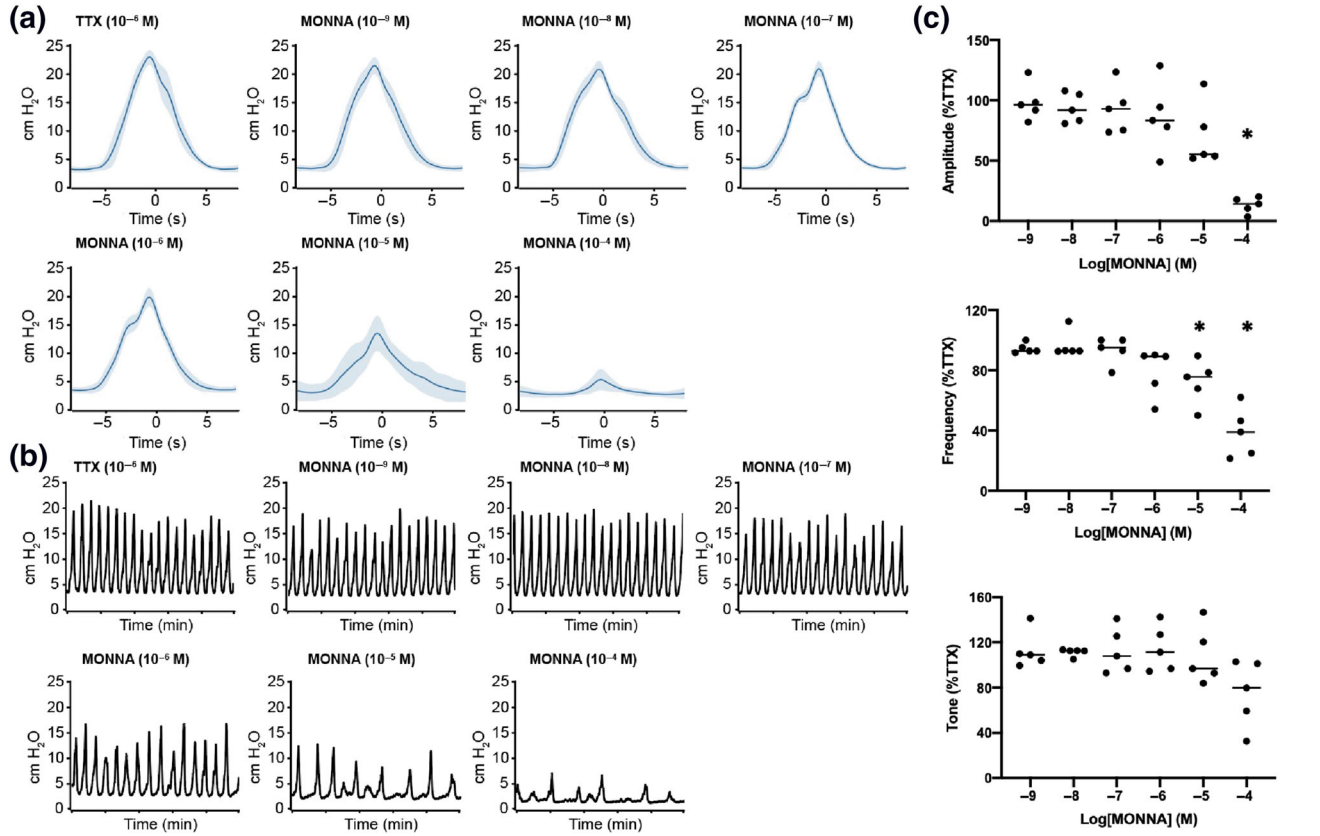

FIGURE 4 Antagonism of mouse stomach myogenic contractions by the calcium activated chloride channel (CaCC) inhibitor (MONNA). (a) Examples of the pressure record averaged following incremental concentrations of MONNA in the presence of tetrodotoxin (TTX; 10^{-6} M; see Section 2.3.2 for analysis). The solid line represents the average, and the shadow illustrates the SEM. Time 0 corresponds to the peak of each contraction. (b) Examples of 5-min segments of original pressure recordings showing the effect of incremental concentrations of MONNA in the presence of TTX (10^{-6} M). (c) Concentration–response curves for the effect of MONNA on the contraction amplitude, frequency and tone relative to the baseline values in the presence of TTX (10^{-6} M). Values are median with all individual values plotted ($n = 5$). Friedman's test/ Dunn's comparison of all concentrations of MONNA with TTX (see Section 2.7); $^{*}P < 0.05$

3.4 | Effect of $CaCC_{inh}$-A01 in the presence of TTX

$CaCC_{inh}$-A01 had no statistically significant effects on contraction amplitude until a concentration of ≥10^{-6} M was reached (Figure 3a,b). $CaCC_{inh}$-A01 at 10^{-5} M significantly reduced the contraction frequency (~50% inhibition) and tone (~40%) (Figure 3c).

3.5 | Effect of MONNA in the presence of TTX

Lower concentrations of MONNA (10^{-9}–10^{-5} M) were without statistically significant effects on contraction amplitude, but frequency was significantly reduced at ≥10^{-5} M. MONNA at 10^{-4} M significantly reduced contraction amplitude (~80% inhibition) and frequency (~60%), also tending to reduce tone (~20%) (Figure 4a–c).

3.6 | Effect of neostigmine in the presence of TTX

Although the myogenic contractions were markedly reduced in amplitude in a concentration-related manner by atropine in the presence of TTX (Section 3.3), additional evidence for an involvement of ACh in these contractions was sought by investigating the effects of the cholinesterase inhibitor neostigmine. Because of closure of the laboratory (Covid-19 pandemic restrictions), these studies were undertaken several months after the other studies reported. Compared with the earlier studies, the baseline contraction amplitude (i.e. post-TTX and pre-neostigmine) and tone were both lower and in the case of amplitude, more variable (respectively for the two groups: 8.0 [5.0–13.0] cmH_2O vs. 1.4 [0.9–27.1] cmH_2O, and 4.3 [3.0–6.3] cmH_2O vs. 3.1 [2.8–4.8] cmH_2O; $n = 20$ original group and 8 new group used to test neostigmine) although the differences did not achieve statistical significance. However, the contraction frequency (respectively 2.8 [2.4–3.4] c.p.m. vs. 3.6 [3.4–3.8] c.p.m.) was significantly greater between the two groups.

In the presence of TTX (10^{-6} M), neostigmine (10^{-5} M; Figure 5a) produced an increase in tone with a rapid onset and was of variable magnitude (δ 1.3 [0.9–1.9] cmH_2O; $n = 8$) upon which were superimposed rhythmic contractions with an increased amplitude (δ 7.6 [6.2–12.6] cmH_2O; $N = 8$) but unchanged frequency (δ 0.00 [−0.15 to 0.00] c.p.m.; $n = 8$). The response lasted at least 30 min.

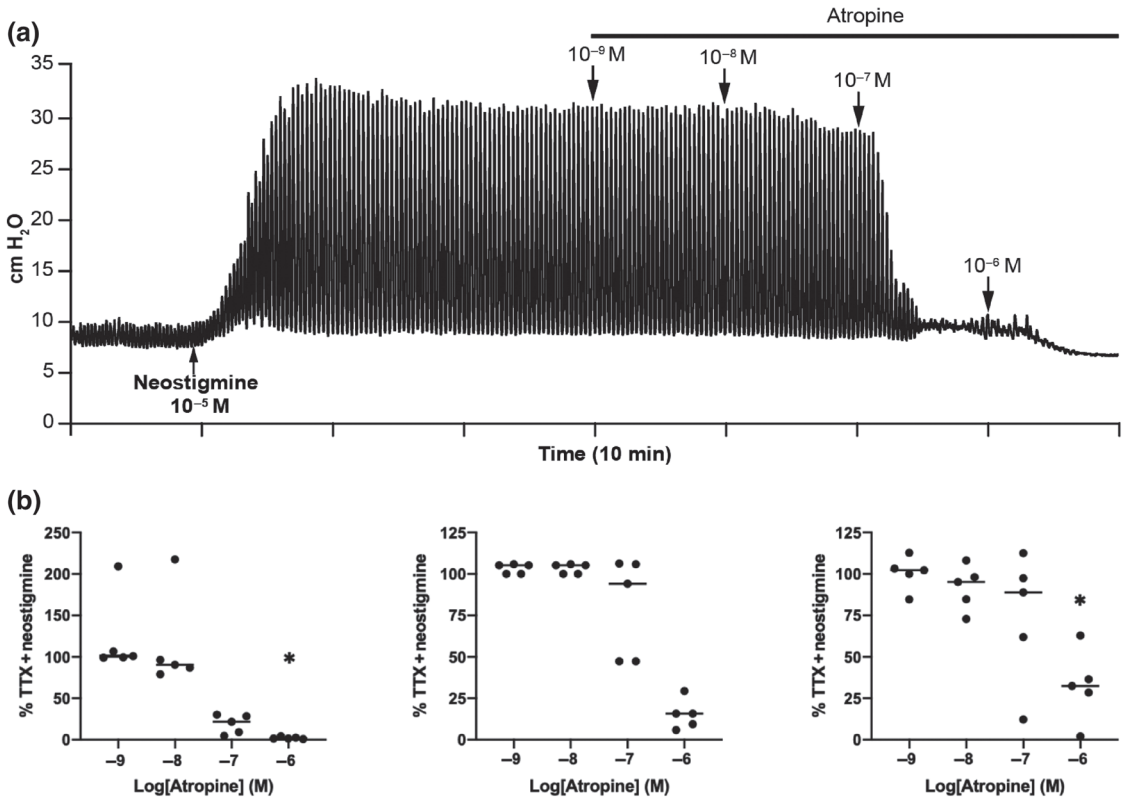

FIGURE 5 Antagonism of neostigmine enhanced myogenic contractile activity by atropine. (a) An original recording selected to illustrate the protracted stimulation of contraction amplitude by neostigmine (10^{-5} M) in the presence of tetrodotoxin (TTX; 10^{-6} M). In this experiment, the effects of atropine (10^{-9}–10^{-6} M) were investigated 30 min after neostigmine. (b) Quantification of the effect of incremental concentrations of atropine (10^{-9}–10^{-6} M) on the response to neostigmine (10^{-5} M) in the presence of TTX (10^{-6} M) with addition of atropine commencing 15 min after neostigmine (see Section 2). The effect of atropine on contraction amplitude, frequency and tone is plotted in relation to the TTX + neostigmine response. Responses are plotted as median with individual values plotted. Friedman's test/Dunn's test comparing responses to incremental concentrations of atropine to the response to neostigmine in the presence of TTX; *$P < 0.05$

3.7 | The effect of atropine and mepyramine on the response to neostigmine in the presence of TTX

In five stomachs treated with neostigmine (see Section 3.6), the effect of incremental concentrations of atropine was investigated (Figure 5a,b). The increase in contraction amplitude and tone induced by neostigmine was significantly reduced by atropine at 10^{-6} M. Contraction frequency tended to be reduced by 10^{-6} M of atropine, but this was not statistically significant (Figure 5b).

As **histamine** is a potent stimulant of gastric contractions (via H_1 receptors) and is present in the stomach in large amounts (see Section 4), we hypothesised that it might also be involved in the non-neuronal responses together with ACh. However, in these preliminary studies using stomachs treated with TTX alone (10^{-6} M), the enhanced contraction amplitude response to neostigmine appeared unaffected by mepyramine (10^{-6} M; $n = 3$), although reduced in an apparently concentration-related manner at higher concentrations (10^{-5}, 10^{-4} M; $n = 3$) (Figure 6a,b); the neostigmine-induced increase in tone and contraction frequency appeared unchanged (Figure 6b).

4 | DISCUSSION

Large amplitude, low frequency (LALF) contractions of mouse whole stomach occurred without TTX-sensitive neuronal control, by a mechanism likely dependent on spontaneous depolarisation of pacemaker cells, perhaps enhanced by the physiological distension of the stomach (mouse stomach intramuscular interstitial cells of Cajal (ICCs) are stretch sensitive; Won et al., 2005). This conclusion has some consistency with Beckett et al. (2003), who recorded slow wave electrical activity in mouse gastric antrum during the presence of TTX. However, in the present study, the contractions were reduced by atropine and increased by neostigmine in a TTX-resistant manner, suggesting additional control involving a TTX-insensitive source of ACh.

In the mouse stomach in the presence of TTX, the most prominent contractions were large amplitude and ~3-c.p.m. frequency. Small amplitude, high frequency (SAHF) contractions were superimposed upon and interspersed between the larger contractions. Combining both (when both present) gave a frequency of ~8 c.p.m. Thus, the 3–8 c.p.m. range covers the values reported for electrical recordings of mouse gastric myoelectric (slow wave) activity in the

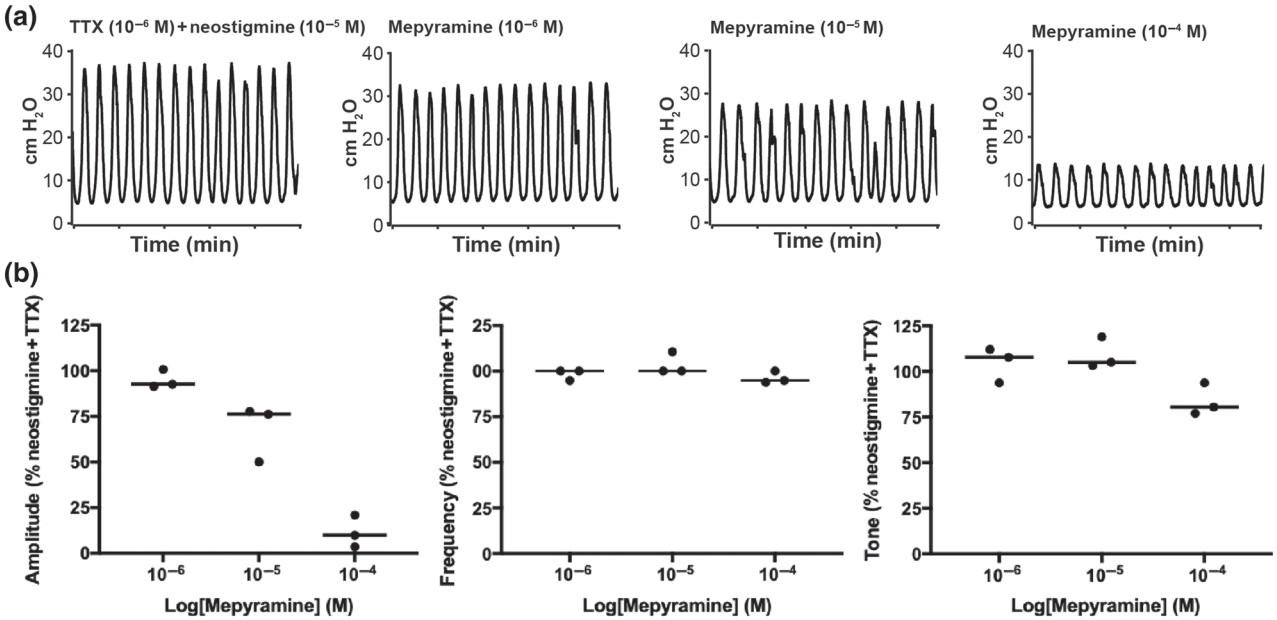

FIGURE 6 Preliminary experiments showing the effect of mepyramine on the response to neostigmine in the presence of tetrodotoxin (TTX). (a) Examples of 5-min segments of original pressure recordings showing the effect of incremental concentrations of mepyramine (10^{-6}–10^{-4} M) on the response to neostigmine (10^{-5} M) in the presence of TTX (10^{-6} M). (b) Concentration–response curves for the effect of mepyramine on the contraction amplitude, frequency and tone relative to values in the presence of neostigmine (10^{-5} M) and TTX (10^{-6} M). Responses are plotted as median with all individual values shown ($n = 3$)

corpus (~6–8/min; Kim et al., 2003; Liu et al., 2019; Worth et al., 2015) and antrum (~3–4/min; Forrest et al., 2006; Hwang et al., 2016; Kim et al., 2003; Sanders, 2020), and also the contraction frequency in antrum circular muscle (3.6 ± 0.5 c.p.m.; Hwang et al., 2019). We hypothesise that the higher frequency SAHF contractions reflect activity originating in the proximal stomach, whereas LALF contractions (see van Helden et al., 2010) reflect antral contractions.

The LALF contractions were myogenic because of insensitivity to TTX and CTX (Costa et al., 2013; Dinning et al., 2012). We confirmed that TTX (10^{-6} M) blocked the inhibitory effect of electrical nerve stimulation on mouse stomach contractility, consistent with blockade of nerve-mediated acid secretion by TTX in mouse stomach (Angus & Black, 1982) and effects on gastrointestinal (GI) movements (e.g. Costa et al., 2013; Hibberd et al., 2017; Mule & Serio, 2002). A limitation of the experiments with CTX was that its ability to block nerve-mediated responses was not confirmed when using the same protocol (but see below for discussion).

The lack of effect of TTX (and CTX) on contractions concords with the slow wave electrical activity in mouse gastric antrum recorded during the presence of TTX (Beckett et al., 2003). Nevertheless, enteric nerve reflexes are triggered during the dynamic phase of gastric distension in guinea pig isolated stomach (Hennig et al., 1997). Accordingly, it remains a possibility that spontaneous enteric nerve activity could occur via TTX-resistant Na_v channels (Bartoo et al., 2005) or calcium channels involved in neurotransmitter release (Waterman, 2000). Further studies with neurotoxins are required (see below).

Pacemaker cells were involved in generating the LALF myogenic contractions. Thus, in the presence of TTX, the CaCC inhibitors MONNA and $CaCC_{inh}$-A01 each reduced contraction amplitude and frequency, with significant effects at the higher concentrations. For $CaCC_{inh}$-A01, the effective concentrations were in the range that reduced frequency and duration of mouse gastric antral electrical slow waves (Hwang et al., 2016, 2019), implicating the ICC in their origin. The highest concentrations of MONNA and $CaCC_{inh}$-A01 also reduced muscle tone. These compounds (10^{-7}–10^{-5} M) have also been shown to relax **noradrenaline** or **U46619**-induced mouse vasoconstriction in a Cl^--free environment, suggesting activity additional to CaCC blockade (Boedtkjer et al., 2015).

ACh may be involved in generation of LALF myogenic contractions. Thus, the >90% reduction in contraction amplitude by atropine in the presence of TTX suggests a tonic release of ACh, possibly from non-neuronal sources. An action on the ICCs is supported by electrophysiological studies of mouse cultured ICC (Kim et al., 2003) and mouse stomach cholinergic transmission (Sung et al., 2018). Elsewhere, in rat isolated stomach, it has been suggested that the generation of contractions is dependent on cholinergic nerve activity (Zhang et al., 2011). However, cholinergic activity was defined by use of only atropine and not by further study with neuronal toxins. In flat sheet preparations of mouse gastric antrum or corpus antrum, measuring slow wave electrical activity in the presence of **nifedipine**, the frequency and propagation of slow waves were increased by electrical field stimulation (EFS) or neostigmine, both blocked by muscarinic antagonism or by TTX/**hexamethonium** (Forrest et al., 2006; Worth et al., 2015). A previous study (Beckett et al., 2003) showed an ability

to detect slow wave activity during the presence of atropine or TTX, so the experiments with neostigmine enabled Forrest et al. (2006) to suggest the existence of an ongoing spontaneous release of ACh (from a neural source and/or affecting neuronal function), the assumption being that endogenous AChE activity 'neutralised' the released ACh during baseline conditions.

In the present experiments, in the presence of TTX, neostigmine increased the amplitude of the LALF contractions. The prolonged facilitation is consistent with a tonic release of ACh, further supported by the atropine sensitivity of the response, which was of comparable magnitude with that of atropine on baseline activity in the presence of TTX. Worth et al. (2015) also found an atropine-sensitive increase by neostigmine of the propagation of contractions in mouse stomach flat sheets. Moreover, the present experiments find similarity to those obtained using rat and guinea pig urinary bladder, in which TTX, and **ω-agatoxin IVA** and CTX (predominantly $Ca_v2.1$ and $Ca_v2.2$ channel blockers)-insensitive spontaneous release of ACh were involved in the genesis of rhythmic contractions (Zagorodnyuk et al., 2009). In summary, therefore, we conclude that in mouse isolated stomach, myogenic activity may be influenced by non-neuronal ACh. Perhaps, the contrast with the conclusions of Forrest et al. (2006) may be explained by the different preparations (whole stomach vs. flat sheet) and measured endpoints (electrical activity vs. muscle contraction).

Non-neuronal sources of ACh are found in gastro-oesophageal epithelia and gastrointestinal immune cells, including tuft cells (Beckmann & Lips, 2013; Cox et al., 2020; Grando et al., 2020; Schneider et al., 2019; Wessler & Kirkpatrick, 2008; Wolf-Johnston et al., 2012). The rat colonic epithelium is also a non-neuronal source of ACh, which is implicated in the regulation of ionic transport and proposed to be an interface between the colonic microbiome and the host (Bader et al., 2019; Bader & Diener, 2018). In addition, intramuscular ICCs may be a source of paracrine substances, based on constitutive expression of **COX-2** (Porcher et al., 2002). Further studies are required in mouse stomach to identify the hypothesised non-neuronal source of ACh.

We considered that the released ACh might affect other cells involved in gastric regulation, such as the enterochromaffin-like or mast cells in the lamina propria and muscle, both of which release histamine (Buhner & Schemann, 2012; Hakanson & Sundler, 1991). Histamine contracts gastric muscle (e.g. Sim et al., 1989) and can enhance rhythmic contractile activity (Naganuma et al., 2018). However, the H_1 receptor antagonist mepyramine, at a concentration likely to substantially antagonise H_1 receptor functions (10^{-6} M), in preliminary experiments had no effect on the motility response to neostigmine in the presence of TTX. Higher concentrations of mepyramine, but with affinity for **muscarinic receptors** (Kubo et al., 1987), reduced the amplitude of the LALF contractions. These data suggest that although H_1 receptors are expressed by cultured ICC from murine intestine (Kim et al., 2013) and elsewhere in the gastrointestinal tract (Sander et al., 2006), they are unlikely to play a role in the control of myogenic activity. We cannot exclude the release of other mediators from the mucosa, such as **5-HT** from gastric enterochromaffin cells.

Although we focus above on the origin of the hypothesised non-neuronal ACh, its potential target(s) should not be overlooked. This is most likely to be muscarinic receptors located on the ICC (Sanders, 2020), but the gastric muscle itself cannot be excluded.

4.1 | Study limitations

Recording of intragastric pressure in mouse isolated stomach has been used infrequently to investigate gastric physiology and pharmacology (e.g., Mule & Serio, 2002). Although the isolated stomach retains a high degree of anatomical and functional integrity, the motor behaviour will inevitably differ from that *in vivo*. Nevertheless, intragastric pressures reflect the overall motor activity (especially when recorded in a small volume), comprising muscle contractions (particularly the LALF) and tone, and can therefore be more representative of gastric physiology than use of muscle strips/sheets, sometimes mucosa free. Additional studies (e.g. high resolution video recording with pressure recording, e.g. Berthoud et al., 2002) are needed to investigate regional motility.

Gastric tone, contraction amplitude and frequency were quantified, but because more than one contraction may present in different stomach regions, the frequency may not equate directly to the frequency of pacemaker activity recorded from ICCs using electrophysiology techniques.

Our primary conclusion relies on the effects of TTX and atropine, supported by findings with other agents presumed to act on the ICCs. The lack of effect of CTX (10^{-7}–1.1×10^{-6} M) on neuronal functions blocked by TTX is acknowledged as a limitation to drawing firmer conclusions. However, CTX is not always fully effective against NANC inhibitory nerve-mediated responses (see Currò, 2010) and lacks efficacy against certain cholinergically mediated responses to vagal nerve stimulation (guinea pig oesophagus; Kerr et al., 1995) or P/Q-type calcium current operating **Ca_v2** channel subunits (Alexander et al., 2021). Further, efficacy may be limited by the use of high frequencies of nerve stimulation (e.g., Van Geldre & Lefebvre, 2004; Zygmunt et al., 1993). Further studies are needed to investigate other neurotoxins to completely exclude involvement of neurones (e.g. botulinum toxin).

The present experiments do not identify the origin of the hypothesised non-neuronal ACh, but potential sources are suggested which could be investigated using immunohistochemistry to identify locations of synthetic and degradative enzymes in addition to receptors.

5 | CONCLUSIONS

The LALF contractions, argued to reflect predominantly antral activity, have myogenic origin involving pacemaker cell activity and muscarinic receptor activation by non-neuronal ACh. This finding is comparable with the bladder (Zagorodnyuk et al., 2009). Key questions remain. What is the origin(s) of the ACh (e.g. TTX-insensitive neurones and epithelium)? What is the stimulus for its release (e.g. antral distension,

muscle contraction, bacteria, luminal hypoxia or pH)? Does ACh act directly (e.g. muscle and ICC) or indirectly via release of other substances? Nevertheless, if replicated, the data indicate that gastric motor control in mice (and potentially other species) is dependent at least in part on non-neuronal ACh interacting with the ICC, most likely those in the distal stomach. The latter has implications in understanding the mechanisms by which gastric motility is disrupted during disorders in which nausea and dysrhythmic gastric electrical activity are reported (O'Grady et al., 2014).

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CONFLICT OF INTEREST

WC, MS, RM, AG and PLRA declare no potential conflict of interest. GJS receives a research grant from Takeda Pharmaceuticals.

AUTHOR CONTRIBUTIONS

All authors planned the studies. WC performed the studies and initial data analysis. MS was involved in the data collection and interpretation of the effects of tetrodotoxin, AG wrote the software enabling analysis of the parameters of contraction, RM used this software to analyse the contractions, WC and PLRA wrote the first draft of the paper, critiqued by GJS with the final draft agreed by all authors, and funding was obtained by GJS.

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 organisations engaged with supporting research.

DATA AVAILABILITY STATEMENT

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

The data that support 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.

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