Received: 13 September 2016 | Revised: 28 January 2017 | Accepted: 18 February 2017

DOI: 10.1111/nmo.13064

ORIGINAL ARTICLE

WILEY Neurogastroenterology & Motility NGM

5-HT_4 receptors facilitate cholinergic neurotransmission throughout the murine gastrointestinal tract

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Funding information
The study was supported with own funds.

Abstract

Background: In the gastrointestinal tract of several species, facilitating 5-HT_4 receptors were proposed on myenteric cholinergic neurons innervating smooth muscle by *in vitro* study of the effect of the selective 5-HT_4 receptor agonist prucalopride on submaximal cholinergic contractions. This was not yet established in the murine gastrointestinal tract.

Methods: In circular smooth muscle strips from murine fundus, jejunum and colon, contractions were induced by electrical field stimulation in the presence of guanethidine, L-NAME and for colon also MRS 2500. Submaximal contractions were induced to study the influence of prucalopride.

Key Results: Electrical field stimulation at reduced voltage induced reproducible submaximal neurogenic and cholinergic contractions as the contractions were abolished by tetrodotoxin and atropine. Hexamethonium had no systematic inhibitory effect but mecamylamine reduced the responses, suggesting that part of the cholinergic response is due to activation of preganglionic neurons. Prucalopride concentration-dependently increased the submaximal cholinergic contractions in the three tissue types, reaching maximum from 0.03 μmol/L onwards. The facilitation in the different series with 0.03 μmol/L prucalopride ranged from 41% to 104%, 30% to 76% and 24% to 74% in fundus, jejunum, and colon, respectively. The effect of 0.03 μmol/L prucalopride was concentration-dependently inhibited by GR 113808.

Conclusions & Inferences: In the murine gastrointestinal tract, activation of 5-HT_4 receptors with prucalopride enhances cholinergic contractions, illustrating facilitation of myenteric cholinergic neurotransmission. The degree of enhancement with prucalopride is of similar magnitude as previously reported in other species, but the effective concentrations are lower than those needed in the gastrointestinal tract of other species.

KEYWORDS
5-HT_4 receptor, cholinergic neurotransmission, gastrointestinal tract, mouse, prucalopride

1 | INTRODUCTION

Although serotonin or 5-hydroxytryptamine (5-HT) is best known for its role in the brain, approximately 95% of the serotonin in the human body is found in the gastrointestinal (GI) tract, mainly synthetized in enterochromaffin cells and to a small extent in serotonergic neurons of the myenteric plexus.[1] In the enteric nervous system multiple 5-HT receptor subtypes are expressed, contributing to the regulation of GI motility and secretion.[2] 5-HT interacts with 7 subtypes of receptors, one being a ligand-gated ion channel (5-HT_3), the 6 others being G-protein-coupled receptors. The 5-HT_4 receptor is present in the human brain, heart, adrenal cortex, bladder, and GI tract. GI 5-HT_4 receptors

have been implicated in intestinal secretion,[3] sensitivity,[4] neurogenesis[5] and –protection,[6] and motility. In the human GI tract, three locations of 5-HT_4 receptors influencing motility have been described: (i) on smooth muscle cells, inducing relaxation[7,8]; (ii) on inhibitory nitrergic neurons, inducing nitric oxide release counteracting contraction[9]; and (iii) by far most extensively established, on myenteric excitatory cholinergic neurons innervating the smooth muscle layer, where activation enhances acetylcholine release and smooth muscle contraction.[9–11] The latter effect was clearly shown *in vitro* for human stomach and colon, as the highly selective 5-HT_4 receptor agonist prucalopride enhanced electrically induced submaximal cholinergic contractions.[9–12] This paradigm also allowed to illustrate 5-HT_4 receptor-mediated facilitation of cholinergic neurotransmission in the rat,[13] guinea-pig,[14] canine[11,15] and porcine[16,17] GI tract. The facilitating effect of prucalopride was observed when either circularly or longitudinally oriented muscle strips were used; when both were studied within the same tissue, facilitation of cholinergic neurotransmission is seen in the circular as well as the longitudinal muscle strips (e.g., in canine stomach[15]). The *in vivo* GI prokinetic effects of 5-HT_4 receptor agonists are attributed to activation of these 5-HT_4 receptors on enteric cholinergic neurons[1] and they are therefore developed for treatment of functional GI hypomotility disorders such as gastroparesis and constipation.[18]

In the murine GI tract, 5-HT_4 receptors were shown at RNA and protein level in the submucosal and myenteric plexus.[19] The receptors also seem to have prokinetic effects. Indeed, gastric emptying and small intestinal transit are delayed in 5-HT_4 receptor knockout mice compared to wild-type mice.[5] Conversely, 5-HT_4 receptor agonists have a GI prokinetic effect in mice as RS67506 shortens whole gut transit time[20] and DA-6886 shortens the colonic transit in normal as well as in loperamide-induced constipated mice.[21] Using Fos expression as a marker for neuronal activation, 5-hydroxytryptophan, the precursor of 5-HT, was shown to activate myenteric cholinergic neurons; it also induced defecation, both effects being abolished by a selective 5-HT_4 receptor antagonist.[22] Still, the facilitation of myenteric cholinergic neurotransmission by 5-HT_4 receptor activation, using the paradigm described above for other species, has not yet been established in the murine GI tract.

This study therefore applies this *in vitro* method to investigate whether 5-HT_4 receptors enhance the function of cholinergic neurons inducing circular muscle contraction in three regions of the murine GI tract, namely the gastric fundus, jejunum, and colon. After establishing the optimal parameters to induce submaximal neurogenic postganglionic cholinergic smooth muscle contractions by electrical field stimulation (EFS) for each tissue type, the effect of prucalopride on these EFS-induced contractions was tested.

Key Points

- *In vitro* studies showed that 5-HT_4 receptor stimulation enhances myenteric cholinergic neurotransmission in gastrointestinal muscle of several species, but not yet in mice.
- In smooth muscle strips of murine fundus, jejunum, and colon, the selective 5-HT_4 receptor agonist prucalopride enhanced electrically induced submaximal cholinergic contractions; this effect was abolished by selective 5-HT_4 receptor antagonism and confirmed with 5-HT, illustrating that 5-HT_4 receptor stimulation also enhances murine myenteric cholinergic neurotransmission.
- This murine *in vitro* model is useful to further investigate the pharmacology and signal transduction of 5-HT_4 receptors increasing the function of myenteric cholinergic neurons.

2 | MATERIALS AND METHODS

2.1 | Animals and tissue preparation

All experimental procedures were approved by the Ethical Committee for Animal Experiments from the Faculty of Medicine and Health Sciences at Ghent University. Male C57BL/6J mice (minimal 7 weeks, body weight 24.2±0.2 g, mean±SEM of *n*=158) were purchased from Janvier Labs (Le Genest-Saint-Isle, France) and maintained on a normal light-dark cycle with food pellets and water *ad libitum*.

After sacrificing the mice by cervical dislocation, the GI tract was isolated and kept in aerated (95% O_2/5% CO_2) Krebs solution (composition in mmol/L: NaCl 118.5, KCl 4.8, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.9, $NaHCO_3$ 25.0, and glucose 10.1). The stomach was emptied from its contents and two full thickness fundus strips were prepared by cutting in the direction of the circular smooth muscle layer. For jejunum, a segment of the small bowel of approximately 5 cm long, starting 10 cm distally to the pylorus, and for distal colon an approximately 4 cm long segment above the pelvic brim was taken. Jejunum and colon segments were opened along the mesenteric border and pinned with the mucosa side up in Krebs solution. By sharp dissection, the mucosa was removed under a microscope and 2 (colon) or 4 (jejunum) smooth muscle strips were cut along the circular axis. To illustrate the weight of the strips, the wet weight (mean±SEM, for each tissue: *n*=18 strips of 9 animals) was 4.84±0.28 mg for fundus, 0.55±0.07 mg for jejunum and 0.51±0.04 mg for colon in the series with construction of voltage-response curves; see protocol.

After a cotton (fundus) or silk thread (jejunum and colon) was attached to both ends of every strip, they were mounted between two platinum plate electrodes (6-8 mm apart) in a 7 or 15 mL organ bath filled with oxygenated Krebs solution kept at body temperature. Muscle contractions were induced by EFS with a 4-channel custom-made stimulator. Changes in muscle tone were registered by isometric tension recording using Grass FT03 (Grass Instrument Co., Quincy, MA, USA) or MLT 050/D force transducers (ADInstruments, Oxford, UK) and recorded on a Powerlab/8sp data recording system (ADInstruments) with Chart v5.5.6 software (ADInstruments).

2.2 | Protocols

Strips were studied at optimal load as determined in preliminary experiments via the contractile response to the muscarinic receptor

agonist carbachol: 1 g for fundus; 0.25 g for jejunum; 0.5 g for colon. Details of determining the optimal load are given in the Data S1.

2.2.1 | Characterization of EFS-induced responses

Experiments were performed in Krebs solution containing 4 μmol/L of the noradrenergic neuron blocker guanethidine and 300 μmol/L of the nitric oxide synthase inhibitor N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME) to avoid relaxant influences due to noradrenaline and nitric oxide, respectively. For colon tissue, 1 μmol/L MRS 2500 ($P2Y_1$ receptor antagonist) was also added to avoid influences of the relaxant neurotransmitter adenosine triphosphate (ATP).[23] After 60 minutes of equilibration with flushing every 15 minutes, voltage-response curves or frequency-response curves were constructed.

At an interval of 5 minutes, 10 seconds trains of EFS (pulse width of 0.5 milliseconds; frequency of 4 Hz; increasing voltage 5-50 V) were applied. The voltage-response curve was repeated in the presence of 3 μmol/L tetrodotoxin (TTX; voltage-gated Na^+ channel blocker; incubated for 30 minutes) or in its absence (parallel control). Frequency-response curves were obtained using the same protocol but now fixing the voltage at 30 V and increasing the frequency from 0.5 to 16 Hz. Instead of TTX, the influence of 1 μmol/L atropine (muscarinic receptor antagonist) was now tested.

2.2.2 | Influence of prucalopride on EFS-induced submaximal cholinergic contractions

All further experiments started with 30 minutes of equilibration in Krebs solution without added substances followed by repetitively studying the contractile response to carbachol until it was stable. The medium was then switched to Krebs solution containing 4 μmol/L guanethidine, 300 μmol/L L-NAME and for colon also 1 μmol/L MRS 2500 and tissues were allowed to further equilibrate for 60 minutes with flushing every 15 minutes. EFS-induced submaximal cholinergic contractions were then obtained as follows. First, 10 seconds trains of EFS with a pulse width of 0.5 milliseconds, an intermediate frequency of 4 (fundus) or 8 (jejunum and colon) Hz and supramaximal voltage (V_{max}) of 30 V were repeated at an interval of 3 (fundus and colon) or 6 minutes (jejunum), yielding reproducible contractions. EFS was then continued with gradual reduction in the voltage until the amplitude of the contraction was approximately 50% of that obtained at V_{max} ($V_{50\%}$). Details on testing the response to carbachol and delineating EFS-induced submaximal cholinergic contractions are given in Data S1.

Once $V_{50\%}$ was selected and a stable response to EFS at $V_{50\%}$ was obtained, EFS at $V_{50\%}$ was repeated at 5 minutes interval in the fundus and colon, and 10 minutes interval in the jejunum. After three contractions were obtained, three parallel strips received 3 μmol/L TTX, 1 μmol/L atropine or 0.5 mmol/L hexamethonium (nicotinic acetylcholine receptor antagonist) and 10 (fundus and colon) or 5 (jejunum) further trains of EFS were applied in the presence of these substances; a 4th parallel control did not receive active compounds.

The same protocol was used to study the influence of three concentrations of prucalopride (first 0.03, 0.1, and 0.3 μmol/L; in a second series 0.003, 0.01, and 0.03 μmol/L); for the colon, the first concentration range was tested with EFS at 4 instead of 8 Hz. The influence of prucalopride was also studied by adding it cumulatively within the same strip (0.3 nmol/L to 1 μmol/L with half log unit concentration increments).

2.2.3 | Influence of compounds on the effect of prucalopride

The responses to carbachol and $V_{50\%}$ were first determined as described above. Then, after obtaining five contractions by EFS at $V_{50\%}$, the compound under study was added in one strip, while the parallel control strip did not receive the compound. A further 20 (fundus and colon) or 10 (jejunum) trains of EFS were then applied, with addition of 0.03 μmol/L prucalopride in both strips after 10 (fundus and colon) or 5 (jejunum) trains. The compounds studied vs prucalopride were MRS 2500 (in fundus and jejunum; 1 μmol/L), the selective $5\text{-}HT_4$ receptor antagonist GR 113808 (0.3 μmol/L) and the nicotinic acetylcholine receptor antagonists hexamethonium (0.5 mmol/L) and mecamylamine (30 μmol/L). Using the same protocol, GR 113808 was also studied vs 0.3 μmol/L 5-HT in the presence of 1 μmol/L methysergide and 0.3 μmol/L granisetron to exclude interaction of 5-HT with other 5-HT receptors than $5\text{-}HT_4$ receptors; additionally, GR 113808 was studied vs prucalopride by adding it cumulatively (0.001-0.3 μmol/L with half log unit concentration increments). As GR 113808 cannot be dissolved in distilled water, parallel control tissues received a corresponding amount of its solvent dimethyl sulfoxide (DMSO).

2.3 | Drugs

Atropine sulfate salt monohydrate, carbamoylcholin chloride, guanethidine sulfate, hexamethonium bromide, L-NAME, mecamylamine hydrochloride and serotonin creatinine sulfate monohydrate (5-HT) were obtained from Sigma-Aldrich (Diegem, Belgium). GR 113808 [(1-[2-[(methylsulfonyl)amino]ethyl)-4-piperidinyl]methyl 1-methyl-1H-indole-3-carboxylate], granisetron hydrochloride, methysergide maleate, MRS 2500 tetraammonium salt [(1R*,2S*)-4-[2-Iodo-6-(methylamino)-9H-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0] hexane-1-methanol dihydrogen phosphate ester tetraammonium salt] and TTX citrate were obtained from Tocris Bioscience (Bristol, UK) and prucalopride succinate from Selleck Chemicals (Houston, TX, USA). All compounds were dissolved and diluted in distilled water, except for GR 113808 which was dissolved in DMSO, yielding a DMSO concentration of 0.03% in the organ bath.

2.4 | Data and statistical analysis

The contraction force induced by carbachol or EFS was expressed as gram.second per milligram wet weight of the tissue (g.s/mg wet weight). It was calculated by determining the area under the curve (AUC) during the contraction (3 minutes for carbachol- and 10 seconds for EFS-induced contractions), reduced with the AUC during a corresponding time period

just before adding carbachol or applying EFS. EFS-induced contractions at $V_{50\%}$ in the presence of substances were expressed as percentage of the mean of the five contractions just before addition (100% reference). For the experiments, testing the influence of MRS 2500, hexamethonium or mecamylamine on the effect of prucalopride, contractions after adding prucalopride were also expressed as percentage of the mean of the five contractions in the presence of MRS 2500, hexamethonium or mecamylamine just before adding prucalopride. Strips with unstable reference responses, defined as when the AUC of minimally one of the five contractions before addition of the substance was outside the range of 75%-125%, were excluded from data analysis. To illustrate the exclusion rate, in the series studying TTX, atropine and hexamethonium on electrically induced submaximal contractions seven of 39, five of 39 and five of 40 strips were excluded in the fundus, jejunum, and colon respectively.

pEC_{50} values for prucalopride and pIC_{50} values for GR 113808 were calculated by linear interpolation. An estimate of the pK_b of GR 113808 was obtained via the functional version of the Cheng-Prusoff equation for analysis of an antagonist inhibition curve.[24] Data are expressed as means±SEM. *n* refers to tissues obtained from different animals. Statistical analysis was performed by use of GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA, USA). The EFS-induced contractions induced by the last train of EFS in parallel groups were assessed with an unpaired *t* test (two groups) or one-way ANOVA followed by a Bonferroni corrected *t* test (more than two groups). A *P*-value less than .05 was considered statistically significant.

3 | RESULTS

3.1 | Characterization of EFS-induced contractions

In the presence of guanethidine, L-NAME and for the colon also MRS 2500, 10 seconds trains of EFS-induced tonic on-contractions that decreased immediately after termination of EFS to reach baseline again within approximately 10 seconds in the fundus (Figure 1A,D) and colon (Figure 1C,F). In jejunum EFS induced an increase in the phasic activity, gradually decreasing after termination of EFS (Figure 1B,E). The voltage-response curves with EFS at 4 Hz indicated that maximal contractile response was obtained at 20 V in the three tissues; the responses at increasing voltage were reproducible (Fig. S1A-C) and abolished by 3 μmol/L TTX except for the responses at 40 and 50 V in the jejunum (Fig. S1D-F). The frequency-response curves with EFS at 30 V showed frequency-dependent responses, still increasing at the highest frequency applied (16 Hz); the responses at increasing frequency were reproducible (Fig. S2A-C) and abolished by 1 μmol/L atropine (Fig. S2D-F). From these experiments, 30 V was determined as supramaximal, and a frequency of 4 (fundus) or 8 Hz (jejunum and colon) was selected as submaximal frequency for further experiments.

3.2 | Characterization of EFS-induced contractions at $V_{50\%}$

Electrical field stimulation with 10 seconds trains (0.5 milliseconds pulse width; supramaximal voltage of 30 V; 4 [fundus] or 8 Hz [jejunum and colon]) at 3 (fundus and colon) or 6 minutes (jejunum) interval induced reproducible contractions. Upon reduction in the stimulation voltage to $V_{50\%}$, reproducible submaximal contractions were obtained. The neurogenic and cholinergic character of the submaximal EFS-induced contractions at 5 (fundus and colon) or 10 minutes (jejunum) interval was confirmed in the three tissues as TTX (3 μmol/L) and atropine (1 μmol/L) abolished the contractions. Hexamethonium (0.5 mmol/L) had no influence on the contractions in jejunum and colon while it significantly enhanced the contractions from 95±7% in controls to 141±17% in the fundus (Figure 2). Results with hexamethonium were quite variable though, as a clear inhibitory effect was obtained in several jejunal strips and an enhancing one in several colonic tissues (Fig. S3).

3.3 | Influence of prucalopride

In a first preliminary series, prucalopride (0.03, 0.1, and 0.3 μmol/L administered in separate strips) enhanced the EFS-induced submaximal cholinergic contractions, but a clear-cut concentration-dependency was not present in this concentration range (Fig. S4).

Tenfold lower concentrations of prucalopride (0.003, 0.01, and 0.03 μmol/L administered in separate strips) concentration-dependently increased the EFS-induced submaximal cholinergic contractions in the fundus (Figure 3A). Although less clear than in the fundus, a trend for a concentration-dependent increase by prucalopride was also present in jejunum and colon (Figure 3B,C). For the last EFS-induced contraction in the presence of, respectively, 0.003, 0.01, and 0.03 μmol/L prucalopride mean values of 133±7%, 181±24% ($P<.05$) and 204±11% ($P<.001$) in fundus, 122±4% ($P<.05$), 124±9% ($P<.01$) and 138±5% ($P<.001$) in jejunum and 133±6%, 136±5% and 152±10% ($P<.01$) in colon were measured (*P*-values obtained from one-way ANOVA with Bonferroni corrected *t* test for the three concentrations of prucalopride vs control). In fundus and jejunum, the facilitating effect of 0.03 μmol/L prucalopride was not influenced by 1 μmol/L MRS 2500 (Fig. S5). In the jejunum, MRS 2500 caused a significant reduction in the EFS-induced contractions (Fig. S5B).

The concentration-dependency of the facilitating effect of prucalopride was corroborated in experiments where prucalopride was added cumulatively within the same strips (Figure 3D-F), showing that the maximal effect was indeed reached from 0.03 μmol/L onwards. pEC_{50} values were 8.37±0.04, 7.83±0.18 and 8.11±0.10 in fundus, jejunum, and colon, respectively.

3.4 | Influence of GR 113808 on the effect of prucalopride and 5-HT

GR 113808, added cumulatively (0.001-0.3 μmol/L) once the facilitating effect of prucalopride on EFS-induced submaximal cholinergic contractions was stable, concentration-dependently reduced and finally abolished the effect of prucalopride, while the effect of prucalopride was maintained in the parallel control strip receiving corresponding amounts of the solvent of GR 113808, DMSO. A representative experiment in fundus strips is shown in Figure 4. pIC_{50}

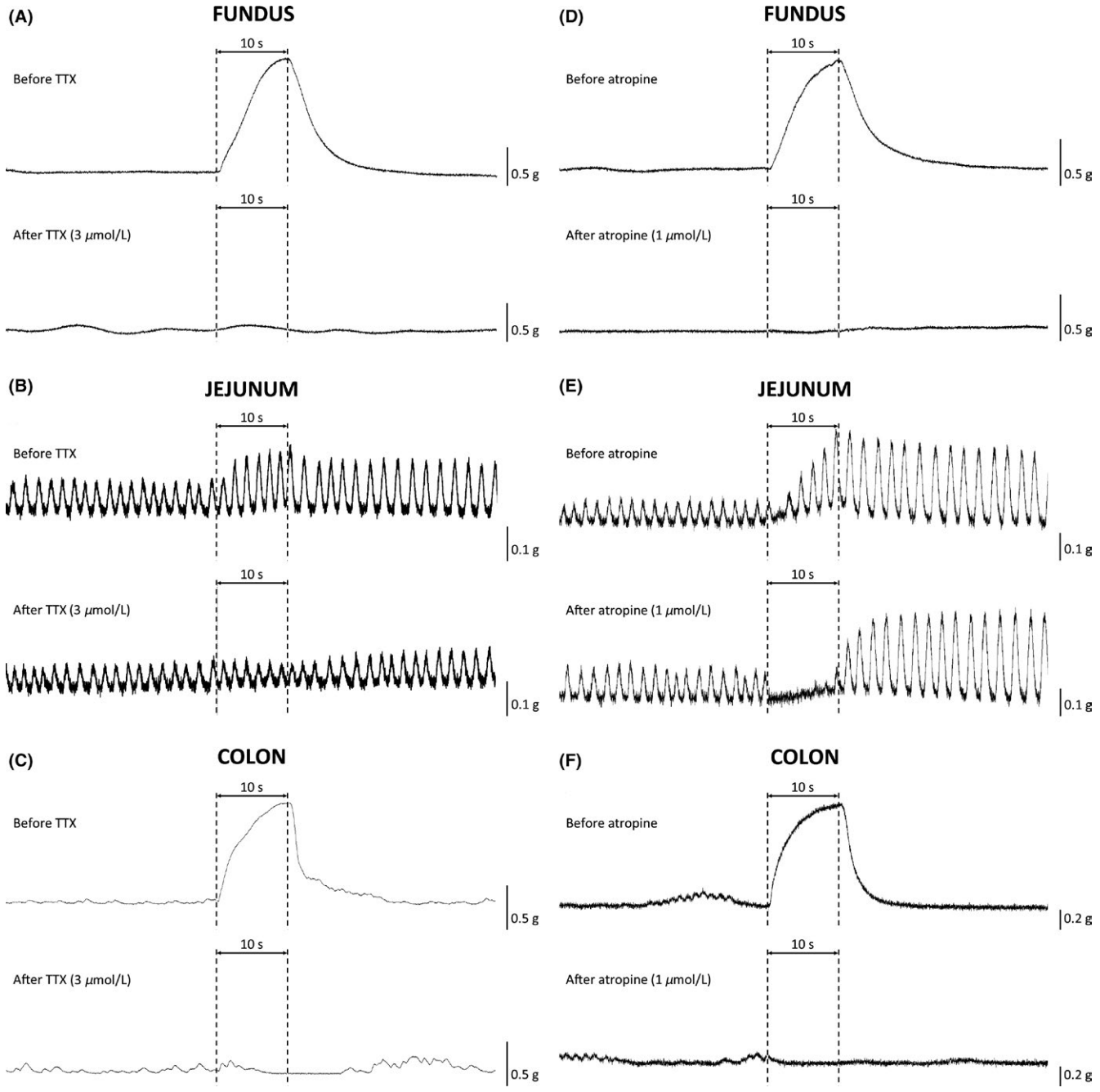

FIGURE 1 Contractile responses to electrical field stimulation before and after adding TTX or atropine. Representative traces showing the influence of 3 μmol/L TTX (A-C) or 1 μmol/L atropine (D-F) on contractions induced by electrical field stimulation (10 seconds trains with 0.5 milliseconds pulse duration) at 30 V and 4 Hz (A-D) or 8 Hz (E,F). Experiments were performed in the continuous presence of 4 μmol/L guanethidine, 300 μmol/L L-NAME and for colon also 1 μmol/L MRS 2500

values were 8.15±0.10, 8.33±0.18, and 8.44±0.21 in fundus, jejunum, and colon, respectively (n=6 in fundus and jejunum and 5 in colon); the pK_b estimates determined from these values were 9.06±0.10, 8.81±0.18 and 9.14±0.21.

When adding prucalopride (0.03 μmol/L) in the presence of DMSO (0.03%), a mean increase in the EFS-induced submaximal cholinergic contractions was observed up to 141±10% in fundus, 130±10% in jejunum and 147±30% in colon (Figure 5A-C). In the presence of 0.3 μmol/L GR 113808, prucalopride (0.03 μmol/L) did not enhance the contractions. In bathing medium containing 1 μmol/L methysergide and 0.3 μmol/L granisetron, also 5-HT (0.3 μmol/L) enhanced the EFS-induced cholinergic contractions to 188±14% in fundus, 136±9% in jejunum and 134±4% in colon; GR 113808 (0.3 μmol/L) abolished the effect of 5-HT in fundus and jejunum, and greatly reduced it in the colon to 107±6% (Figure 5D-F). Neither GR 113808 (0.3 μmol/L) nor its solvent DMSO (0.03%) as such influenced the EFS-induced submaximal cholinergic contractions (Figure 5).

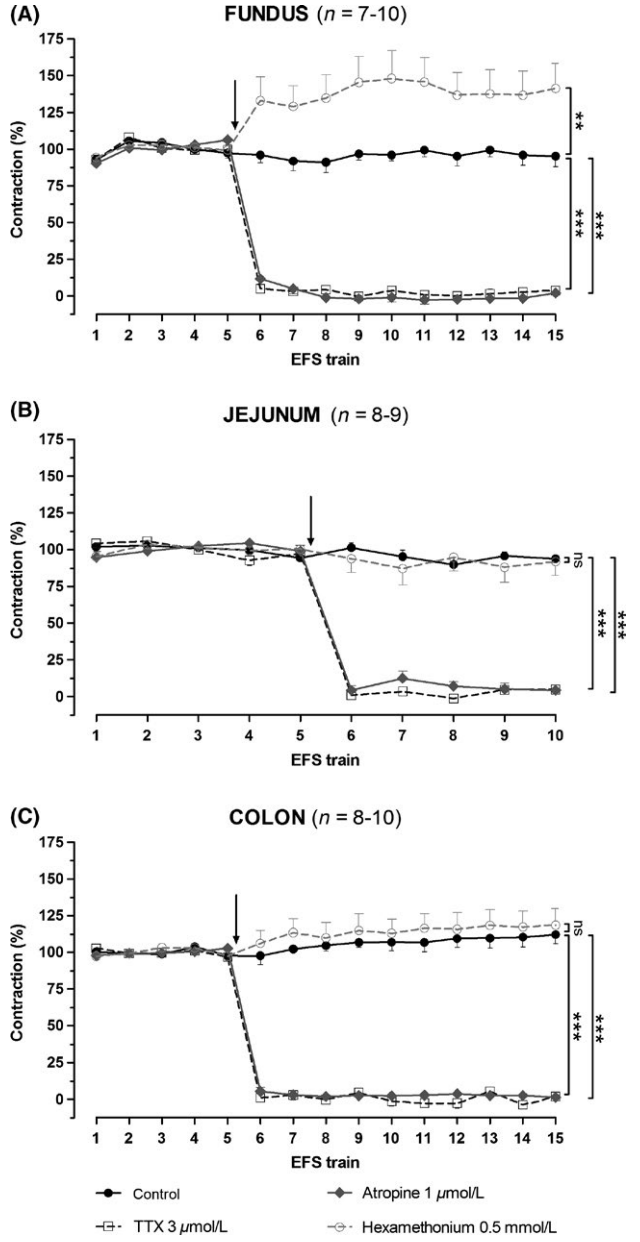

FIGURE 2 Characterization of electrically induced submaximal contractions with TTX, atropine and hexamethonium. Influence of 3 μmol/L TTX, 1 μmol/L atropine and 0.5 mmol/L hexamethonium on submaximal electrically induced contractions at $V_{50\%}$ (10 seconds trains at 4 Hz [fundus] or 8 Hz [jejunum and colon], 0.5 milliseconds, interval of 5 [fundus and colon] or 10 minutes [jejunum]) in murine fundus (A), jejunum (B) and colon (C). Contractions are expressed as percentage of the mean of the five contractions before addition of TTX, atropine, or hexamethonium. Experiments were performed in the continuous presence of 4 μmol/L guanethidine, 300 μmol/L L-NAME and for colon also 1 μmol/L MRS 2500. Means±SEM; ns not significant, $^{**}P<.01$, $^{***}P<.001$ vs control (one-way ANOVA with Bonferroni corrected *t* test)

3.5 | Influence of hexamethonium and mecamylamine on the effect of prucalopride

In the series where hexamethonium (0.5 mmol/L) was tested vs prucalopride, it increased the EFS-induced submaximal cholinergic contractions as such in fundus but also in colon and decreased them in jejunum (Fig. S6A-C). In view of this effect of hexamethonium as such, the responses in the presence of prucalopride were also expressed as percentage of the mean of the five responses in the presence of hexamethonium just before adding prucalopride; with this analysis the facilitating effect of prucalopride (0.03 μmol/L) on EFS-induced submaximal cholinergic contractions was not significantly influenced by hexamethonium although a decreasing trend was present in fundus and jejunum (Fig. S6D-F).

In view of the varying effect of hexamethonium on EFS-induced contractions, another nicotinic receptor blocker was studied. Mecamylamine (30 μmol/L) consistently reduced EFS-induced submaximal cholinergic contractions (Figure 6A-C). In the presence of mecamylamine, prucalopride still enhanced the EFS-induced contractions. When expressing the responses as percentage of the mean of the five responses in the presence of mecamylamine just before adding prucalopride, the effect of prucalopride was not influenced in jejunum and colon, and significantly reduced in fundus (Figure 6D-F).

4 | DISCUSSION

This study investigated the influence of prucalopride on EFS-induced submaximal cholinergic contractions in murine circular smooth muscle of the fundus, jejunum and colon to elucidate whether 5-HT_4 receptors also facilitate myenteric cholinergic neurotransmission in the murine GI tract. To induce pure contractile responses by 10 seconds trains of EFS, guanethidine, L-NAME and for colon also MRS 2500 had to be added. Although also in murine stomach[25] (antrum) and jejunum[26] evidence for purinergic inhibitory neurotransmission has been provided, we previously showed that non-adrenergic non-cholinergic relaxations induced by EFS in murine fundus and jejunum with similar stimulation parameters as in the actual study were fully nitrergic.[27] In the series where the influence of MRS 2500 on the effect of prucalopride was studied, it was also clear that this $P2Y_1$ receptor antagonist did not influence EFS-induced cholinergic contractions in the fundus and even decreased them in the jejunum. Although there is no clear-cut explanation for the latter effect, the results with MRS 2500 in fundus and jejunum confirm that there is no inhibitory purine counteracting the contractile neurotransmitter during EFS as the response should then increase in the presence of MRS 2500. In the conditions used, the neurogenic nature of the contractions induced at increasing voltage with the frequency fixed at 4 Hz was demonstrated by their abolishment with TTX; only in the jejunum, some contractile response persisted with EFS at 40 and 50 V but these voltages were not used in the following experiments. The involved neurotransmitter is acetylcholine, as the contractions induced at increasing frequency with the voltage fixed at 30 V were abolished by the non-selective muscarinic receptor antagonist atropine. The neurogenic cholinergic nature was confirmed when testing TTX and atropine on submaximal contractions at $V_{50\%}$ and 4 or 8 Hz. To investigate to what extent activation of preganglionic neurons might contribute to the EFS-induced contractions, the influence of the nicotinic receptor

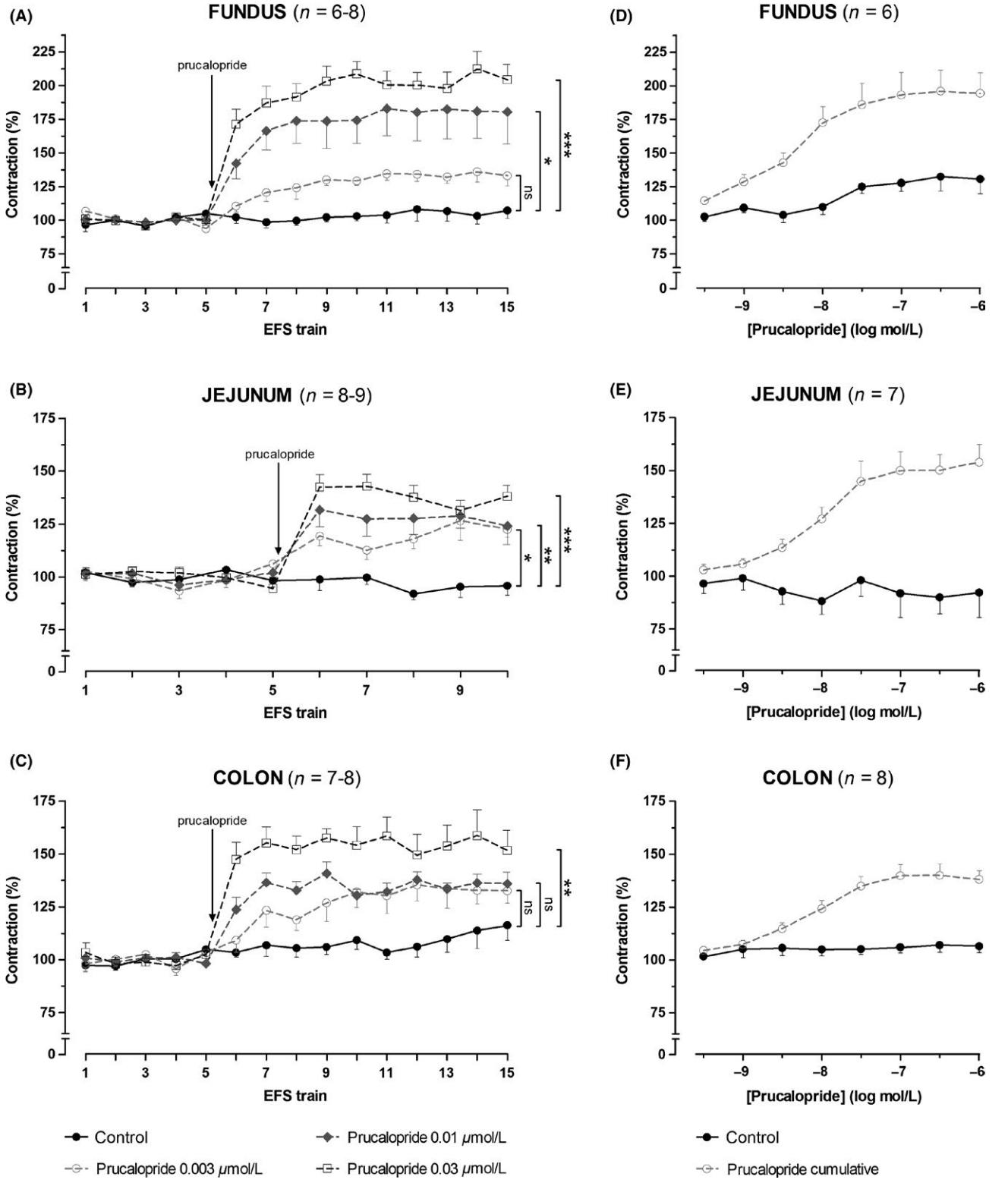

FIGURE 3 Concentration-dependent facilitation by prucalopride of electrically induced submaximal cholinergic contractions. Influence of 0.003, 0.01 and 0.03 μmol/L prucalopride, administered to separate strips (A-C) and of 0.3 nmol/L to 1 μmol/L prucalopride, added cumulatively (D-F) on submaximal electrically induced cholinergic contractions at $V_{50\%}$ (10 seconds trains at 4 Hz [fundus] or 8 Hz [jejunum and colon], 0.5 milliseconds, interval of 5 [fundus and colon] or 10 minutes [jejunum]) in murine fundus (A,D), jejunum (B,E) and colon (C,F). Contractions are expressed as percentage of the mean of the five contractions before adding prucalopride. Experiments were performed in the continuous presence of 4 μmol/L guanethidine, 300 μmol/L L-NAME and for colon also 1 μmol/L MRS 2500. Means±SEM; ns not significant, **P*<.05, ***P*<.01, ****P*<.001 vs control (one-way ANOVA with Bonferroni corrected *t* test)

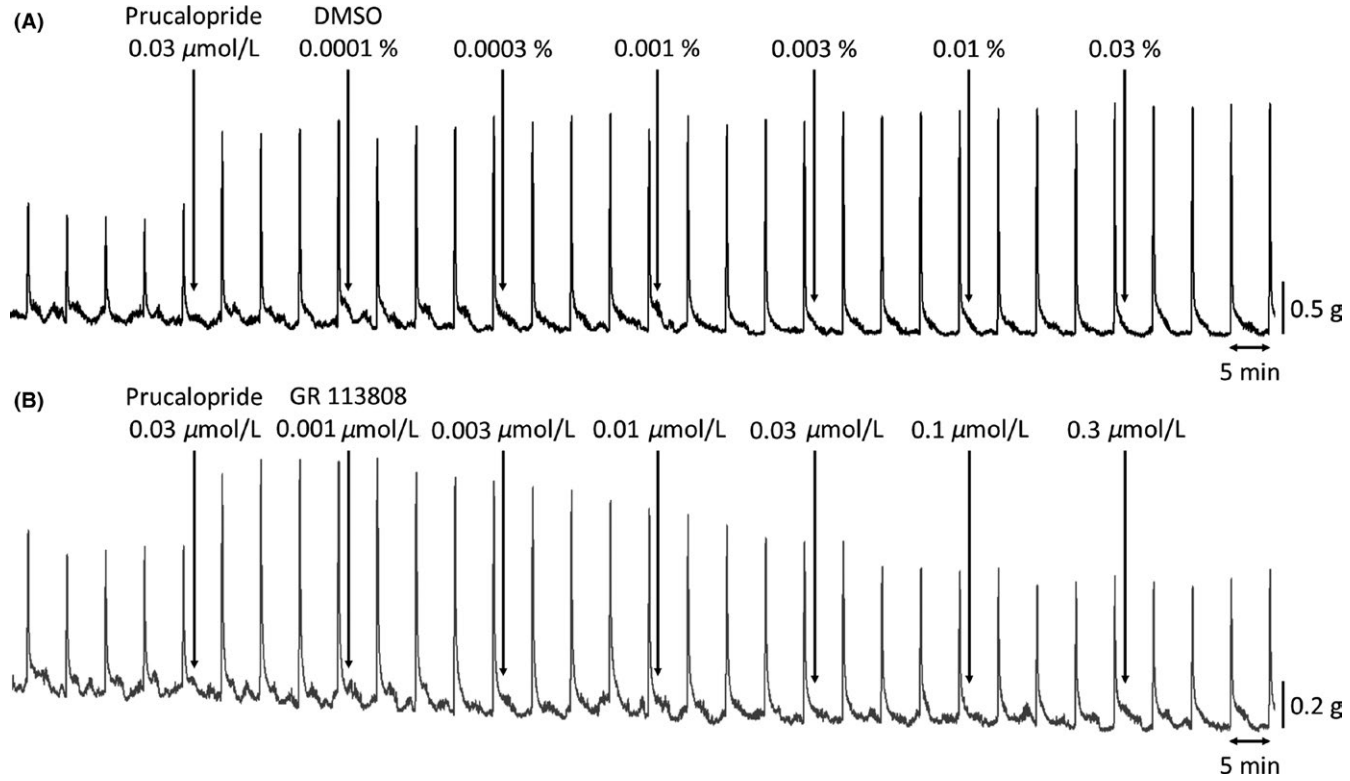

FIGURE 4 Antagonism of prucalopride-induced facilitation by cumulatively added GR 113808. Representative traces showing the response to repetitive electrical field stimulation (10 seconds trains at 4 Hz, 0.5 milliseconds, $V_{50\%}$) in the continuous presence of 4 μmol/L guanethidine and 300 μmol/L L-NAME in two parallel circular smooth muscle strips of the murine fundus. Both received 0.03 μmol/L prucalopride; when the facilitating effect of prucalopride on the contractions was stable, either GR 113808 (B) or its solvent DMSO (A) was added cumulatively

blocker hexamethonium was tested on the submaximal cholinergic contractions; it had a tissue-dependent influence as it increased the EFS-induced submaximal contractions in the fundus and in some colonic tissues while reducing them clearly in some jejunal tissues. In the series where hexamethonium was tested vs prucalopride, clear-cut increase in the EFS-induced contractions in the fundus and colon and decrease in the jejunum was observed. In view of these variable results, the influence of another nicotinic receptor antagonist mecamylamine was tested; it antagonized neuronal nicotinic receptors in the myenteric plexus with a 30-fold greater potency than hexamethonium.[28] Mecamylamine reduced the EFS-induced submaximal cholinergic contractions in the three tissues, illustrating a contribution of preganglionic neurons via nicotinic receptors. The increase in the submaximal cholinergic contractions by hexamethonium in the fundus and colon is puzzling. This cannot be due to removal of a preganglionically activated inhibitory input, as the adrenergic, nitrergic and when relevant purinergic inhibitory neurotransmission was ruled out. A similar enhancing effect of hexamethonium on electrically induced cholinergic contractions was observed in the guinea-pig ileum longitudinal muscle-myenteric plexus preparation.[29] Based on electrophysiological data obtained in cultured enteric neurons[30] the possibility of a functional cross-inhibition between nicotinic receptors and P2X receptors on myenteric neurons was suggested; blockade of the nicotinic receptors would then strengthen ganglionic transmission onto the myenteric cholinergic neurons via P2X receptors. But if this mechanism would be present, one would also expect it to occur with mecamylamine which was not the case. Interestingly, in rabbit gastric fundus EFS-evoked contractile responses were significantly enhanced by mecamylamine but not by hexamethonium.[31]

Prucalopride was first tested at the concentration range of 0.03-0.3 μmol/L, which showed concentration-dependent enhancement of submaximal cholinergic contractions in porcine stomach (isolated administration[17]) and canine stomach (cumulative administration[15]). In the three murine tissues, these three concentrations enhanced the submaximal cholinergic contractions but without concentration-dependency suggesting that the maximal response might already be obtained from 0.03 μmol/L onwards. The range was therefore reduced 10-fold (0.003-0.03 μmol/L), yielding clear-cut concentration-dependent enhancement in the mouse gastric fundus, and a trend for it in jejunum and colon. The concentration-dependency of the facilitation by prucalopride was clearly illustrated for the three tissues when it was added cumulatively, also showing that the maximal effect of prucalopride was nearly reached from 0.03 μmol/L onwards. The pEC_{50} values (from 8.37 in the fundus to 7.83 in the jejunum, roughly corresponding with an EC_{50} of 0.004-0.015 μmol/L), were similar to those reported for canine (pEC_{50} 7.9)[15] and porcine stomach (pEC_{50} 8.25)[32] but clearly lower than those reported for rat forestomach (EC_{50} 1.1 μmol/L),[13] human colon (EC_{50} 2.3 μmol/L)[13] and human gastric fundus (pEC_{50} 5.6).[33] The facilitating effect of 0.03 μmol/L prucalopride was concentration-dependently depressed by GR 113808 and abolished

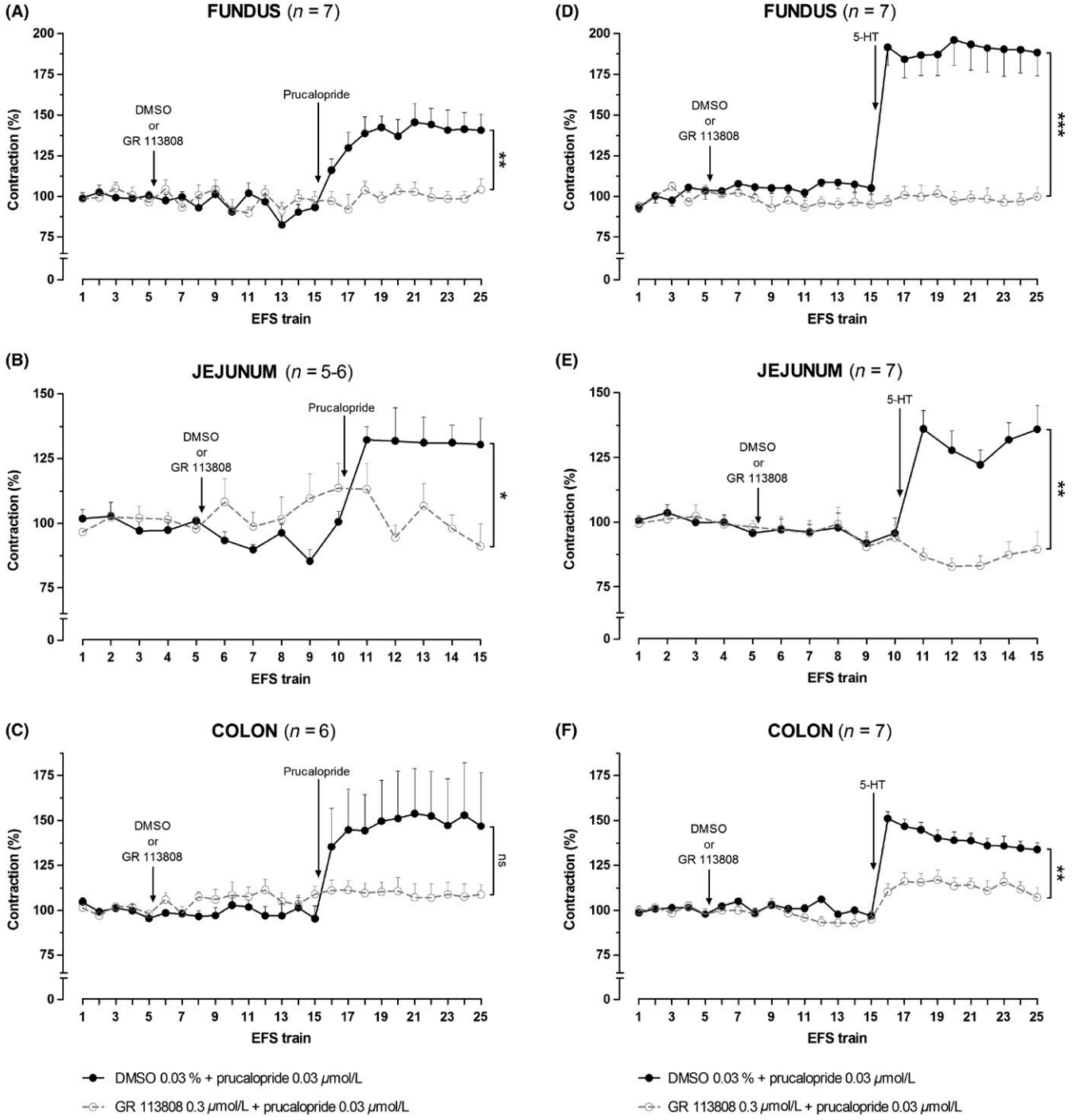

FIGURE 5 Antagonism of prucalopride- and 5-HT-induced facilitation by GR 113808. Influence of 0.3 μmol/L GR 113808 on the facilitating effect of 0.03 μmol/L prucalopride (A-C) and 0.3 μmol/L 5-HT (D-F) on submaximal electrically induced cholinergic contractions at $V_{50\%}$ (10 seconds trains at 4 Hz [fundus] or 8 Hz [jejunum and colon], 0.5 milliseconds, interval of 5 [fundus and colon] or 10 minutes [jejunum]) in murine fundus (A,D), jejunum (B,E), and colon (C,F). Contractions are expressed as percentage of the mean of the five contractions before adding GR 113808. Experiments were performed in the continuous presence of 4 μmol/L guanethidine, 300 μmol/L L-NAME and for colon also 1 μmol/L MRS 2500; to study the effect of 5-HT, the Krebs solution also contained 1 μmol/L methysergide and 0.3 μmol/L granisetron. Means±SEM; ns not significant, **P*<.05, ***P*<.01, and ****P*<.001 vs prucalopride or 5-HT in the presence of 0.03% DMSO (unpaired *t* test)

by 0.3 μmol/L GR 113808. The selective competitive 5-HT_4 receptor antagonist GR 113808 has a high affinity with originally described pA_2 values between 9.2 and 9.7.[34] Few affinity estimates of GR 113808 have been reported for its interaction with 5-HT_4 receptors enhancing cholinergic neurotransmission. When tested vs prucalopride in canine stomach, a pA_2 estimate of 9.4 was obtained; testing vs 5-HT yielded a pK_b estimate of 9.1.[15] When tested vs the facilitating effect of 5-HT on EFS-induced cholinergic contractions in isolated human detrusor

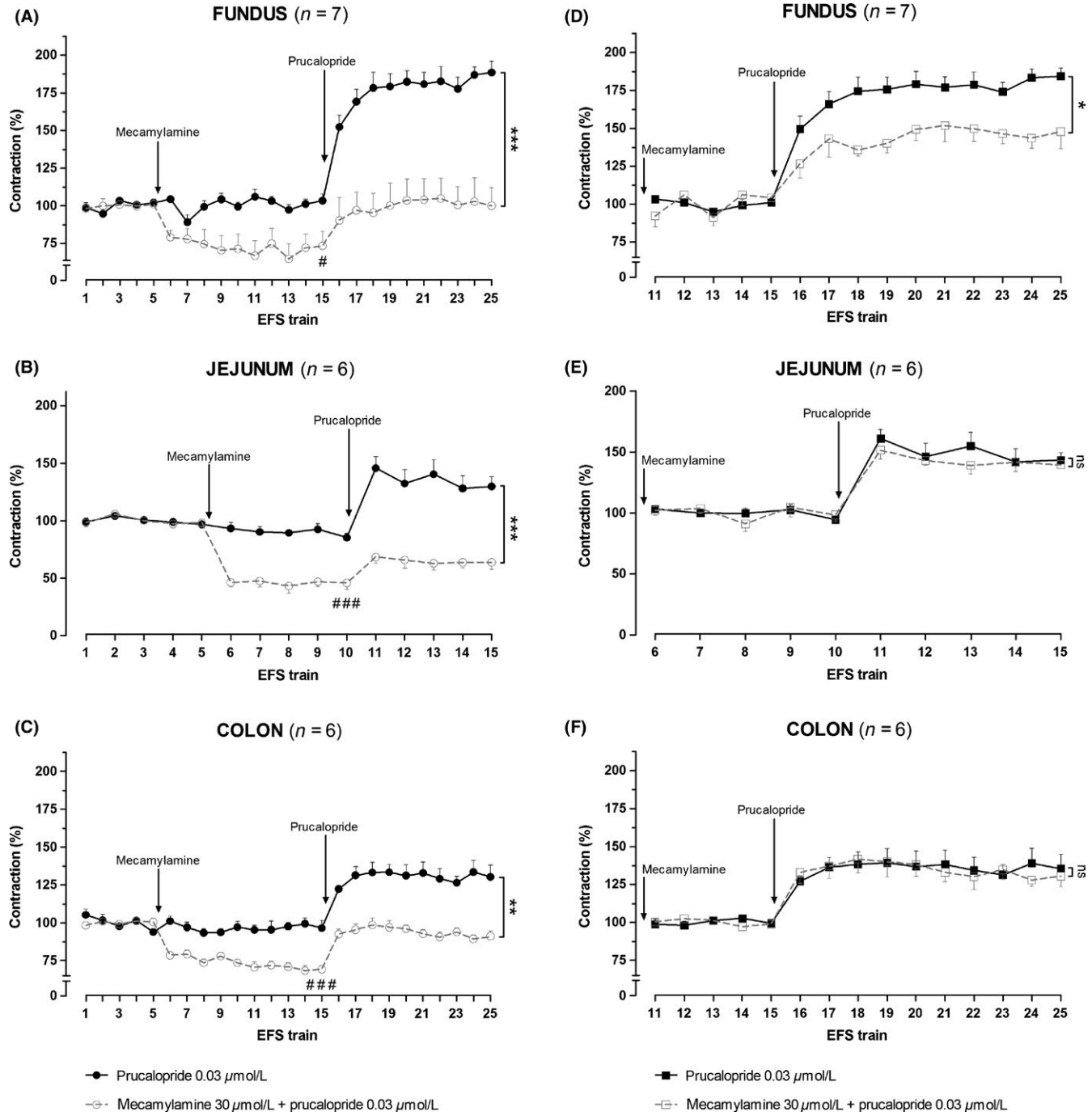

FIGURE 6 Effect of mecamylamine on the facilitating effect of prucalopride. Influence of 30 μmol/L mecamylamine on the facilitating effect of 0.03 μmol/L prucalopride on submaximal electrically induced cholinergic contractions at $V_{50\%}$ (10 seconds trains at 4 Hz [fundus] or 8 Hz [jejunum and colon], 0.5 milliseconds, interval of 5 [fundus and colon] or 10 minutes [jejunum]) in murine fundus (A,D), jejunum (B,E), and colon (C,F). Contractions are expressed as percentage of the mean of the five contractions before adding mecamylamine (A-C) or of the five contractions in the presence of mecamylamine just before adding prucalopride (D-F). Experiments were performed in the continuous presence of 4 μmol/L guanethidine, 300 μmol/L L-NAME and for colon also 1 μmol/L MRS 2500. Means±SEM; ns not significant, $^{*}P<.05$, $^{**}P<.01$, and $^{***}P<.001$ vs prucalopride in the absence of mecamylamine (unpaired *t* test), $^{\#}P<.05$ and $^{\#\#\#}P<.001$ vs control not treated with mecamylamine (unpaired *t* test)

muscle, a pA_2 estimate of 8.9 was obtained.[35] The pK_b estimates of GR 113808 obtained in the murine GI tissues (8.81-9.14) correspond with these values. Full blockade of the effect of prucalopride by a concentration of GR 113808 approximately 300-fold higher than its affinity can then indeed be expected and further underlines interaction with 5-HT_4 receptors. Enhancement of cholinergic neurotransmission in the murine GI tissues was confirmed with the endogenous agonist 5-HT in the presence of antagonists to inhibit the other 5-HT receptors than

5-HT_4. Only one high concentration of 5-HT (0.3 μmol/L) was studied to proof the principle, which might explain that its effect was not completely abolished in colon strips.

In view of the variable effects of hexamethonium as such on EFS-induced contractions but the consistent inhibitory effect of mecamylamine, illustrating a contribution of preganglionic neurons, we concentrate on the results with mecamylamine vs prucalopride to discuss whether interaction with 5-HT_4 receptors on preganglionic neurons contributes to the facilitating effect of prucalopride on cholinergic neurotransmission. When expressing results as percentage of the responses before adding mecamylamine, the response to prucalopride was significantly reduced in the three tissues. However, when taking in account the effect of mecamylamine as such on the contractions by expressing the results as percentage of the responses before adding prucalopride, significant reduction in prucalopride's effect was only maintained in the fundus. This suggests that at least in the fundus, part of the enhancing effect of prucalopride is related to activation of 5-HT_4 receptors on preganglionic myenteric neurons activating subsequently cholinergic motor neurons via nicotinic receptors.

The facilitating effect of 0.03 μmol/L prucalopride on submaximal cholinergic contractions varied from series to series; in the fundus, jejunum, and colon, respectively, increases from 41% to 104%, 30% to 76%, and 24% to 74% were measured. Variation in the enhancing effect of a given concentration of prucalopride has also been observed before.[36] In the fundus, the lowest value for the effect of prucalopride was obtained in the series where the control strips received prucalopride in the presence of the solvent of GR 113808, DMSO; but this was not the case for jejunum and colon. DMSO added cumulatively after reaching a stable effect with 0.03 μmol/L prucalopride showed no effect so that it is unlikely that DMSO suppresses the effect of prucalopride at 5-HT_4 receptors. When the concentration-dependency of the facilitating effect of prucalopride on cholinergic contractions was studied in rat forestomach,[13] canine stomach,[15] porcine stomach,[17,32] human stomach[33] and human colon,[9] a similar degree of maximal facilitation was observed as in the murine GI tract but higher concentrations than 0.03 μmol/L prucalopride were required to obtain this maximal effect, even in tissues with a similar pEC_{50}.[15,32] This suggests a higher number and/or a more effective coupling of the 5-HT_4 receptors enhancing myenteric cholinergic neurotransmission in the murine GI tract, at least fundus and colon, as no comparative data for prucalopride are available for the small intestine in the literature.

5 | CONCLUSION AND FUTURE PERSPECTIVES

In conclusion, in murine fundus, jejunum and colon, 5-HT_4 receptors enhance myenteric cholinergic neurotransmission, inducing circular smooth muscle contraction when activated by prucalopride. The magnitude of the effect of prucalopride in the murine GI tract is similar to that in other species, but the concentration of prucalopride required to induce maximal effects are overall lower than those needed at 5-HT_4 receptors in the rat, canine, porcine and human GI tract.

The gastroprokinetic effect of 5-HT_4 receptor agonists is attributed to activation of 5-HT_4 receptors on myenteric cholinergic neurons toward the smooth muscle layer. To reduce the risk of side effects of gastroprokinetic drugs such as prucalopride, interest exists for lowering the dose by combination therapy. Acetylcholinesterase inhibition[13] and more recently phosphodiesterase inhibition[17,36] were put forward to combine with 5-HT_4 receptor agonists. As the presence of 5-HT_4 receptors enhancing myenteric cholinergic neurotransmission toward the circular smooth muscle layer is now confirmed in the murine GI tract, the murine *in vitro* model described can be used as an easy accessible and less expensive model than canine or porcine tissue for further investigation of these 5-HT_4 receptors and their signaling pathway.

ACKNOWLEDGMENTS

The authors thank Mrs. Els Van Deynse for technical support.

DISCLOSURE

The authors have no competing interests.

AUTHOR CONTRIBUTION

RL designed the study; VP performed experiments and data analysis. Both interpreted the findings and wrote the manuscript.

REFERENCES

1. Gershon MD, Tack J. The serotonin signaling system: from basic understanding to drug development-for functional GI disorders. *Gastroenterology*. 2007;132:397-414.
2. Galligan JJ, Parkman H. Recent advances in understanding the role of serotonin in gastrointestinal motility and functional bowel disorders. *Neurogastroenterol Motil*. 2007;19:1-4.
3. Kellum JM, Albuquerque FC, Stoner MC, Harris RP. Stroking human jejunal mucosa induces 5-HT release and Cl- secretion via afferent neurons and 5-HT4 receptors. *Am J Physiol Gastrointest Liver Physiol*. 1999;277:G515-G520.
4. Greenwood-Van Meerveld B, Venkova K, Hicks C, Dennis E, Crowell MD. Activation of peripheral 5-HT4 receptors attenuates colonic sensitivity to intraluminal distension. *Neurogastroenterol Motil*. 2006;18:76-86.
5. Liu MT, Kuan YH, Wang JW, Hen R, Gershon MD. 5-HT4 receptor-mediated neuroprotection and neurogenesis in the enteric nervous system of adult mice. *J Neurosci*. 2009;29:9683-9699.
6. Bianco F, Bonora E, Natarajan D, et al. Prucalopride exerts neuroprotection in human enteric neurons. *Am J Physiol Gastrointest Liver Physiol*. 2016;310:G768-G775.
7. McLean PG, Coupar IM. Further investigation into the signal transduction mechanism of the 5-HT4-like receptor in the circular smooth muscle of human colon. *Br J Pharmacol*. 1996;118:1058-1064.
8. Prins NH, Shankley NP, Welsh NJ, et al. An improved *in vitro* bioassay for the study of 5-HT4 receptors in the human isolated large intestinal circular muscle. *Br J Pharmacol*. 2000;129:1601-1608.
9. Cellek S, John AK, Thangiah R, et al. 5-HT4 receptor agonists enhance both cholinergic and nitrergic activities in human isolated colon circular muscle. *Neurogastroenterol Motil*. 2006;18:853-861.

10. Leclere PG, Prins NH, Schuurkes JAJ, Lefebvre RA. 5-HT4 receptors located on cholinergic nerves in human colon circular muscle. *Neurogastroenterol Motil*. 2005;17:366-375.
11. Prins NH, Akkermans LMA, Lefebvre RA, Schuurkes JAJ. 5-HT4 receptors on cholinergic nerves involved in contractility of canine and human large intestine longitudinal muscle. *Br J Pharmacol*. 2000;131:927-932.
12. Leclere PG, Lefebvre RA. Presynaptic modulation of cholinergic neurotransmission in the human proximal stomach. *Br J Pharmacol*. 2002;135:135-142.
13. Cellek S, Thangiah R, Jarvie EM, Vivekanandan S, Lalude O, Sanger GJ. Synergy between 5-HT4 receptor activation and acetylcholinesterase inhibition in human colon and rat forestomach. *Neurogastroenterol Motil*. 2008;20:539-545.
14. Tonini M, Candura SM, Onori L, Coccini T, Manzo L, Rizzi CA. 5-Hydroxytryptamine4 receptor agonists facilitate cholinergic transmission in the circular muscle of guinea-pig Ileum - Antagonism by tropisetron and Dau-6285. *Life Sci*. 1992;50:Pl173-Pl178.
15. Prins NH, van der Grijn A, Lefebvre RA, Akkermans LMA, Schuurkes JAJ. 5-HT4 receptors mediating enhancement of contractility in canine stomach; an *in vitro* and in vivo study. *Br J Pharmacol*. 2001;132:1941-1947.
16. Priem EKV, Lefebvre RA. Investigation of neurogenic excitatory and inhibitory motor responses and their control by 5-HT4 receptors in circular smooth muscle of pig descending colon. *Eur J Pharmacol*. 2011;667:365-374.
17. Priem E, Van Colen I, De Maeyer JH, Lefebvre RA. The facilitating effect of prucalopride on cholinergic neurotransmission in pig gastric circular muscle is regulated by phosphodiesterase 4. *Neuropharmacology*. 2012;62:2126-2135.
18. Galligan JJ, Vanner S. Basic and clinical pharmacology of new motility promoting agents. *Neurogastroenterol Motil*. 2005;17:643-653.
19. Liu M, Geddis MS, Wen Y, Setlik W, Gershon MD. Expression and function of 5-HT4 receptors in the mouse enteric nervous system. *Am J Physiol Gastrointest Liver Physiol*. 2005;289:G1148-G1163.
20. Nagakura Y, Ito H, Kiso T, Naitoh Y, Miyata K. The selective 5-hydroxytryptamine (5-HT)(4)-receptor agonist RS67506 enhances lower intestinal propulsion in mice. *Jpn J Pharmacol*. 1997;74:209-212.
21. Lee MJ, Cho KH, Park HM, Sung HJ, Choi S, Im W. Pharmacological profile of DA-6886, a novel 5-HT4 receptor agonist to accelerate colonic motor activity in mice. *Eur J Pharmacol*. 2014;735:115-122.
22. Wang L, Martinez V, Kimura H, Tache Y. 5-Hydroxytryptophan activates colonic myenteric neurons and propulsive motor function through 5-HT4 receptors in conscious mice. *Am J Physiol Gastrointest Liver Physiol*. 2007;292:G419-G428.
23. Gallego D, Gil V, Martinez-Cutillas M, Mane N, Martin MT, Jimenez M. Purinergic neuromuscular transmission is absent in the colon of P2Y1 knocked out mice. *J Physiol*. 2012;590:1943-1956.
24. Craig DA. The Cheng-Prusoff relationship: something lost in the translation. *Trends Pharmacol Sci*. 1993;14:89-91.
25. Gil V, Martinez-Cutillas M, Mane N, Martin MT, Jimenez M, Gallego D. P2Y(1) knockout mice lack purinergic neuromuscular transmission in the antrum and cecum. *Neurogastroenterol Motil*. 2013;25:e170-e182.
26. De Man JG, De Winter BY, Seerden TC, De Schepper HU, Herman AG, Pelckmans PA. Functional evidence that ATP or a related purine is an inhibitory NANC neurotransmitter in the mouse jejunum: study on the identity of P2X and P2Y purinoceptors involved. *Br J Pharmacol*. 2003;140:1108-1116.
27. Cosyns SMR, Dhaese I, Thoonen R, et al. Heme deficiency of soluble guanylate cyclase induces gastroparesis. *Neurogastroenterol Motil*. 2013;25:e339-e352.
28. Torocsik A, Oberfrank F, Sershen H, Lajtha A, Nemesy K, Vizi ES. Characterization of somatodendritic neuronal nicotinic receptors located on the myenteric plexus. *Eur J Pharmacol*. 1991;202:297-302.
29. Donnerer J, Liebmann I, Holzer-Petsche U. Hexamethonium-induced augmentation of the electrical twitch response in the guinea-pig ileum longitudinal muscle-myenteric plexus strip. *Neurosci Lett*. 2014;577:34-37.
30. Zhou XP, Galligan JJ. Non-additive interaction between nicotinic cholinergic and P2X purine receptors in guinea-pig enteric neurons in culture. *J Physiol*. 1998;513:685-697.
31. Vural IM, Ozturk Fincan GS, Bozkurt NB, Ercan ZS, Sarioglu Y. Role of nicotinic acetylcholine receptor subtypes on nicotine-induced neurogenic contractile response alternation in the rabbit gastric fundus. *Eur J Pharmacol*. 2009;602:395-398.
32. De Maeyer JH, Prins NH, Schuurkes JAJ, Lefebvre RA. Differential effects of 5-hydroxytryptamine(4) receptor agonists at gastric versus cardiac receptors: an operational framework to explain and quantify organ-specific behavior. *J Pharmacol Exp Ther*. 2006;317:955-964.
33. Broad J, Hughes F, Chin-Aleong J, Sifrim D, Sanger GJ. Regionally dependent neuromuscular functions of motilin and 5-HT4 receptors in human isolated esophageal body and gastric fundus. *Neurogastroenterol Motil*. 2014;26:1311-1322.
34. Gale JD, Grossman CJ, Whitehead JWF, Oxford AW, Bunce KT, Humphrey PPA. GR113808 - a novel, selective antagonist with high-affinity at the 5-HT4 receptor. *Br J Pharmacol*. 1994;111:332-338.
35. Tonini M, Messori E, Franceschetti GP, et al. Characterization of the 5-HT receptor potentiating neuromuscular cholinergic transmission in strips of human isolated detrusor muscle. *Br J Pharmacol*. 1994;113:1-2.
36. Priem EKV, De Maeyer JH, Lefebvre RA. Influence of phosphodiesterases on basal and 5-HT4 receptor facilitated cholinergic contractility in pig descending colon. *Eur J Pharmacol*. 2013;705:156-163.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Pauwelyn V, Lefebvre RA. 5-HT_4 receptors facilitate cholinergic neurotransmission throughout the murine gastrointestinal tract. *Neurogastroenterol Motil*. 2017;00:e13064. https://doi.org/10.1111/nmo.13064