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Effects and sites of action of a M1 receptor positive allosteric modulator on colonic motility in rats and dogs compared with 5-HT_4 agonism and cholinesterase inhibition

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Running title: Sites, mechanisms and comparison of M1PAMs with other colokinetics

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Abstract

Background: Muscarinic receptor 1 positive allosteric modulators (M1PAMs) enhance colonic propulsive contractions and defecation through the facilitation of M1 receptor (M1R) mediated signaling. We examined M1R expression in the colons of 5 species and compared colonic propulsion and defecation caused by the M1PAM, T440, the 5-HT_4 agonist, prucalopride, and the cholinesterase inhibitor, neostigmine, in rats and dogs.

Methods: M1R expression was profiled by immunostaining and in-situ hybridization. *In vivo* studies utilized male SD rats and beagle dogs. Colonic propulsive contractions were recorded by manometry in anaesthetized rats. Gut contractions in dogs were assessed using implanted force transducers in the ileum, proximal, mid and distal colons.

Key Results: M1R was localized to neurons of myenteric and submucosal plexuses and the epithelium of the human colon. A similar receptor localization was observed in rat, dog, mouse and pig. T440 enhanced normal defecation in rats in a dose-dependent manner. Prucalopride also enhanced defecation in rats, but the maximum effect was half that of T440. Neostigmine and T440 were similarly effective in enhancing defecation, but the effective dose of neostigmine was close to its lethal dose. In rats, all 3 compounds induced colonic contractions, but the associated propulsion was strongest with T440. In dogs, intestinal contractions elicited by T440 propagated from ileum to distal colon. Prucalopride and neostigmine also induced intestinal contractions, but these were less well coordinated. No loss of effectiveness of T440 on defecation occurred after 5-days of repeated dosing.

Conclusion and Inferences: These results suggest that M1PAMs produce highly coordinated propagating contraction by actions on the enteric nervous system of the colon. The localization of M1R to enteric neurons in both animals and humans suggests that the M1PAM effects would be translatable to human. M1PAMs provide a potential novel therapeutic option for constipation disorders.

KEYWORDS: muscarinic positive allosteric modulator, prucalopride, neostigmine, enteric nervous system, colonic motility, prokinetics, defecation

Key Points

- The M1 muscarinic receptor was localized to neurons of the myenteric and submucosal plexuses and the epithelium of the colon in human, rat, dog, mouse and pig.
- Comparison of the effectiveness of the M1PAM, T440, the 5-HT_4 agonist, prucalopride and the anticholinesterase, neostigmine, to enhance normal defecation in rats revealed that T440 was superior to the other agents. In dogs, intestinal contractions elicited by T440 propagated from ileum to distal colon. Intestinal contractions induced by prucalopride or neostigmine were less well coordinated.
- No desensitization occurred when T440 was administered on 5 successive days.
- The results indicate that M1PAMs produce well-coordinated propagating contraction in the colon by actions on the enteric nervous system.

1 Introduction

Constipation is a common disorder that is difficult to treat for many patients, with about 50% of patients being dissatisfied with their treatment.[1] Although a range of colokinetic agents that act at different sites have been tested to relieve constipation, there is a lack of head-to head comparisons of their actions.[1] Drug therapies include anticholinesterases, such as neostigmine,[2] 5-HT_4 receptor agonists, such as prucalopride,[3, 4] and recently, muscarinic receptor 1 positive allosteric modulators (M1PAMs) have been suggested to be provide a possible treatment.[5-7] PAMs have provoked much interest as therapeutic agents because, in comparison to direct agonists that stimulate receptors independent of their physiological state of engagement, PAMs have effects in proportion to physiological levels of receptor activation.[8] Thus, PAMs are anticipated to be more effective in augmenting physiological patterns of activity than are direct agonists. However, this assumption has not been tested for M1PAM effects on the colorectum by matched comparison of colokinetics that act as direct agonists of receptors and colokinetics that act indirectly to enhance receptor activation.

In this study, we have compared a direct agonist, prucalopride, that acts at 5-HT_4 receptors on enteric neurons in the colorectum, with neostigmine, that enhances acetylcholine (ACh) action by blocking its degradation, thus enhancing cholinergic transmission, and with a M1PAM, T440, that augments transmitter action specifically at M1 ACh receptors (M1R). We have investigated the

distribution of M1R in the human colon and compared the distribution to that in rodents, dogs and pigs.

2 Materials and methods

Experiments were conducted on rats and dogs. Further tissue for localization studies were collected from human, mouse, rat, dog and pig. Procedures were approved by the Experimental Animal Care and Use Committee of Takeda Pharmaceutical Company Limited (approval AU-00020311), the University of Melbourne Animal Ethics Committee (approval 1613996), the South Australian Health and Medical Research Institute (approval SAM195), and Nissei Bilis Co. Ltd. (approval 1901-01).

2.1 Test compounds

The M1PAM, T440 (1,5-anhydro-2,3-dideoxy-3-[6-({3-fluoro-4-[(2-methoxyethyl)carbamoyl]phenyl}methyl)-7,8-dimethyl-4-oxoquinazolin-3(4H)-yl]-L-threo-pentitol), was synthesized by the Takeda Pharmaceutical Company. The potency of T440 was measured using CHO-K1 cells transfected with the human, mouse or canine muscarinic acetylcholine receptor M1r or M2r-5r genes. T440 was a highly potent M1PAM with *in vitro* potency and selectivity as follows. M1PAM activity at species specific receptors was: human, EC_{50}=6.3 nM; rat, EC_{50}=5.9 nM; dog, EC_{50}=5.1 nM. Agonist activities at the M1r for each species were all greater than 10000 nM. T440 had no PAM or agonist effects at the M2, 3, 4 or 5 receptors; EC_{50} values at these receptors were all greater than 10000 nM for PAM or agonist activity. T440 was orally active and bioavailability was 40% in rats and 31% in dogs after oral administration (data not shown). Prucalopride was also synthetized by the Takeda Pharmaceutical Company. Neostigmine bromide (Prostigmine®) was purchased from Abcam, Japan.

2.1 Localization studies

Tissue processing: For immunohistochemistry, human gastrointestinal tissue was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 4-12 hours followed by cryo- protection in 30% sucrose for 24 hours at 4°C, prior to freezing in optimal cutting medium (OCT Compound, Sakura® Finetek, Netherlands). Similarly, mouse, rat, dog and pig gastrointestinal tract and brain tissue were collected and fixed for 18-20 hours followed by cryo-protection. Tissue for *in situ* hybridization histochemistry was post-fixed in 4% paraformaldehyde for 24 hours followed by cryo-protection in graded sucrose (10%, 20% then 30%) in 0.1 M phosphate buffer at a temperature of 4°C until the

tissue sank to the bottom of the vessel. Tissue was then frozen in OCT prior to cryosectioning. Sections were 10-12 μm thick.

2.1.1 M1R immunostaining:

Labelling: Sections were dried for 1 hour at room temperature (RT) followed by 3 x 5 minute washes in 0.2% Triton-X in phosphate buffered saline (PBS-T; Sigma-Aldrich). Sections were then incubated for 30 minutes at room temperature with 5% normal chicken serum (CHBX0010; Applied Biological Products, SA, Australia) in PBS-T to reduce non-specific binding of secondary antibodies. This was followed by overnight incubation (18-24 hours) at 4°C with rabbit anti-muscarinic receptor (1:100; recognizing aa 443-458 of the intracellular C-terminal; AMR010 from Alomone Laboratories, Jerusalem, Israel) in PBS-T. Following this, sections were washed (3 x 5 minutes washes in PBS-T) before being incubated for 1 hour at room temperature with chicken anti-rabbit Alexa Fluor 594 (1:200, A-21442 Molecular Probes, ThermoFisher Scientific, MA, USA). Sections were washed (3 x 5 minute washes in PBS-T) then mounted with ProLong Diamond Antifade with DAPI (P36962 Molecular Probes, ThermoFisher Scientific) and dried at room temperature for 24 hours prior to visualization.[9]

Pre-absorption controls: Primary antibody was absorbed with the immunogen peptide (supplied by Alomone) in a 1:3 antibody to peptide ratio as per recommendations.

2.1.2 M1R *in situ* hybridisation (ISH)

Probes: To detect M1R expression RNAScope (Advanced Cell Diagnostics BioTechne, CA, USA) probes against CHRM1 were used for mouse (ADV495291; https://www.ncbi.nlm.nih.gov/nuccore/NM_001112697.1 target region: 851 – 1994) or human (ADV407951; http://www.ncbi.nlm.nih.gov/nuccore/NM_000738.2 target region: 34 – 2807) were used. RNAScope probes for peptidylprolyl isomerase B (Ppib; human PN 313901 NM_000942.4; mouse PN 313911 NM_011149.2) were used as positive control probes and dapB (bacterial protein) was used as a negative control probe. Ppib expression has been shown to be greatest in colonic epithelial cells and glandular tissue with weak expression in neural tissue.[10]

Labelling: RNAscope ISH Brown Reaction Kits (mouse ADV322371 and human ADV322370; Advanced Cell Diagnostics BioTechne) were used as per manufacturer instructions.

Sample preparation: Sections were mounted on SuperFrost slides and air dried for 20 minutes before undergoing phosphate buffered saline washes followed by 10 minute incubation with RNAscope hydrogen peroxide followed by rinses with MilliQ (MQ) water before undergoing target

retrieval using RNAscope retrieval solution at 99°C for 5 minutes. Sections were then washed with MQ water followed by a 100% ethanol wash prior to air drying for 5 minutes. Sections were then pre-treated with RNAscope Protease Plus for 30 minutes at 40°C in a hybridization oven.

Assay: Sections were washed with MQ water and the probes were applied for 2 hours at 40°C. Sections were washed with RNAscope wash buffer followed by a series of incubations with RNAscope hybridization amplifiers (as per manufacturer's instructions).

Signal detection: RNAscope DAB-A and DAB-B substrates were mixed in equal volumes then applied to sections for 10 minutes followed by washes with tap water. Sections were mounted in 80% glycerol and stored in the fridge overnight.

Counterstain: Sections were washed with water and counterstained with diluted Meyer's haematoxylin in water (1:1 ratio) for 2 minutes then rinsed well with water followed by a quick rinse in 0.02% ammonia water. Sections were then rinsed with water prior to ethanol washes (2 minutes in 70% ethanol, 95% ethanol then 100% ethanol) followed by 5 minutes in xylene. Slides were then mounted with DPX mountant (Sigma-Aldrich) and dried prior to visualization.

Microscopy: Immunofluorescence labelling was viewed using an epifluorescent microscope (Olympus BX51; Olympus Australia Pty Ltd, Vic, Australia). ISH DAB reaction was viewed using a Zeiss Axio Observer Z1 microscope (Carl Zeiss Pty Ltd, NSW, Australia).

2.2 Fecal output and scoring

Male Sprague Dawley (SD) rats (7 wks-old, IGS, Charles River, Ibaraki, Japan) were used. Rats were acclimated to the experimental environment by handling 2-3 times in a week before the experiment. Rats were moved to a mesh bottom cage from their regular home cage the day before the experiment. On the day of the experiment, test compounds were given by oral gavage, animals were placed in the cages and the feces produced were collected and counted for up to 2 hours after the administration of drug. Collected feces were inspected for scoring and weighed immediately after collection. Water content in feces was measured by drying the pellets overnight at 65°C in an oven and reweighing the tubes. Scoring of the fecal condition was made by an observer blinded to the treatment of the rats, and the person doing the scoring was not the person collecting the feces. We used a scoring scale with well-formed externally dry pellets (as produced normally by healthy mice) = 1; well-formed but moist = 2; unformed adhering moist pellets = 3; unformed wet pellets

(diarrhea-form) = 4. These correspond, approximately, to human Bristol scale 3, 4, 5 and 6, respectively.

For defecation studies, T440 was suspended in 0.5% methylcellulose solution (Metolose®, SM-100, Shin-etsu Chemicals, Tokyo, Japan) and administered orally at 5 mL.kg^{-1}. Prucalopride was dissolved in 2% dimethylsulphoxide (Wako, Tokyo, Japan) and 0.5% hydroxymethylcellulose (Wako, Tokyo, Japan) and administered orally at 5 mL.kg^{-1}. Neostigmine bromide was dissolved in saline and administered subcutaneously at 2mL.kg^{-1}.

2.3 Experiments in anesthetized rats

Experiments were conducted as previously described [11]. Rats were sedated with ketamine hydrochloride from Troy Laboratories, Glendenning, Australia (50-60 mg.kg^{-1}, i.m.), following which anesthesia was induced with α-chloralose (Troy Laboratories; 60 mg.kg^{-1}, i.v.). The femoral artery was then cannulated for the infusion of anesthetic and blood pressure recording, and the femoral vein was cannulated for delivery of drugs. Blood pressure and heart rate were recorded with a Power Lab recording system using Chart 5 software (both from ADInstruments, Sydney, Australia). Anesthesia was maintained by intra-arterial infusion of α-chloralose (12-20 mg.kg^{-1}.h^{-1}) plus ketamine (3-5 mg.kg^{-1}.h^{-1}) in phosphate buffered saline (PBS; 0.15M NaCl containing 0.01M sodium phosphate buffer, pH 7.2). For colorectal recording, the distal colon was cannulated at the colonic flexure, and a second cannula was inserted into the anus. The colon remained *in situ*, and the abdominal muscle and skin were closed around the proximal cannula. The oral cannula was connected to a Mariotte bottle filled with warm PBS, and the distal cannula to a pressure transducer via a one-way valve. The baseline intraluminal pressure was maintained at 7-8 mmHg by adjusting the heights of the Mariotte bottle and outlet. Expelled fluid was collected in a cylinder distal to the one-way valve and measured by weighing with a force transducer. At the end of each experiment, the rat was killed with a lethal dose of sodium pentobarbitone (300 mg/kg, i.v.), while still under anesthesia. T440 and prucalopride were dissolved in dimethylacetamide (DMA): polyethelene glycol400: dH_20, 1:1:3 and neostigmine was dissolved in dH2O. Compounds were given intravenously at a rate of 1mL/kg body weight.

2.4 Experiments on dogs

Eight adult male beagle dogs (~10 kg, Kitayama Labes, Nagano, Japan) were used for colonic motility studies. These dogs were surgically implanted with force transducers prior to the experiments, as previously described [7]. Once the animals recovered to a stable state (wound healed and GI motility recovered by observed fecal output), colonic motility was assessed using telemetry. Dogs (n=4) that showed stable intestinal contraction wave patterns were selected for further studies.

These animals were housed in individual cages and were provided with solid food every morning. On the day of experiment, food was withheld in the morning and dogs were fed later in the day when the experiment was completed. Colonic motility was measured by the telemetry system with the dogs in a freely moving state. After stabilization of the baseline, T440 or prucalopride suspended with vehicle (0.5% methylcellulose) or vehicle alone was administered by oral gavage at a volume of 10 mL/kg and colonic motility was measured for 4 hours after administration. In the case of neostigmine, the compound was dissolved in saline and administered subcutaneously (s.c.) at volume of 0.5 mL/kg. The numbers of high amplitude contractions and defecation episodes were quantified.

2.5 Data and statistics

Data are expressed as mean ± SEM. Statistical comparisons are made using Student's t-test, one-way ANOVA with Tukey's multiple comparisons test or by Shirley-Williams multiple comparison test followed by one-way ANOVA, as indicated in the text. The statistical packages were Prism 7.0 (GraphPad, San Diego, CA, USA) and Exsus 8.0 (CAC Croix Co, Tokyo, Japan). Results were considered to differ significantly when $P \leq 0.05$.

3 Results

3.1 Localization of M1 receptor (M1R) expression

M1R protein (immunostaining) and mRNA expression (*in situ* hybridization histochemistry) were both localized in sections of colon from human (Fig. 1), rat, dog, mouse and pig (Fig. 2). In human colon (Fig. 1) M1R-immunoreactivity (M1R-IR) was localized to the mucosa, specifically within mucosal epithelial cells lining the lumen and crypts (Fig. 1A, 1D) and within the submucosal (Fig. 1B) and myenteric ganglia (Fig. 1A, 1B). M1R-IR was also evident in nerve fibre-like structures in the circular muscle (Fig. 1A, 1B). Pre-absorption of the M1R antiserum with the immunizing peptide abolished labelling of epithelial cells, neurons and nerve-fibres (Fig. 1C, 1E), whereas non-specific labelling of cells within the lamina propria of the mucosa remained (Fig. 1E). In human colon, similar to the pattern of M1R-IR, *in situ* hybridization localization of M1R was present within mucosal epithelial cells (Fig. 1F) and in myenteric ganglia (Fig. 1H), which was absent in negative probe (dapB) controls (Fig. 1G, 1I).

In the colons of rat (Fig. 2A), dog (Fig. 2B-2D), mouse (Fig. 2E-2G) and pig (not shown), M1R-IR and M1R mRNA were localized to mucosal epithelial cells lining the lumen and crypts (Fig 2A, 2B,

2E and 2F) and within myenteric ganglia (Fig. 2A, 2D, 2E-2G and 2I) and submucosal ganglia (Fig. 2C, 2H).

3.2 Effects of colokinetics on defecation in rat

T440 given by oral gavage to conscious rats increased fecal pellet output with a threshold dose of ~0.1 mg/kg (Fig 3; Table 1). Doses of 0.3 and 1 mg/kg caused the number of fecal pellets produced in the 2 hours after drug administration to increase 2.5-fold. There were no discernable behavioral side-effects of this M1PAM at doses up to 30 mg/kg in conscious rats; that is, no indications of aversion, stress or discomfort. Prucalopride also increased the fecal pellet output, but the maximum number of fecal pellets was fewer than the maximum produced after M1PAM administration. This can be compared to a significant effect of prucalopride on the transit of a charcoal meal in the rat stomach and small intestine with a dose of 2 mg/kg [12] and increases in bead propulsion in the rat colon with 4 mg/kg.[13] In our experiments, the threshold for prucalopride to increase fecal pellet output appeared to be between 3 and 10 mg/kg, consistent with Dalziel[13], but the dose-response relation was shallow, with 100 mg/kg being required to cause a 60% increase above control. There were no discernable behavioural side effects of prucalopride in the conscious rats. Subcutaneous administration of neostigmine also caused an increase in fecal pellet output, with a threshold of about 0.03 mg/kg and more than doubling at 0.3 mg/kg. However, neostigmine caused substantial side effects, notably uncoordinated skeletal muscle contraction at 0.3 mg/kg; and at 1 mg/kg it was lethal. The fecal scores, a measure of increased moisture in the stool, were elevated by T440 and neostigmine, but not by prucalopride (Table 1).

3.3 Effects of intravenous administration of colokinetics in rat

Each of the 3 colokinetics increased the numbers of contractile events and the propulsion of colonic content (Fig 4). T440 elicited a steep dose response relation, with a threshold below 3 µg/kg and a strong effect at 30 µg/kg. Prucalopride elicited a bell-shaped curve, with a strong effect on the numbers of contraction, but a lesser effect on propulsion. The maximum effects of prucalopride on contractions and propulsion were less than maximum effects elicited by T440 or neostigmine. The concentration-response relation was steep for neostigmine, with 2.5 µg/kg being near the bottom of the curve and 7.5 µg/kg producing a maximum effect. In contrast to T440, increasing the dose did not increase its effectiveness in propelling the contents.

The relative efficiency of the three colokinetics was assessed by determining the amount of fluid propelled per contraction greater than 6 mmHg, at the dose for maximum effect for each compound (Fig 5). T440 at a dose of 10 µg/kg expelled 233 ± 47 µL per contraction (n = 6), whereas

prucalopride at the maximum effective dose of 1 mg/kg expelled 22 ± 9 μL per contraction (n = 6) and neostigmine at 25 μg/kg expelled 25 ± 6 μL per contraction (n = 6). Thus, contractions evoked by the M1PAM are more efficient in causing propulsion. Comparisons of the increased amount of fluid expelled in 60 minutes after drug administration with the most effective doses were: T440 (10 mg/kg), 4.9 ± 1.1 mL expelled; prucalopride (1 mg/kg), 1 ± 0.5 mL expelled, and neostigmine (25 μg/kg), 1.8 ± 0.5 mL expelled.

3.4 Effects in conscious dogs

High amplitude propagated contractions (HAPCs) that were conducted from the distal ileum to the mid-colon were monitored by using force transducers implanted on the colonic walls in dogs. Oral administration of the M1PAM, T440 (0.03mg/kg) to conscious dogs, evoked prominent contractions with high amplitudes (Fig. 6). These propagating contractions were highly correlated in time with defecation episodes. There were no discernable behavioral side-effects (aversion, stress or discomfort) of oral T440 at doses up to 0.3 mg/kg in conscious dogs.

A low dose of neostigmine (0.1 mg/kg) caused sustained trains of contractions and the basal tone was elevated. Neostigmine at 0.03 mg/kg, s.c., induced colonic contraction and defecation, but the net number of contractions was fewer than with T440. A higher dose of neostigmine, 0.1 mg/kg s.c., caused irregular contractions and that did not correlate in time with defecation (Fig 6 F), and the dogs vomited. Prucalopride also induced colonic contractions (Fig 6 E). Some contractions were correlated with defecation episodes, but the number of these propagating contractions was fewer than evoked by T440, rather, many uncoordinated contractions occurred. HAPCs were also observed after prucalopride administration (0.3mg/kg), but they were less well coordinated than after T440. Only a limited number of defecation episodes could be attributed to administration of prucalopride. The dose response relations for defecation episodes are compared in Fig. 6A, B and C.

3.4 Repeated administration of T440 in rats

There is often a decline in response when applications of agonists of GPCRs are repeated. To test whether this occurred for T440, it was administered once daily for 5 days and defecation at day 1 and 5 was monitored. As illustrated in Fig 2A, T440 enhanced normal defecation in rats in a dose-dependent manner on day 1. On day 5, a similar enhancement of defecation was observed, and no marked reduction of the efficacy was observed (Fig 7).

4 Discussion

We investigated the expression of the M1R in the colon of human, rat, dog, mouse and pig and compared the colokinetic effects of the M1R positive allosteric modulator, T440, with that of a direct enteric neuron agonist, prucalopride, and the anti-cholinesterase, neostigmine. The M1R was expressed by neurons of the myenteric and submucosal plexuses of human, rat, dog, mouse and pig colon. Previous studies of guinea-pig and human colon have localized M1R-IR to cholinergic enteric neurons, specifically to myenteric neurons positive for choline acetyltransferase and to vesicular acetylcholine transferase positive nerve fibres in the circular muscle.[14, 15] Moreover, functional experiments show that activation of the M1R increases enteric neuron excitability and transmitter release.[16] Thus, the M1R is deduced to be expressed by excitatory muscle motor neurons, although this has not been shown directly in dog and rat, and M1PAMs would be predicted to enhance physiologically-mediated cholinergic transmission to the muscle. We also observed M1R expression by colonic epithelial cells across all species investigated.

In experiments on conscious rats, the M1PAM, T440, enhanced fecal pellet output. The maximum effect was an increase of 2.5-fold the response to vehicle. T440 also increased the moisture content of the feces as indicated by the increased fecal score. Taken together with the fact that M1PAMs have been reported to enhance contractions of colon muscle and water secretion in isolated tissues,[5, 7] it is deduced that M1PAMs have a dual mode of action as prokinetics and secretory stimulants.[7] Neostigmine also enhanced defecation, but there was a very small safety margin for neostigmine between its prokinetic effect and severe, even fatal, side effects. Prucalopride is efficacious in human and is approved for chronic idiopathic constipation in the EU and the US.[17] As expected, prucalopride enhanced defecation in a dose dependent manner, but it was less effective than T440 or neostigmine. This is consistent with the essential role of cholinergic transmission in excitatory pathways to the muscle, and the lesser role of 5-HT.[18, 19]

In order to investigate the basis for the differences of the effectiveness of the 3 compounds to elicit defecation, we investigated colonic propulsive contractions in anesthetized rats. T440 increased the number of contractions in parallel with increased propulsion of fluid from the colon. Prucalopride also increased the number of contractions and fluid outflow, but it was less efficient than T440, in that there was less fluid propelled per contraction. This relationship is consistent with prucalopride eliciting a smaller effect than T440 on defecation. Furthermore, prucalopride exhibited a bell-shaped dose response relation, which agrees with previous studies of 5-HT_4 agonist effects on intestinal motility,[20] and with 5-HT_4 agonists having inhibitory effects on pre-contracted intestine.[21] The bell-shaped relation, and the dual excitatory and inhibitory effects of 5-HT_4 agonists, may limit their effectiveness. By contrast, cholinergic neurotransmission that is enhanced by M1PAMs is excitatory. The M1R occurs on both choline acetyltransferase (ChAT) positive

excitatory motor neurons and neuronal nitric oxide synthase (nNOS) positive inhibitory motor neurons.[14] Thus, the M1PAMs are suggested to maintain co-ordination of the oral contractions and the anal relaxations of propulsive reflexes. Neostigmine, like prucalopride, was less efficient in causing propulsion than T440, even though the maximum numbers of colonic contractions elicited were almost the same as with T440. The efficiency of propulsive contraction estimated by the ratio between fluid outflow and number of colonic contractions indicates a greater effectiveness of the M1PAM. Thus, the outcome from our manometry studies is well correlated with that from our defecation studies.

Because neostigmine acts by enhancing the actions of released ACh, not simply directly stimulating receptors, it might be expected to be similarly effective to the M1PAM. We speculate that neostigmine may be less effective because neostigmine prolongs as well as enhances responses at synapses. This means that the correct temporal relationships of excitation at cholinergic synapses in the enteric circuit might be retained when an M1PAM enhances transmission, but not when an anticholinesterase that extends the timecourse of action of ACh is used. Neostigmine also enhances transmission at all cholinergic synapses, nicotinic and muscarinic, and possibly permits ACh that would be metabolized by cholinesterases to overflow the synapses and access other sites.

Force transducers implanted on the serosal surface of the colon were used to detect high amplitude propagating contractions (HAPCs). This enabled us to investigate colonic motility, in conscious dogs, without the anaesthesia necessary for the rat studies. Our previous report demonstrated that M1PAMs enhanced HAPCs, and that the HAPCS were highly correlated with defecation episodes.[7] In the present study, both neostigmine and prucalopride induced colonic contractions in conscious dogs, but only a few defecation episodes were observed, compared with those elicited by the M1PAM. This relative effectiveness is highly consistent with the rat manometry study. Both compounds induced colonic contractions in dogs, but many of the contractions were uncoordinated. Since the HAPC itself is spontaneously induced by endogenous stimuli,[22] it is considered that positive allosteric modulation would maintain coordination of the colonic motility, while enhancing contraction amplitudes. 5-HT_4 agonism and cholinesterase inhibition activate the enteric motor system directly and with each of these the coordination of colonic contractions is lost to some extent.

Finally, we investigated the repeated administration of the M1PAM on defecation in rats, in order to investigate if tolerance occurred. In the case of T440, when it was administered orally once daily for 5 days, the fecal pellet output was almost identical on day 1 and day 5, suggesting no tolerance occurred. Generally, long lasting activation of GPCR causes desensitization of the receptor by internalization.[8] In the case of the M1PAM, at least in the rat study, lack of tolerance

suggests it could be utilized in the clinical setting as a daily medication for chronic constipation. This needs to be tested.

In conclusion, an M1PAM can induce coordinated colonic propulsive activity and induce or enhance defecation. The level of coordination of colonic contraction by the M1PAM was superior to that of prucalopride and neostigmine.

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Disclosures

Yasuhiro Tsukimi was an employee of the Takeda Pharmaceutical Company Limited at the time this study was conducted. The other authors declare no conflict of interest.

Author contributions

YT, JBF, SMB and RVP conceptualized and designed the study. RVP, MDN, JCM performed animal studies and analysed the data. AHM, S G-C, SMB conducted localisation studies and analysed the data. JBF and YT integrated data and wrote the manuscript. All authors contributed discussion and approved the manuscript.

Table 1. Effects of T440, prucalopride and neostigmine on fecal pellet output and nature of feces. Two hours after compound administration, the feces were collected and weighed. Fecal scores, wet weight and dry weight were determined.

	Dose mg/kg	Number of rats (n)	Fecal weight		Score	Water content (%)
			Wet wt (g)	Dry wt (g)		
T440	0	12	1.14 ± 0.30	0.43 ± 0.11	1.01	62.3
	0.13	12	1.08 ± 0.16	0.44 ± 0.07	1.32	59.3
	0.1	12	1.70 ± 0.29	0.65 ± 0.11	1.25	61.8
	0.3	12	3.08 ± 0.27	1.03 ± 0.06	1.45	66.6
	1	12	1.36 ± 0.40	1.08 ± 0.12	1.73	67.9

Prucalopride	0	10	1.17 ± 0.24	0.44 ± 0.09	1.10	62.4
	1	10	0.79 ± 0.23	0.34 ± 0.10	1.10	57.0
	3	10	0.96 ± 0.14	0.39 ± 0.06	1.20	59.4
	10	10	1.36 ± 0.18	0.56 ± 0.08	1.30	58.8
	30	10	1.77 ± 0.14	0.66 ± 0.04	1.50	62.7
	100	10	1.71 ± 0.21	0.70 ± 0.09	1.20	59.1

Neostigmine	0	8	0.94 ± 0.17	0.42 ± 0.09	1.00	55.3
	0.01	8	0.81 ± 0.22	0.38 ± 0.10	1.13	53.1
	0.03	8	1.86 ± 0.42	0.74 ± 0.14	1.13	60.2
	0.1	8	1.76 ± 0.29	0.76 ± 0.13	1.25	56.8
	0.3	8	3.16 ± 0.24	1.19 ± 0.06	1.75	62.3
	1	6	death	-	-	-

Figure Descriptions

Figure 1: Localization of Muscarinic 1 receptor (M1R) in human colon. A, B: Sections of human colon immunostained for M1R protein (M1R-IR, cyan), showing M1R-IR localized within the colonic mucosa, myenteric ganglia (mg) and submucosal ganglia (smg). **C:** Control peptide pre-absorbed with M1R antisera (M1r-pre-ab), showing a lack of M1R-IR. **D:** M1R-IR (cyan) in colonic epithelial cells with their nuclei labelled with DAPI (magenta). **E:** Pre-absorbed M1R antisera resulted in a lack of M1R-IR within colonic epithelial cells, whilst non-specific labelling of cells within the lamina propria of the mucosa remained. **F:** In situ hybridization (ISH) shows M1R mRNA **(**m1r, brown**)** in colonic epithelial cells of human colon. **G:** The negative control probe, dapB, shows a lack of staining. **H:** ISH shows M1R mRNA **(**m1r, brown**)** in myenteric ganglia, positive labelling indicated by arrows, which is not present when the negative control probe, dapB, was used (**I**). Scale bars are all 50□m. cm: circular muscle; lm: longitudinal muscle.

Figure 2: Localization of Muscarinic 1 receptor (M1R) in rat, dog, mouse and pig colon. Sections of colon from rat (A), dog (B-D), mouse (E-G) and pig (H and I) immunostained for M1R protein (M1R-IR, cyan; A-E, H and I) and nuclei label DAPI (magenta; B-D, H and I) or in situ hybridization labelled for m1r mRNA (m1r, brown; F and G). Data collectively shows consistent M1R protein and mRNA expression within mucosal epithelial cells (A, B, E and F) and within the myenteric ganglia (D, E, F, G and I) across a range of species, with M1R-IR also evident in submucosal ganglia (C and H). Scale bars are all 50□m. mg: myenteric ganglia; smg: submucosal ganglia; cm: circular muscle; lm: longitudinal muscle.

Figure 3: Effects of T440 (po), prucalopride (po) and neostigmine (sc) on fecal output in conscious rats. Test compounds were administered PO or SC and fecal pellet output was counted for 2 hours. The maximum number of fecal pellets were doubled by T440 and neostigmine over a 10-30 fold doses range. In contrast, prucalopride had a shallow dose response relation and between 3 and 100 mg/kg, there was less than a doubling. The highest dose of neostigmine tested, 1 mg/kg, was toxic and at 0.3 mg/kg there were substantial side effects, particularly uncoordinated skeletal muscle contraction. Data are presented as the mean ± SEM, n = 6. Asterisks: Significantly different from vehicle control (blue bars).

Figure 4: Effects of intravenous administration of T440, prucalopride and neostigmine on colorectal propulsion in anaesthetized rats. A: T440 caused a substantial, dose-dependent, increase in colorectal contractions that was accompanied by propulsion of colon content. B: Prucalopride also caused a substantial increase in colorectal contractions, but the efficiency in causing propulsion of content was less, and the dose-response relation was bell-shaped. C: Neostigmine, like T440, substantially increased numbers of contractions, but was less efficient than T440 in causing propulsion. D, E: Records of propulsive contractions and propulsion in response to T440, 3 μg/kg.

Figure 5: Relative efficiency of colorectal contractile events in causing propulsion of colorectal content. Data are μL of fluid expelled through the anal cannula per pressure wave with amplitude > 6 mmHg in the first 60 min after compound administration, i.v. Compounds were added at doses that gave maximum numbers of contractions (see Fig. 4). T440 caused expulsion of significantly more fluid per contraction, compared to prucalopride or neostigmine.

Figure. 6: Comparisons of numbers of defecation events and contractile activity elicited in dogs by administration of the colokinetics. The dogs were given T440 (PO), prucalopride (PO) or neostigmine (SC) and intestinal smooth muscle contractions were monitored in freely moving conscious dogs. Defecation episodes were also counted. Data are presented as the mean ± SEM from 4 dogs.

Figure. 7: Comparisons of effects of initial and repeated oral dosing with T440. In A is the dose response in rats that had not been previously exposed to T440 (day 1). In B are the responses on day 5 for rats previously exposed to the same doses of T440, on days 1 to 4. Data are presented as the mean + SEM from 10 animals. Note that no diminution of the enhancement of defecation occurred after 5 days treatment.

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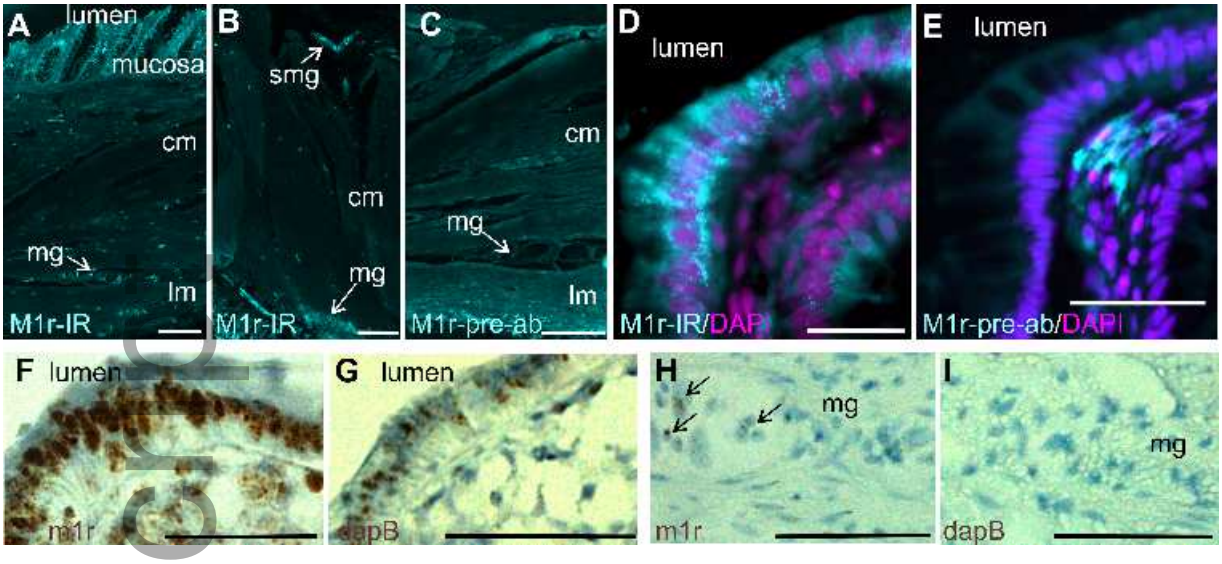

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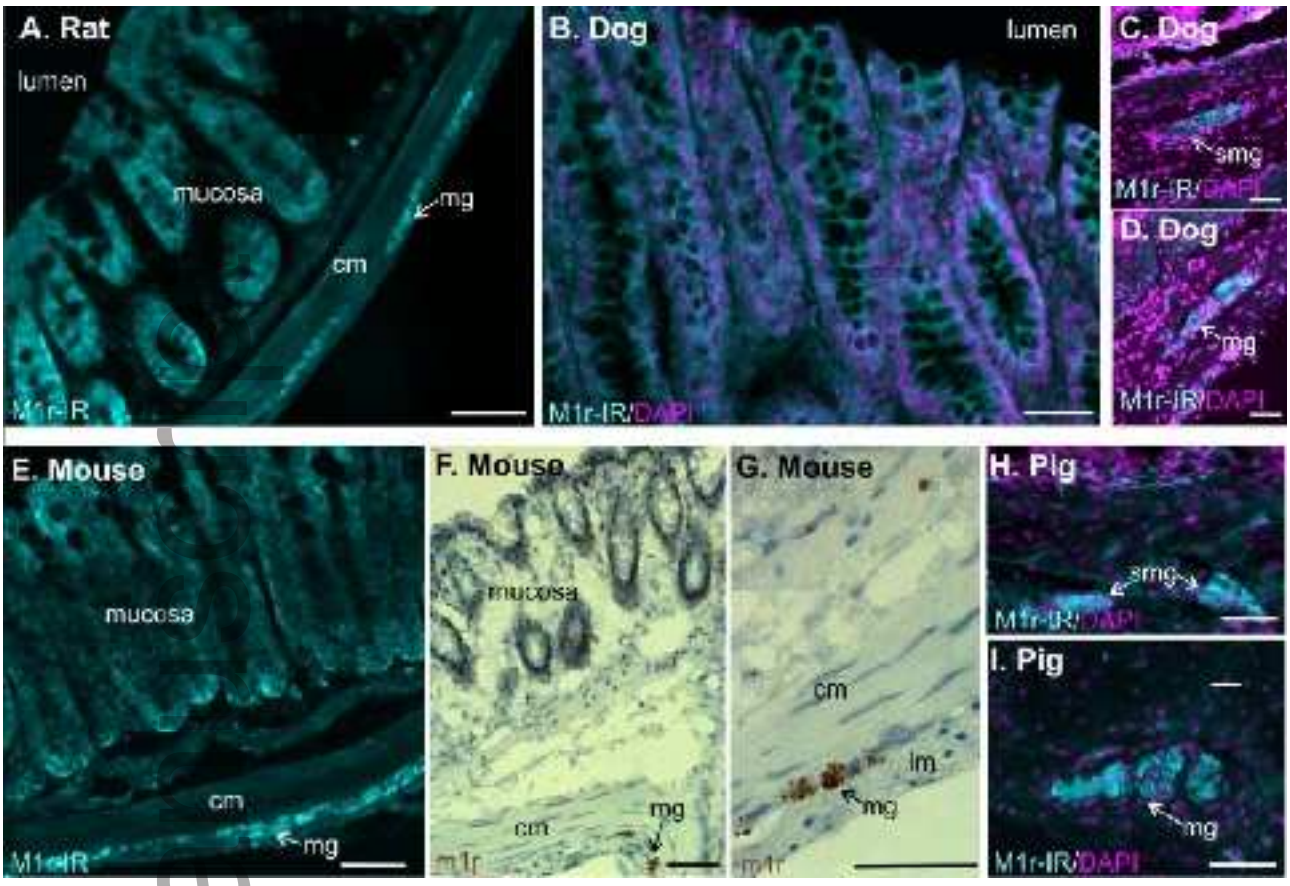

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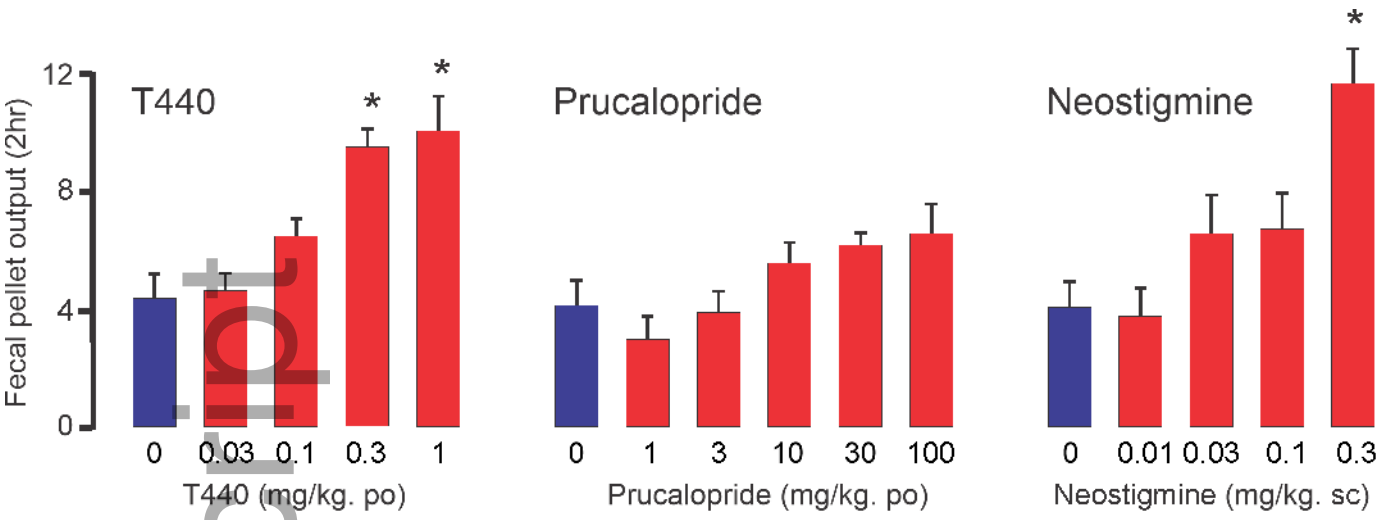

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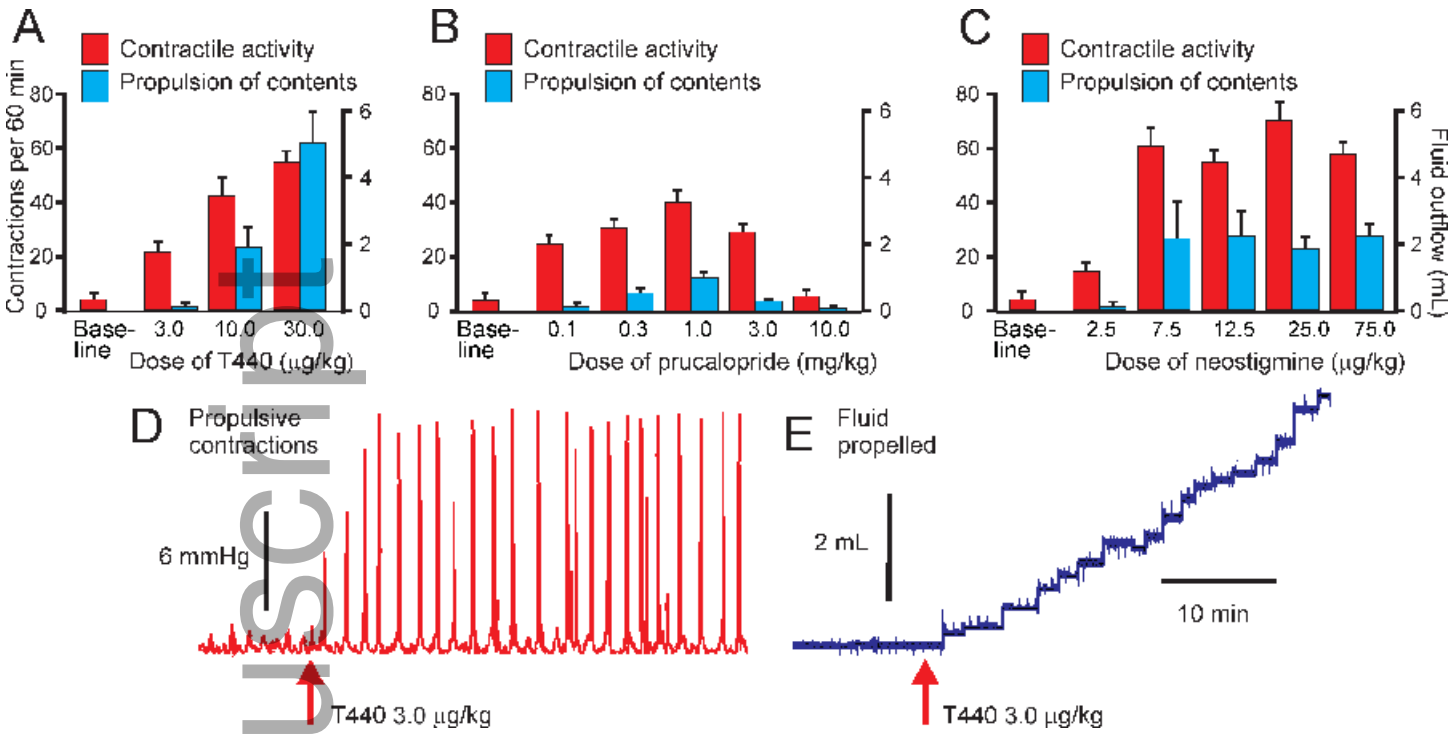

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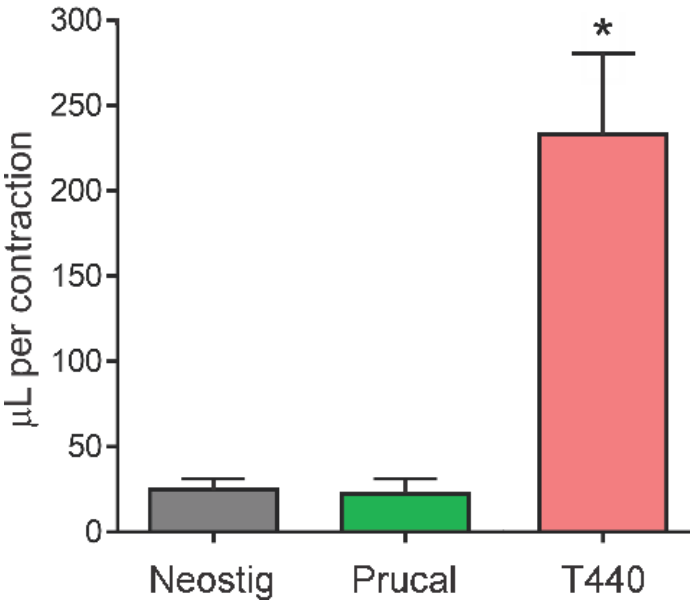

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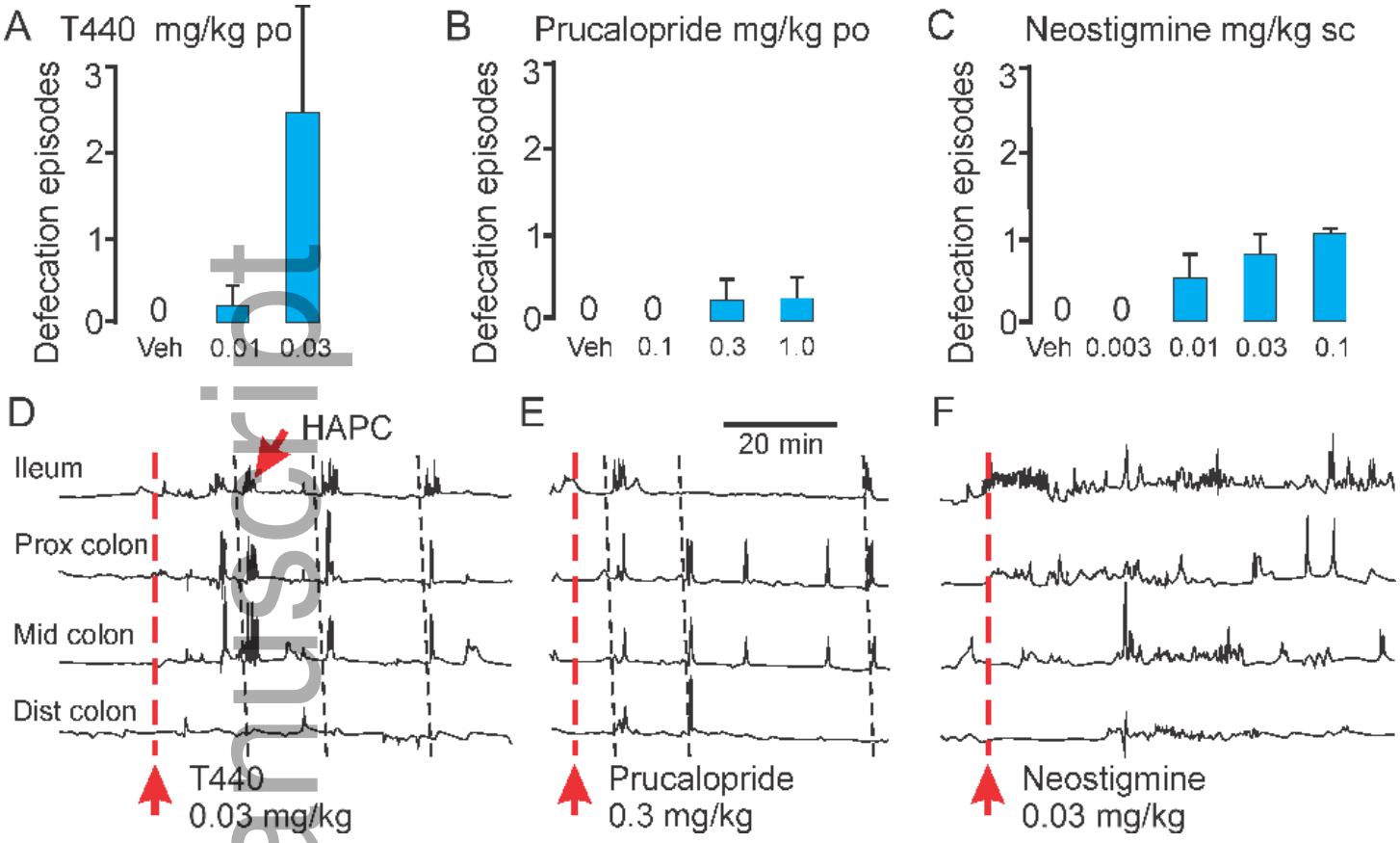

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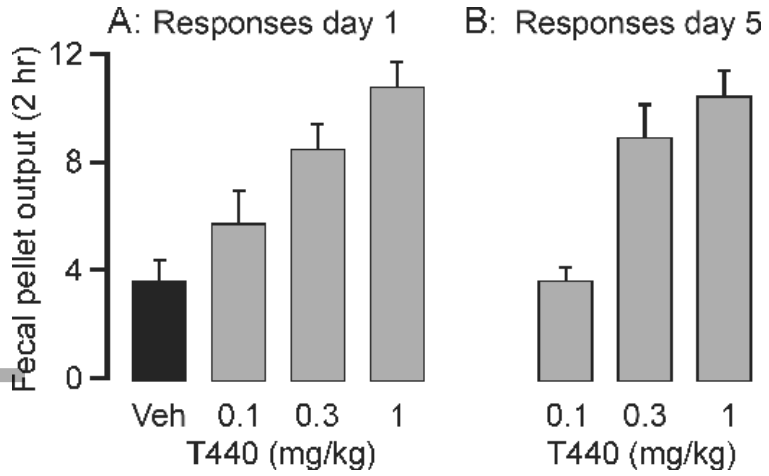

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