Cell and Tissue Research

Expression of the relaxin family peptide 4 receptor by enterochromaffin cells of the mouse large intestine

--Manuscript Draft--

Manuscript Number:	CTRE-D-22-00074R1
Full Title:	Expression of the relaxin family peptide 4 receptor by enterochromaffin cells of the mouse large intestine
Article Type:	Regular Article
Funding Information:	National Health and Medical Research Council (APP1145686) — Dr. John Furness Medical Research Council (MRC_MC_UU_12012/3) — Dr Frank Reimann Wellcome Trust (220271/Z/20/Z) — Dr Fiona M Gribble
Abstract:	The gastrointestinal hormone, insulin-like peptide 5 (INSL5), is found in large intestinal enteroendocrine cells (EEC). One of its functions is to stimulate nerve circuits that increase propulsive activity of the colon through its receptor, the relaxin family peptide 4 receptor (RXFP4). To investigate the mechanisms that link INSL5 to stimulation of propulsion, we have determined the localisation of cells expressing Rxfp4 in the mouse colon, using a reporter mouse to locate cells expressing the gene. The fluorescent signal indicating the location of Rxfp4 expression was in EEC, the greatest overlap of Rxfp4 dependent labelling being with cells containing 5-HT. In fact, >90% of 5-HT cells were positive for Rxfp4 labelling. A small proportion of cells with Rxfp4 dependent labelling was 5-HT negative, 11-15% in the distal colon and rectum, and 35% in the proximal colon. Of these, some were identified as L-cells by immunoreactivity for oxyntomodulin. Rxfp4 dependent fluorescence was in a sparse population of nerve endings, where it was colocalised with CGRP. We used the RXFP4 agonist, INSL5-A13, to activate the receptor and probe the role of the 5-HT cells in which it is expressed. INSL5-A13 administered by i.p. injection to conscious mice caused an increase in colorectal propulsion that was antagonised by the 5-HT 3 receptor blocker, alosteron, also given i.p. We conclude that stimuli that excite INSL5-containing colonic L-cells release INSL5 that, through RXFP4, excites 5-HT release from neighbouring endocrine cells, which in turn acts on 5-HT 3 receptors of enteric sensory neurons to elicit propulsive reflexes.
Corresponding Author:	John Furness Unimelb AUSTRALIA
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Unimelb
Corresponding Author's Secondary Institution:	
First Author:	Ada Koo
First Author Secondary Information:	
Order of Authors:	Ada Koo Ruslan V Pustovit Orla RM Woodward Jo E Lewis Fiona M Gribble Mohammed Akhter Hossain

	Frank Reimann
	John Furness
Order of Authors Secondary Information:	
Author Comments:	

Responses to Editors and Reviewers' comments
Manuscript No: CTRE-D-22-00074
Title: Expression of the relaxin family peptide 4 receptor by enterochromaffin cells of the mouse large intestine
By: Ada Koo; Ruslan V Pustovit; Orla RM Woodward; Jo E Lewis; Fiona M Gribble; Mohammed Akhter Hossain; Frank Reimann; John Furness

Editor:
We have responded to all comments by the reviewers. The reviewers' comments are copied below and after each comment we have added our response in *italic*. In the revised manuscript the changes are marked in *red.*

Reviewer: #1
Comments to the author:
This study was conducted to determine what types of enterochromaffin (EC) cells express the RXFP4, the receptor for insulin-like peptide, and whether stimulation of these receptors has a prokinetic effect via a serotonergic mechanism. A combination of immunohistochemistry in tissue from an Rxfp4-dependent reporter mouse along with in vivo colonic motility studies were used. The study was well-designed, the results are sound and clearly illustrated, and the conclusions are well founded. There are a small number of issues that the authors should address.
Response: We thank the reviewer for the comments. The issues raised are commented on below.

1) The parts of the Results section that cover the immunocytochemistry should be edited to make it explicitly clear what staining the authors are referring to. For example, page 5, line 46, the authors are talking about tissue co-stained for GFP and 5-HT, and give numbers for "positive cells". Are they referring to GFP-positive, 5-HT-positive, or double labeled? This is not the instance where it is confusing.
Response: We thank the reviewer for pointing out the ambiguity, which we have now rectified.
2) The authors should indicate how they avoided counting mast cells, which express 5-HT in the mouse.
Response: It is interesting that in healthy mice there are essentially no mast cells in the mucosa of the mouse colon, and definitely no intra-epithelial mast cells. So we were able to unambiguously identify the enteroendocrine cells.
3) Page 3, line 20/21, I believe the word ""of" should be changed to "or"..
Response: This typographic error has been fixed .
4) Page 4, line 55/56, "greated" should be changed to "greater".
Response: This typographic error has been fixed.
5) Page 4, line 57/58, "deviation" should be changed to "deviations".
Response: This error has been fixed.
6) Page 11, line 51, "ealier" should be changed to "earlier".
Response: This typographic error has been fixed.

Reviewer: #2
Comments to the author:
This manuscript presents new, interesting findings on the localisation and potential function of the intestinal hormone, Insl5 and its cognate receptor RXFP4. The Furness group have performed immunohistochemical and an in vivo bead excretion assay, to indicate that RXFP4 agonism induces endogenous 5HT3-mediated promotile efficacy that involves enterochromaffin cells and sensory neurons within the colon.

There are some important omissions, as follows.

1. Introduction, page 2, lines 46-48. Please explain which hormones are released from L cells to cause defecation, and how this was established. Context is missing here too and readers could be reminded that L cell-derived hormones, GLP-1 and PYY slow upper GI transit, the latter mediating ileal brake.
Response: We have added, with references, that there is no evidence that either GLP-1 or PYY is involved in defecation control, but that they have roles in slowing gastric and upper intestinal transit.
2. Methods, page 3, line 48. Include 'UK' and 'Australia' after mentioning respective University cities.
Response: Done
3. Page 5, in vivo studies, line 12. Were female mice used as in immunohistochemical studies? Either way, include the sex of the animals used please.
Response: The sex has been added, we used males for the in vivo studies.
4. Lines 12 - 24. You do not explain the experimental design of in vivo data shown in Figure
Response: This has been added in the revision.
5. Details of vehicle controls and the sequential testing of mice (presumably females?) must be included here.
Response: We have added to the text and Fig 5 the vehicle controls and that the same mice were used for successive tests, one week apart.
6. Why is the maximum time allowed for bead expulsion 30 min? This may appear in previous publications but this should be explained to this readership. Is it correct to record 30 min, when expulsion times were longer? This too requires explanation.
Response: We have explained the reason in the revision.
7. Results, page 5 lines 50 onwards. Please include comments on the EEC morphology here. From the images presented in Fig 1, long processes appear to be more evident in the distal colonic region specifically - not so in proximal or rectal?
Response: The reviewer is correct that cells with long processes were more prominent in the distal colon and rectum than in the proximal colon. We have modified the text to report this observation and have indicated that this reflects the relative abundances of 5-HT cells with different morphologies in the three regions that had been previously reported (Koo et al., 2021)
8. Page 7, legend to Fig 2. Please include the n numbers of animals from which provide the mean ± SEM.
Response: Cell counts were from 4 mice. This is now included in the figure legend.
9. Legend to Fig 5. Presumably vehicle controls are 'no drug' in blue? See comment above about lack of text explaining this in vivo experimental design.
Response: That is correct. To make it clear, we have changed 'no drug' to 'vehicle in the figure.
10. Page 12. Discussion, the last sentence (line 24-25) does not follow logically from the preceding text. So what? Please bring this final section together better. It is an abrupt ending!
Response: We have written a new concluding paragraph that brings together the main points of discussion.
11. Some references are incomplete: Lewis et al (2020) and (2021) for example.
Response: All references are now complete
12. Other corrections required;
Page 3 line 21 'with short, or no basal processes…'
Response: Corrected
Page 3, line 50, then sections of proximal colon.…
Response: Corrected
Page 4, line 47, please correct the spelling of super-resolution, and on lines 56-58, correct the grammar and spelling in this sentence.
Response: The spelling has been corrected and the sentence has been re-written as two sentences.
Page 5, lines 4 and 7 - correct spelling mistakes.
Response: Corrected

Page 11, line 51. Please correct the spelling mistake here.
Response: Corrected

Expression of the relaxin family peptide 4 receptor by enterochromaffin cells of the mouse large intestine

Ada Koo[1], Ruslan V Pustovit[1,2], Orla RM Woodward[3], Jo E Lewis[3], Fiona M Gribble[3], Mohammed Akhter Hossain[2,4], Frank Reimann[3], John B Furness[1,2]

[1]Department of Anatomy & Physiology, University of Melbourne, Parkville, VIC 3010, Australia

[2]Florey Institute of Neuroscience and Mental Health, Parkville, VIC 3010, Australia

[3]Wellcome Trust-MRC Institute of Metabolic Science-Metabolic Research Laboratories, University of Cambridge, Cambridge CB2 OQQ, UK

[4]Department of Biochemistry and Pharmacology, University of Melbourne, Parkville, VIC 3010, Australia

ORCID numbers:

AK: 0000-0003-2414-0852

RVP: 0000-0002-9221-1811

JBF: 0000-0002-0219-3438

*Proofs and Correspondence to:

Dr John B Furness

Dept Anatomy and Physiology

University of Melbourne

Parkville, VIC 3010

Australia

Email: j.furness@unimelb.edu.au

Abstract

The gastrointestinal hormone, insulin-like peptide 5 (INSL5), is found in large intestinal enteroendocrine cells (EEC). One of its functions is to stimulate nerve circuits that increase propulsive activity of the colon through its receptor, the relaxin family peptide 4 receptor (RXFP4). To investigate the mechanisms that link INSL5 to stimulation of propulsion, we have determined the localisation of cells expressing *Rxfp4* in the mouse colon, using a reporter mouse to locate cells expressing the gene. The fluorescent signal indicating the location of *Rxfp4* expression was in EEC, the greatest overlap of *Rxfp4* dependent labelling being with cells containing 5-HT. In fact, >90% of 5-HT cells were positive for *Rxfp4* labelling. A small proportion of cells with *Rxfp4* dependent labelling was 5-HT negative, 11-15% in the distal colon and rectum, and 35% in the proximal colon. Of these, some were identified as L-cells by immunoreactivity for oxyntomodulin. *Rxfp4* dependent fluorescence was also found in a sparse population of nerve endings, where it was colocalised with CGRP. We used the RXFP4 agonist, INSL5-A13, to activate the receptor and probe the role of the 5-HT cells in which it is expressed. INSL5-A13 administered by i.p. injection to conscious mice caused an increase in colorectal propulsion that was antagonised by the 5-HT_3 receptor blocker, alosetron, also given i.p. We conclude that stimuli that excite INSL5-containing colonic L-cells release INSL5 that, through RXFP4, excites 5-HT release from neighbouring endocrine cells, which in turn acts on 5-HT_3 receptors of enteric sensory neurons to elicit propulsive reflexes.

Keywords: INSL5; 5-HT; enteroendocrine cells, enteric nervous system, colonic reflexes

Introduction

Amongst gut endocrine cell products, insulin-like peptide 5 (INSL5) is confined to the distal large intestine, where it occurs in L-type enteroendocrine cells (EEC) that also contain glucagon-like peptide 1 (GLP-1) and peptide YY (PYY), both of these being costored with INSL5 in the same secretory vesicles of these EEC (Grosse et al. 2014, Billing et al. 2018, Vahkal et al. 2021). The administration of an INSL5 mimetic and stimulation of hormone release from colonic L cells using DREADD technology both cause defecation, but there is no evidence that either GLP-1 or PYY is involved in defecation control (Diwakarla et al. 2020, Lewis et al. 2020; Pustovit et al. 2021). On the other hand, these peptides, GLP-1 and PYY, have roles in slowing gastric and upper intestinal transit (Lin et al. 1996, Holst 2007). The INSL5 mimetic had no effect in mice in which the receptor for INSL5, the relaxin family peptide 4 receptor (RXFP4) was knocked out (Diwakarla et al. 2020), implying that RXFP4 is downstream of the L cell release of INSL5. The L cells express receptors for microbial products, including free fatty acid receptor (FFAR) 2 and OLFR78 (Karaki et al. 2006, Karaki

et al. 2008, Husted et al. 2017, Billing et al. 2019), and instillation of a short chain fatty acid mixture into the colon accelerates colonic emptying (Yajima 1985, Fukumoto et al. 2003). Acceleration of colonic propulsion by SCFAs in mice was inhibited by the RXFP4 receptor antagonist, INSL5-A13NR, implying that INSL5 has a physiological role to stimulate propulsion (Pustovit et al. 2021). There is also likely to be an involvement of 5-HT, acting through 5-HT_3 receptors, because enhanced propulsion caused by SCFAs or by DREADD-mediated stimulation of L cells was inhibited by 5-HT_3 receptor antagonists (Fukumoto et al. 2003, Lewis et al. 2020).

A feasible interpretation of these results is that INSL5 released from L cells acts on RXFP4 of adjacent enterochromaffin cells, causing release of 5-HT that stimulates the enteric nervous system to evoke propulsive reflexes (Pustovit et al. 2021). Supporting this interpretation, a recent study has located *Rxfp4*-dependent fluorescence to EEC of the mouse colon, 65% of these also expressing 5-HT (Lewis et al. 2021). The 5-HT containing enteroendocrine cells in the mouse colon have a variety of shapes that can be best revealed in thick sections (Koo et al. 2021, Kuramoto et al. 2021). Amongst these are enterochromaffin cells with basal processes as long as several 100 µm, some of which form close relationships with L cells and cells with short or no basal processes (Koo et al. 2021).

In the current study we have utilised an *Rxfp4*-dependent reporter mouse (Lewis et al. 2021) to investigate which of the types of 5-HT containing EEC express *Rxfp4* throughout the mouse colon, and we have also investigated whether the stimulation of colonic propulsion using an RXFP4-specific agonist is inhibited by a 5-HT_3 receptor blocker.

Methods

Tissue preparation

Tissue was harvested from $RXFP4^{EYFP}$ mice that were generated by crossing *Rxfp4*-Cre mice with GFP-based fxSTOPfx reporter mice (Lewis et al. 2021). Four female $RXFP4^{EYFP}$ mice were anesthetised and perfused through the heart with 4% paraformaldehyde (PFA). The large intestine, from the caecum to the internal anal sphincter was removed and placed in the same fixative at 4^{o}C overnight. Fixative was removed by 5 washes in phosphate buffered saline (PBS; 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH7.2). Fixed large intestine tissues from four female Rxfp4-Cre:Het Rosa26-GCaMP3:Hom mice, 8-10 weeks old, stored in PBS-sucrose-azide (0.1% w/v sodium azide and 30% w/v sucrose in PBS) on cool pack, were transferred from the Cambridge UK to the Melbourne Australia laboratories. Tissues were transferred to a 1:1 ratio of PBS-sucrose-azide and OCT compound, then sections of proximal colon, distal colon and rectum were embedded in 100% OCT compound and frozen in isopentane cooled with liquid nitrogen.

Immuohistochemistry

Sections of 60 µm thickness were cut using a cryostat and placed in PBS. Tissues were blocked in normal horse serum (10% v/v in PBS with 1% Triton X-100) for 1 hour at room temperature and then incubated with a mixture of primary antibodies (Table 1) for 3 nights at 4°C. Sections were washed three times with PBS, 15 minutes each, followed by incubation with a mixture of secondary antibodies overnight at 4°C. Sections were washed twice with PBS, 10 minutes each, and quenched with quenching buffer (5 mM copper sulfate and 50 mM ammonium acetate, pH5.0) for 1 hour at room temperature. Sections were washed once with PBS and twice with distilled water, 5 minutes each, followed by an incubation with Hoechst 33258 (10 µg/mL; Sigma-Aldrich, Sydney, NSW, Australia) for 45 minutes at room temperature. Sections were washed 3 times with distilled water, 5 minutes each, and then mounted on microscope slides in non-fluorescent mounting medium (Dako, Carpinteria, CA, USA).

Image acquisition and analysis

Images were captured using a super-resolution confocal microscope (LSM880 Airyscan Fast, Carl Zeiss, Sydney, NSW, Australia) using a 20x air objective or 63x oil objective. Captured images were deconvoluted using Airyscan Processing in Zeiss Zen (black edition) software prior to analysis. Brightness and contrast were adjusted using Fiji Image J (https://imagej.nih.gov/ij/), then cells were selected based on immunoreactivity and intensity and were exported for cell count analysis. Approximately 100 cells were counted for each region from each of the 4 animals. A cell was considered immuopositive when intensity was greater than background mean plus two standard deviations. Example images were converted to RGB colour before exporting as TIFF files using Fiji Image J.

Synthesis of RXFP4 agonist, INSL5-A13

INSL5-A13 was synthesised in house by our previoulsy published method (Patil et al. 2016). The A- and B-chains were each chemically assembled on solid phase support. Following this, the disulfide bridges between the chains were formed in solution and the two chain compound was purified.

In vivo studies

Mice were injected with vehicle, loperamide (1.0 mg/kg s.c.), or loperamide plus the 5-HT_3 receptor antagonist, alosetron (1.0 mg/kg i.p.), then 5 min later with the RXFP4 receptor antagonist, INSL5-A13 (6 ug/kg i.p.) or vehicle. After a further 20 min, colorectal propulsion was assessed using the bead expulsion test. Loperamide (Sigma-Aldrich, Sydney, NSW, Australia) was prepared in 1% Tween-80 in distilled water; alosetron HCl (Sigma-Aldrich) and INSL5-A13 were dissolved in distilled water. To measure bead expulsion, male mice, 20-30 g body weight, were briefly anaesthetized with 2% (v/v) isoflurane in 1L/min O_2 for a maximum of 15 seconds following induction with 4% isoflurane in 1L/min O_2 (Pustovit et al. 2021). A

3-mm round bead was inserted 2 cm into the distal colon using a flexible, plastic rod. After bead insertion, mice were placed in individual clean cages. The time taken from bead insertion to bead expulsion was recorded. The maximum time allowed for bead expulsion was 30 min. This was a practical choice; in control, bead expulsion times were less than 100 sec, and it was decided that 1800 sec was a sufficiently longer time to test for the effectiveness of loperamide to delay bead expulsion. If bead expulsion time was greater than 30 min, the mouse was left undisturbed in a quiet place and the bead was recovered 5 or 10 min later. The time was recorded as 30 min and the data included. Agonist and antagonist experiments were conducted in the period 8.00 am to 1.00 pm. The same mice were used for successive tests, one week apart.

Results

Colocalisation of Rxfp4-GFP, 5-HT, and oxyntomodulin (OXM)

A high degree of overlap of *Rxfp4*-dependent GFP and 5-HT was observed in EEC of the large intestine, with the greatest proportion of *Rxfp4*-GFP cells that expressed 5-HT being in the distal colon (Fig. 1). Of 5-HT cells, the great majority expressed *Rxfp4*-GFP; 93.5 ± 1.6% expressed *Rxfp4*-GFP in the proximal colon, 98.0 ± 1.5% in the distal colon, and 94.3 ± 2.9% in the rectum (Fig 1). The co-localization encompassed all morphologies of 5-HT cells, in particular, 5-HT cells with long basal processes that have been recently described in the mouse large intestine (Kuramoto et al. 2021) expressed *Rxfp4*-GFP. It is notable that EEC with long processes were a greater proportion of 5-HT/ *Rxfp4* cells in the distal colon and rectum, compared to the proximal colon. This reflects relative abundances of 5-HT cells with different morphologies in the three regions (Koo et al. 2021).

In tissues from *Rxfp4*-GFP mice co-stained for GFP and 5-HT, 34.6 ± 7.1% of cells that were positive for either marker only expressed *Rxfp4*-GFP in the proximal colon, 10.9 ± 5.0% in the distal colon, and 15.5 ± 6.5% in the rectum (Fig 1). When all staining was considered, in the proximal colon, co-expression of *Rxfp4*-GFP and 5-HT accounted for 59.0 ± 8.3% of all immunoreactive cells, (Fig. 1a‴), 87.0 ± 5.5% of all immunoreactive cells exhibited co-expression in the distal colon (Fig. 1b‴), and 78.7 ± 5.7% in the rectum (Fig. 1c‴).

We next examined the expression of OXM, as a marker for L cells, in relation to *Rxfp4*-GFP and 5-HT positive cells in the proximal colon, distal colon, and rectum. Interestingly, we observed scattered co-expression of OXM and *Rxfp4*-GFP amongst EEC (Fig. 2a-b‴), which was more commonly found in proximal colon (4.8 ± 1.0 cells/mm^2) than distal colon (2.0 ± 1.2 cells/mm^2) and rectum (1.4 ± 1.4 cells/mm^2). There were also some OXM and 5-HT double immunoreactive, but *Rxfp4*-GFP negative, cells in

the proximal colon (7.9 ± 3.8 cells/mm^2) and the distal colon (1.4 ± 0.8 cells/mm^2), but none in the rectum. Amongst OXM, *Rxfp4*-GFP and 5-HT positive cells, the majority of immunopositive cells were those that co-expressed *Rxfp4*-GFP and 5-HT (Fig. 2c).

Rxfp4-GFP postive nerve fibres

Nerve fibres with *Rxfp4*-GFP immunoreactivity were observed in all regions of the large intestine and were primarily found in the external muscle layers and submucosa (Fig 3a-c). Triple staining for GFP, 5-HT and CGRP was performed to further examine the relationship of *Rxfp4*-GFP positive fibres and CGRP containing fibres in the mucosa. *Rxfp4*-GFP positive fibres were not observed in the mucosa of the proximal colon, however, they were found in distal colon and rectum (Fig. 3b,c). The rectum was more densely innervated by *Rxfp4*-GFP and CGRP fibres than distal colon (Fig. 3c-c″). Higher power super-resolution microscopy was performed to examine the possible colocalisation of *Rxfp4*-GFP and CGRP, which revealed double labeled varicosities in the distal colon and rectum (Fig. 4). Interestingly, 5-HT immunoreactivity was also detected in some of the double labeled varicosities and appeared to be stored in vesicles (Fig. 4a‴,b‴), unlike the diffused labelling of CGRP. The co-expression of *Rxfp4*-GFP and CGRP indicated that *Rxfp4*-GFP postive fibres could be sensory nerve fibres orginating from the dorsal root ganglion, in which the expression of *Rxfp4* has been reported (Lewis et al. 2021).

Effect of the 5-HT$_3$ receptor antagonist, alosetron, on RXFP4 agonism

We used colorectal bead expulsion in conscious mice to investigate whether 5-HT is involved in the acceleration of colorectal propulsion that is evoked by stimulation of RXFP4. The same mice were investigated in successive weeks. In week 1, control bead expulsion times were determined and in week 2 the slowing by loperamide (opiate agonist) was measured. Loperamide (1 mg/kg, s.c.) slowed bead expulsion times, determined 25 minutes after loperamide, approximately 8-fold (Fig. 5). When the RXFP4 agonist, INSL5-A13 (6 µg/kg, i.p.), was given 5 minutes after loperamide, expulsion times measured 20 minutes later were reduced (Fig. 5). This acceleration of bead expulsion is due to action at RXFP4, as it is not observed in animals in which RXFP4 is knocked out (Diwakarla et al. 2020). The effect of INSL5-A13 to accelerate colonic bead expulsion was prevented by the 5-HT$_3$ receptor antagonist, alosetron (1 mg/kg, i.p.), given at the same time as loperamide (Fig. 5, $p < 0.05$). In the week after (week 5), the loperamide effect was tested again because there are sometimes changes in sensitivity to opiate agonists. There was no difference between bead expulsion times with the two loperamide applications (yellow columns, Fig. 5).

Discussion

The current work revealed that over 90% of 5-HT cells in the murine large intestine express *Rxfp4*, which implies that the 5-HT cells are downstream of the L cells that release the RXFP4 natural agonist, INSL5. In the mouse large intestine there are two subtypes of L cells, those in the proximal colon express PYY, GLP-1 and neurotensin, but rarely INSL5 (L^{Nts} cells), and those in the distal colon express PYY, GLP-1 and INSL5 (L^{Insl5} cells) (Billing et al. 2019). In human, neurotensin is not expressed in the large intestine, but, like mouse, expression of *Insl5* is higher distally in the large intestine and it is absent from the small intestine (Wang et al. 2020). When mice in which L cells of the distal colon (L^{Insl5} cells) that expressed a DREADD under the control of the *Insl5* promotor were stimulated with clozapine N-oxide (CNO; i.p.), there was an increase in defecation that was inhibited by a 5-HT_3 receptor antagonist (Lewis et al. 2020). Colonic L cells express receptors for short chain fatty acids (SCFA) (Karaki et al. 2006, Karaki et al. 2008, Husted et al. 2017, Billing et al. 2019) and in a previous study we showed that administration of a SCFA mix into the lumen of the large intestine caused an increase in colorectal bead expulsion and defecation that was blocked by an antagonist of the RXFP4 receptor (Pustovit et al. 2021). In the current study, we found a similar degree of antagonism of colorectal propulsion with the 5-HT_3 antagonist, alosetron, when the RXFP4 agonist, INSL5-A13 was used to stimulate colorectal propulsion. These data confirm previous studies that enteric motility reflexes can be initiated through 5-HT_3 receptors. The receptors are on the terminals of enteric intrinsic primary afferent neurons (IPANs) and are activated by 5-HT applied to the mucosa (Bertrand et al. 2000). The terminals of IPANs form a rich network of fibres beneath the mucosal epithelium where the 5-HT containing endocrine cells are located (Furness et al. 1990, Furness et al. 2004).

INSL5-containing L cells of the mouse colon express functional receptors for a number of other GPCRs including receptors for bile acids, amino acids and peptones, angiotensin-II, vasopressin and bombesin, all of which cause INSL5 release from EEC (Billing et al. 2018). Thus, like SCFAs, each of these is likely to increase the release of 5-HT indirectly through the INSL5/ RXFP4 system.

Possible overlapping roles of colonic enterochromaffin (EC) cells

Enterochromaffin (5-HT-containing) cells in the large intestine, like L cells, also express microbial metabolite receptors, *Ffar2*, *Olfr78*, and *Olfr558*, as well as the bile acid receptor, *Gpbar1* (Lund et al. 2018, Billing et al. 2019). This implies that there are both indirect, via L cell release of INSL5, and direct effects of microbial metabolites on EC cells. Moreover, the mechanosensitive ion channel *Piezo2* is expressed by about 58 ± 5% of colonic EC cells (Alcaino et al. 2018), which is consistent with earlier observations that mechanical stimulation of the mucosa causes 5-HT release (Bülbring and Crema 1959, Grider et al. 1996). A higher proportion of EC cells expressed *Piezo2* in the distal compared to the proximal colon (Billing et al.

2019). Thus, the majority of 5-HT cells in the distal colon, where > 95% exhibit *Rxfp4*-dependent labelling, are predicted to respond to both mechanical distortion and SCFAs. In the distal colon, about 20% of EC cells have prominent long basal processes and 58% have long or intermediate length basal processes (Kuramoto et al. 2021). Many of these cells must express *Rxfp4*. The long processes might be assumed to be associated with mechansensitivity, although EC cells in the small intestine which express *Piezo2* and are mechanosensitive (Alcaino et al. 2018), do not have basal processes (Koo et al. 2021).

RXFP4 nerve fibres

We observed a small population of nerve fibres positive for *Rxfp4*-GFP in the mucosa and adjacent submucosa that were immunoreactive for CGRP. We have not specifically investigated the origins of these fibres, but the majority of spinal afferent (dorsal root ganglion) fibres that supply the gastrointestinal tract in mice and other mammals are CGRP immunoreactive (Tan et al. 2010). Furthermore, *Rxfp4* dependent fluorescence is observed in small diameter nerve cells, of the type that express CGRP, in the dorsal root ganglia of mice (Lewis et al. 2021). *Rxfp4*-GFP labelling was not found in enteric neurons (Lewis et al. 2021), which are therefore deduced not to be a source of *Rxfp4*/CGRP nerve fibres in the colon.

Concluding remarks: Integrative roles of 5-HT cells

As discuused above, the 5-HT cells receive signals from L-cells through INSL5 and its receptor, RXFP4, also express receptors for bacterial metabolites and secondary bile acids, and are mechanoreceptive. The L-cells themselves have bacterial metabolite receptors. Thus it appears that a range of stimuli, such as SCFAs, bile metabolites and mechanical distortion, are integrated by the 5-HT-secreting EC cells, which may be a common pathway for different stimuli that influence colonic and rectal motility. Studies in which combinations of stimuli are applied may assist in unravelling how responses to different stimuli are integrated by the 5-HT cell population.

Acknowledgements

We thank Dr Linda Fothergill for her assistance with the figures. Confocal microscopy was undertaken at the Biological Optical Microscopy Platform, University of Melbourne.

Funding

These studies were supported by an NHMRC ideas grant (APP1182996) to MAH and RP and an NHMRC project grant (APP1145686) to JBF. Work in the Reimann/Gribble laboratory was funded by the MRC-UK (MRC_MC_UU_12012/3) and the Wellcome Trust (220271/Z/20/Z).

Conflicts of interest

Authors declare no conflict of interests.

Ethics approval

Tissues were harvested in accord with UK Home Office project licences 70/7824 and PE50F6065 and with approval by the University of 359 Cambridge Animal Welfare and Ethical Review Body.

Authors' contributions

AK, RVP, ORMW and JEL conducted experimental investigations; FR produced the reporter mouse; MAH synthesized and validated the agonist; AK, ORMW and JBF contributed to study design; AK, RVP and JBF analysed the data; JBF and AK wrote the manuscript and prepared the illustrations; all authors contributed to and approved the final manuscript.

References

Alcaino C, Knutson KR, Treichel AJ, Yildiz G, Strege PR, Linden DR, Li JH, Leiter AB, Szurszewski JH, Farrugia G, Beyder A (2018) A population of gut epithelial enterochromaffin cells is mechanosensitive and requires Piezo2 to convert force into serotonin release. Proc Nat Acad Sci USA 115:E7632-E7641

Bertrand PP, Kunze WAA, Furness JB, Bornstein JC (2000) The terminals of myenteric intrinsic primary afferent neurons of the guinea-pig ileum are excited by 5-hydroxytryptamine acting at 5-hydroxytryptamine-3 receptors. Neuroscience 101:459-469

Billing LJ, Larraufie P, Lewis J, Leiter A, Li J, Lam B, Yeo GSH, Goldspink DA, Kay RG, Gribble FM, Reimann F (2019) Single cell transcriptomic profiling of large intestinal enteroendocrine cells in mice - identification of selective stimuli for insulin-like peptide-5 and glucagon-like peptide-1 co-expressing cells. Mol Metab 29:158-169

Billing LJ, Smith CA, Larraufie P, Goldspink DA, Galvin S, Kay RG, Howe JD, Walker R, Pruna M, Glass L, Pais R, Gribble FM, Reimann F (2018) Co-storage and release of insulin-like peptide-5, glucagon-like peptide-1 and peptideYY from murine and human colonic enteroendocrine cells. Mol Metab 16:65-75

Bülbring E, Crema A (1959) The release of 5-hydroxytryptamine in relation to pressure exerted on the intestinal mucosa. J Physiol (Lond) 146:18-28

Diwakarla S, Bathgate RAD, Zhang X, Hossain MA, Furness JB (2020) Colokinetic effect of an insulin-like peptide 5-related agonist of the RXFP4 receptor. Neurogastroenterology & Motility 32:e13796

Fukumoto S, Tatewaki M, Yamada T, Fujimiya M, Mantyh C, Voss M, Eubanks S, Harris M, Pappas TN, Takahashi T (2003) Short-chain fatty acids stimulate colonic transit via intraluminal 5-HT release in rats. Am J Physiol Regul Integr Comp Physiol 284:R1269-R1276.

Furness JB, Robbins HL, Xiao J, Stebbing MJ, Nurgali K (2004) Projections and chemistry of Dogiel type II neurons in the mouse colon. Cell Tissue Res 317:1-12

Furness JB, Trussell DC, Pompolo S, Bornstein JC, Smith TK (1990) Calbindin neurons of the guinea-pig small intestine: quantitative analysis of their numbers and projections. Cell Tissue Res 260:261-272

Grider JR, Kuemmerle JF, Jin JG (1996) 5-HT released by mucosal stimuli initiates peristalsis by activating 5-HT4/5-HT1p receptors on sensory CGRP neurons. Am J Physiol 270:G778-G782

Grosse J, Heffron H, Burling K, Hossain MA, Habib AM, Rogers GJ, Richards P, Larder R, Rimmington D, Adriaenssens AA, Parton L, Powell J, Binda M, Colledge WH, Doran J, Toyoda Y, Wade JD, Aparicio S, Carlton MBL, Coll AP, Reimannk F, O'Rahilly S, Gribble FM (2014) Insulin-like peptide 5 is an orexigenic gastrointestinal hormone. Proc Natl Acad Sci USA 111:11133-11138

Holst JJ (2007) The physiology of glucagon-like peptide 1. Physiol Rev 87:1409-1439

Husted AS, Trauelsen M, Rudenko O, Hjorth SA, Schwartz TW (2017) GPCR-mediated signaling of metabolites. Cell Metab 25:777-796

Karaki S-I, Mitsui R, Hayashi H, Kato I, Sugiya H, Iwanaga T, Furness JB, Kuwahara A (2006) Short-chain fatty acid receptor, GPR43, is expressed by enteroendocrine cells and mucosal mast cells in rat intestine. Cell Tissue Res 324:353-360

Karaki S-I, Tazoe H, Hayashi H, Kashiwabara H, Tooyama K, Suzuki Y, Kuwahara A (2008) Expression of the short-chain fatty acid receptor, GPR43, in the human colon. J Mol Hist 39:135-142

Koo A, Fothergill LJ, Kuramoto H, Furness JB (2021) 5‐HT containing enteroendocrine cells characterised by morphologies, patterns of hormone co‐expression, and relationships with nerve fibres in the mouse gastrointestinal tract. Histochem Cell Biol 155:623-636

Kuramoto H, Koo A, Fothergill LJ, Hunne B, Yoshimura R, Kadowaki M, Furness JB (2021) Morphologies and distributions of 5‐HT containing enteroendocrine cells in the mouse large intestine. Cell Tissue Res 384:275-286

Lewis JE, Miedzybrodzka EL, Foreman RE, Woodward ORM, Kay RG, Goldspink DA, Gribble FM, Reimann F (2020) Selective stimulation of colonic L cells improves metabolic outcomes in mice. Diabetologia 63:1396-1407

Lewis JE, Woodward ORM, Smith CA, Adriaenssens AE, Billing L, Brighton C, Phillips BU, Tadross JA, Kinston SJ, Ciabatti E, Göttgens B, Tripodi M, Hornigold D, Baker D, Gribble FM, Reimann F (2021) Relaxin/insulin-like family peptide receptor 4 (Rxfp4) expressing hypothalamic neurons modulate food 1 intake and preference in mice. bioRxiv 2021, doi.org/10.1101/2021.06.26.450020.

Lin HC, Zhao X-T, Wang L, Wong H (1996) Fat-induced ileal brake in the dog depends on peptide YY. Gastroenterology 110:1491-1495

Lund ML, Egerod KL, Engelstoft MS, Dmytriyeva O, Theodorsson E, Patel BA, Schwartz TW (2018) Enterochromaffin 5-HT cells - a major target for GLP-1 and gut microbial metabolites. Mol Metab 11:70-83

Patil NA, Hughes RA, Rosengren KJ, Kocan M, Ang SY, Tailhades J, Separovic F, Summers RJ, Grosse J, Wade JD, Bathgate RAD, Hossain MA (2016) Engineering of a novel simplified human insulin-like peptide 5 agonist. J Med Chem 59:2118-2125

Pustovit RV, Zhang X, Liew JJM, Praveen P, Liu M, Koo A, Oparija-Rogenmozere L, Ou Q, Kocan M, Nie S, Bathgate RAD, Furness JB, Hossain MA (2021) A novel antagonist peptide reveals a physiological role of insulin-like peptide 5 in control of colorectal function. ACS Pharmacol Transl Sci 4:1665-1674

Tan LL, Bornstein JC, Anderson CR (2010) The neurochemistry and innervation patterns of extrinsic sensory and sympathetic nerves in the myenteric plexus of the C57BL6 mouse jejunum. Neuroscience 166:564-579

Vahkal B, Yegorov S, Onyilagha C, Donner J, Reddick D, Shrivastav A, Uzonna J, Good SV (2021) Immune system effects of insulin-like peptide 5 in a mouse model. Frontiers in Endocrinology 11:610672

Wang Y, Song W, Wang J, Wang T, Xiong X, Qi Z, Fu W, Yang X, Chen Y-G (2020) Single-cell transcriptome analysis reveals differential nutrient absorption functions in human intestine. J Exp Med 217:e20191130

Yajima T (1985) Contractile effect of short-chain fatty acids on the isolated colon of the rat. J Physiol (Lond) 368:667-678

Table

Table 1. Primary and secondary antibodies used and their dilutions.

	Target	Catalogue Number	Source	Species	Dilution	RRID
Primary	GFP	Ab13970	Abcam, Melbourne, Australia	Chicken	1:5000	AB_300798
	5-HT	20079	ImmunoStar, Hudson, Wi, USA	Goat	1:5000	AB_572262
	Oxyntomodulin	AB-323-AO010	Ansh Labs, Webster, Tx, USA	Mouse	1:1000	—
	CGRP	T4032	Peninsula Labs, Santa Cruz, Ca, USA	Rabbit	1:500	AB_518147
Secondary	Chicken IgG	703-545-155, Alexa Fluor® 488	Jackson ImmunoResearch Lab, West Grove, Pa, USA	Donkey	1:500	AB_2340375
	Goat IgG	A21432, Alexa Fluor® 555	Thermo Fisher Scientific, Scoresby, Australia	Donkey	1:800	AB_2535853
	Mouse IgG	A31571, Alexa Fluor® 647	Molecular Probes, Eugene, Or, USA	Donkey	1:2000	AB_162542
	Rabbit IgG	A32795, Alexa Fluor® 647	Thermo fisher Scientific	Donkey	1:1000	AB_2762835

Figures and Figure Descriptions

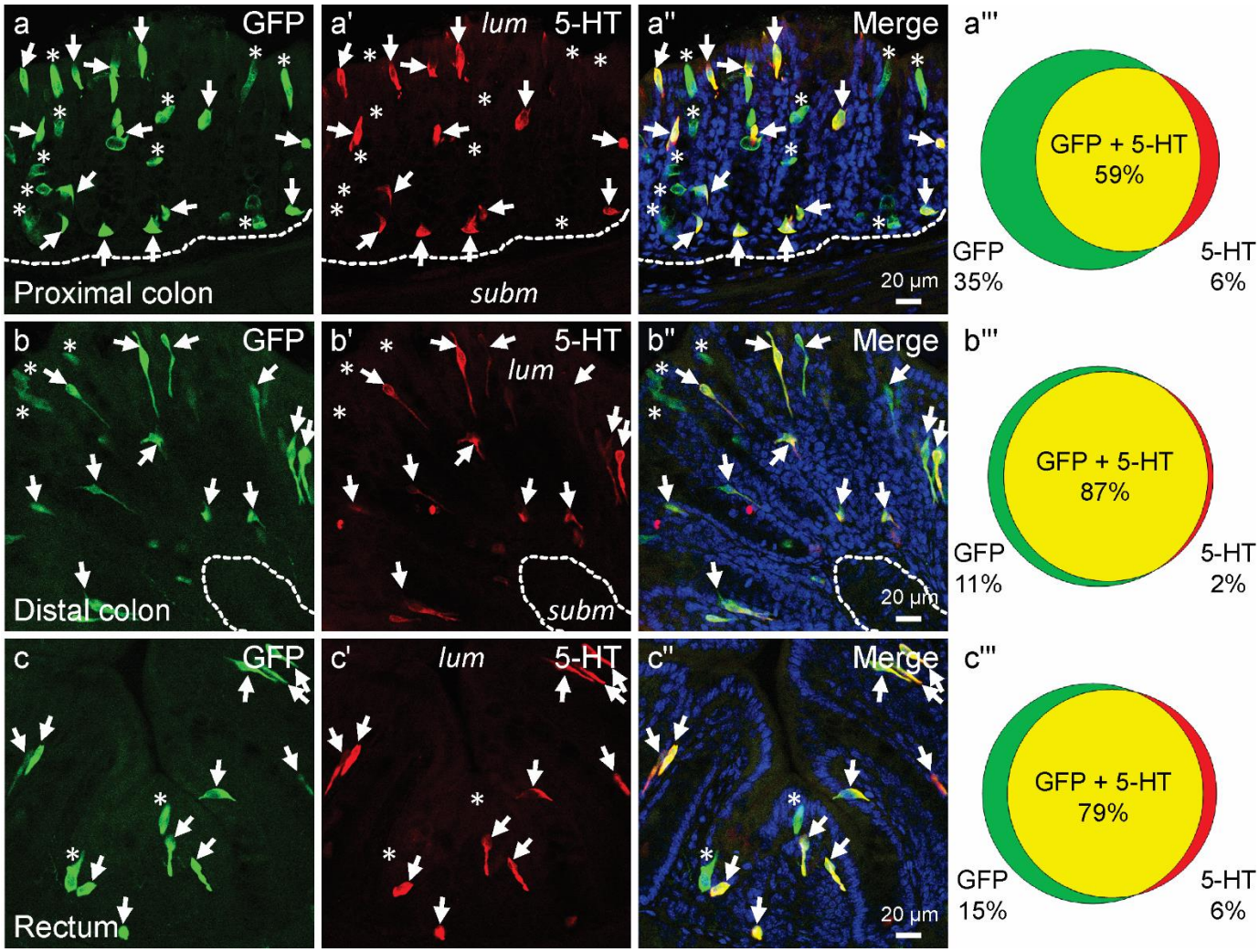

Fig. 1 Co-expression of *Rxfp4*-GFP and 5-HT in EEC of the proximal colon (**a-a‴**), distal colon (**b-b‴**), and rectum (**c-c‴**). These transverse sections through the mucosa show EEC of various morphologies, including cells with long basal processes. The luminal (*lum*) and submucosal (*subm*) aspects of the mucosa are indicated in the 5-HT images. Nuclei are revealed by Hoechst 33258 stain in the merged images. Double immunopositive cells for *Rxfp4*-GFP and 5-HT are marked by *arrows* and cells expressed only *Rxfp4*-GFP are indicated by *asterisks*. Venn diagrams show the proportions of *Rxfp4*-GFP, 5-HT, or double immunoreactive cells of approximately 100 cells in each region from each of the 4 animals.

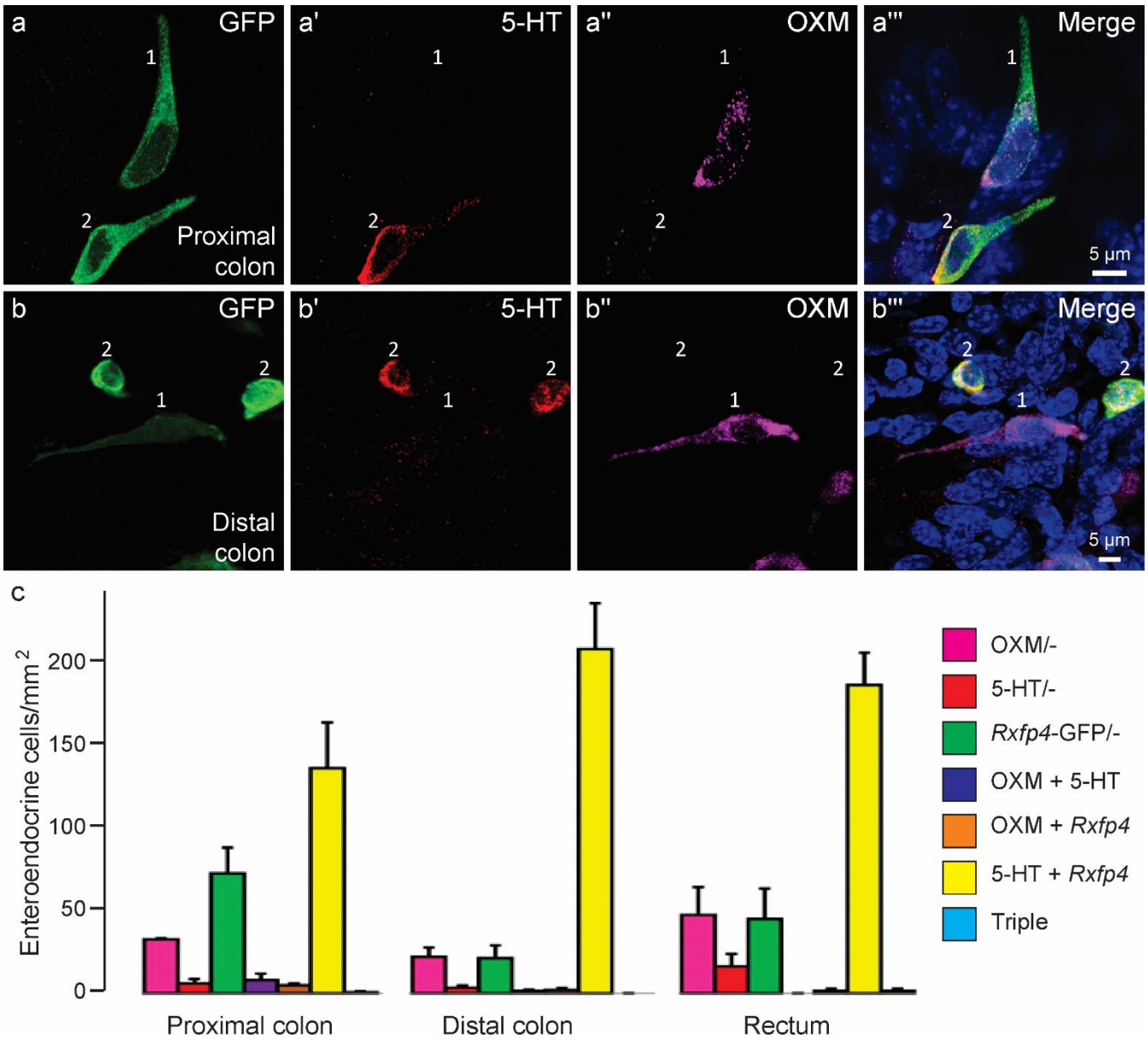

Fig. 2 Colocalisation of *Rxfp4*-GFP, 5-HT, and OXM in EEC of the large intestine. Shown are examples of double labelled *Rxfp4*-GFP and OXM cells (1) and double labelled *Rxfp4*-GFP and 5-HT cells (2) in the proximal colon (**a-a‴**) and the distal colon (**b-b‴**). **c**: Quantification of immuoreactive cells, from 4 mice, expressed as numbers of cells per total tissue area in mm^2. OXM/- indicates oxyntomodulin only (L cells without *Rxfp4*-GFP or 5-HT), etc. The most numerous cell type is 5-HT cells showing fluorescence for *Rxfp4*-GFP (yellow columns) . Data are mean ± SEM.

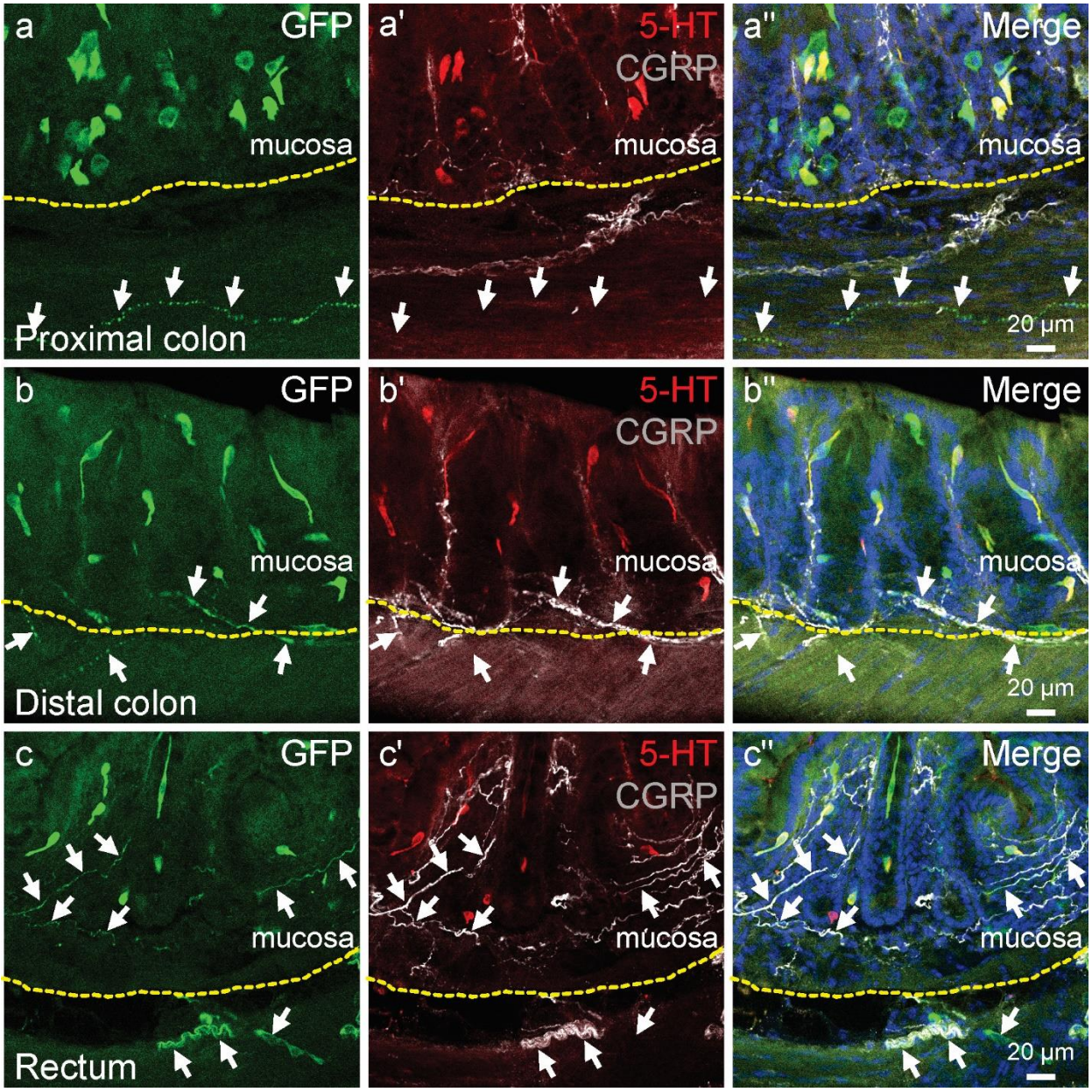

Fig. 3 *Rxfp4*-GFP positive nerve fibres in the muscle layer and submucosa in the proximal colon (**a-a″**), distal colon (**b-b″**), and rectum (**c-c″**) and the relations of 5-HT and CGRP to *Rxfp4*-GFP. Many of the *Rxfp4*-GFP positive fibres were CGRP immunoreactive. At this magnification it is difficult to identify 5-HT immunoreactive nerve fibres. *Arrows* indicate *Rxfp4*-GFP positive fibres.

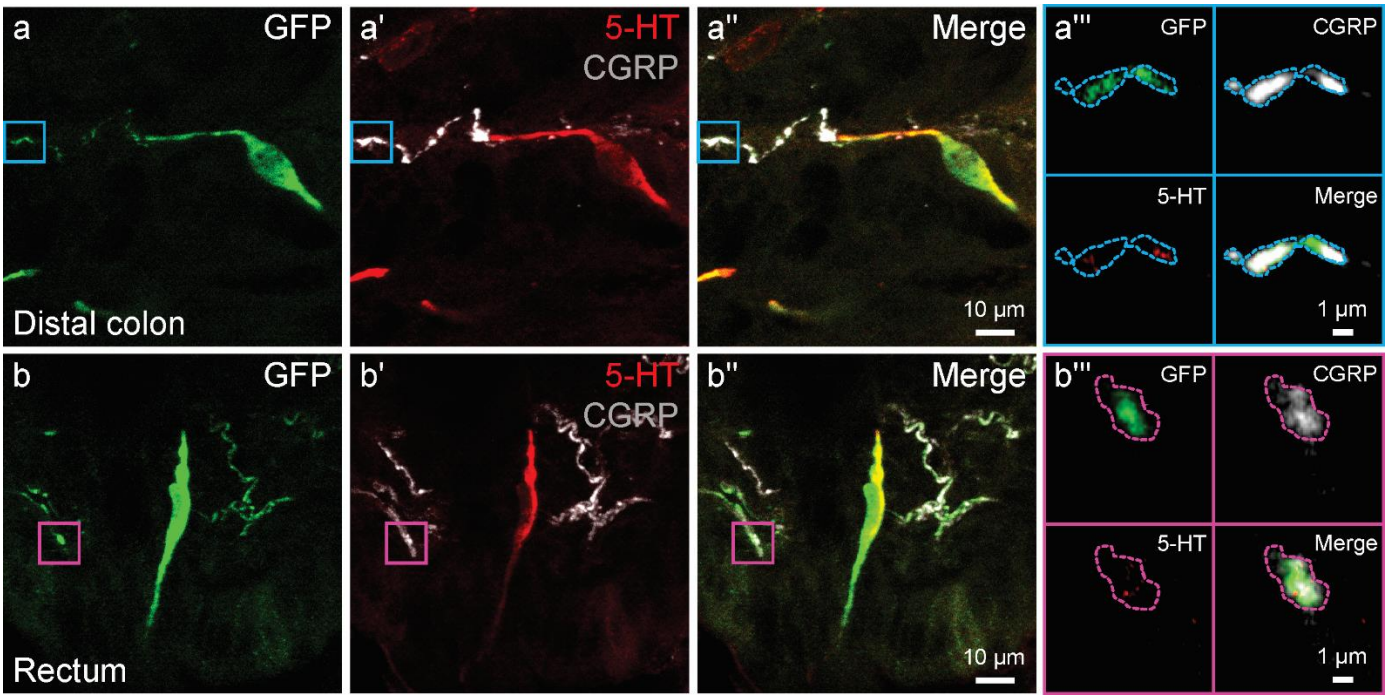

Fig. 4 Colocalisation of *Rxfp4*-GFP, CGRP and 5-HT in nerve fibre varicosities in the distal colon (**a-a″**) and rectum (**b-b″**). **a‴ and b‴**: Selected zoomed region of a-a″ and b-b″ with corresponding coloured box, showing overlap of *Rxfp4*-GFP and CGRP with 5-HT puncta within varicosities that are outlined by dotted lines. Note that the localisations of GFP, CGRP immunoreactivity and 5-HT immunoreactivity within the varicosities are different.

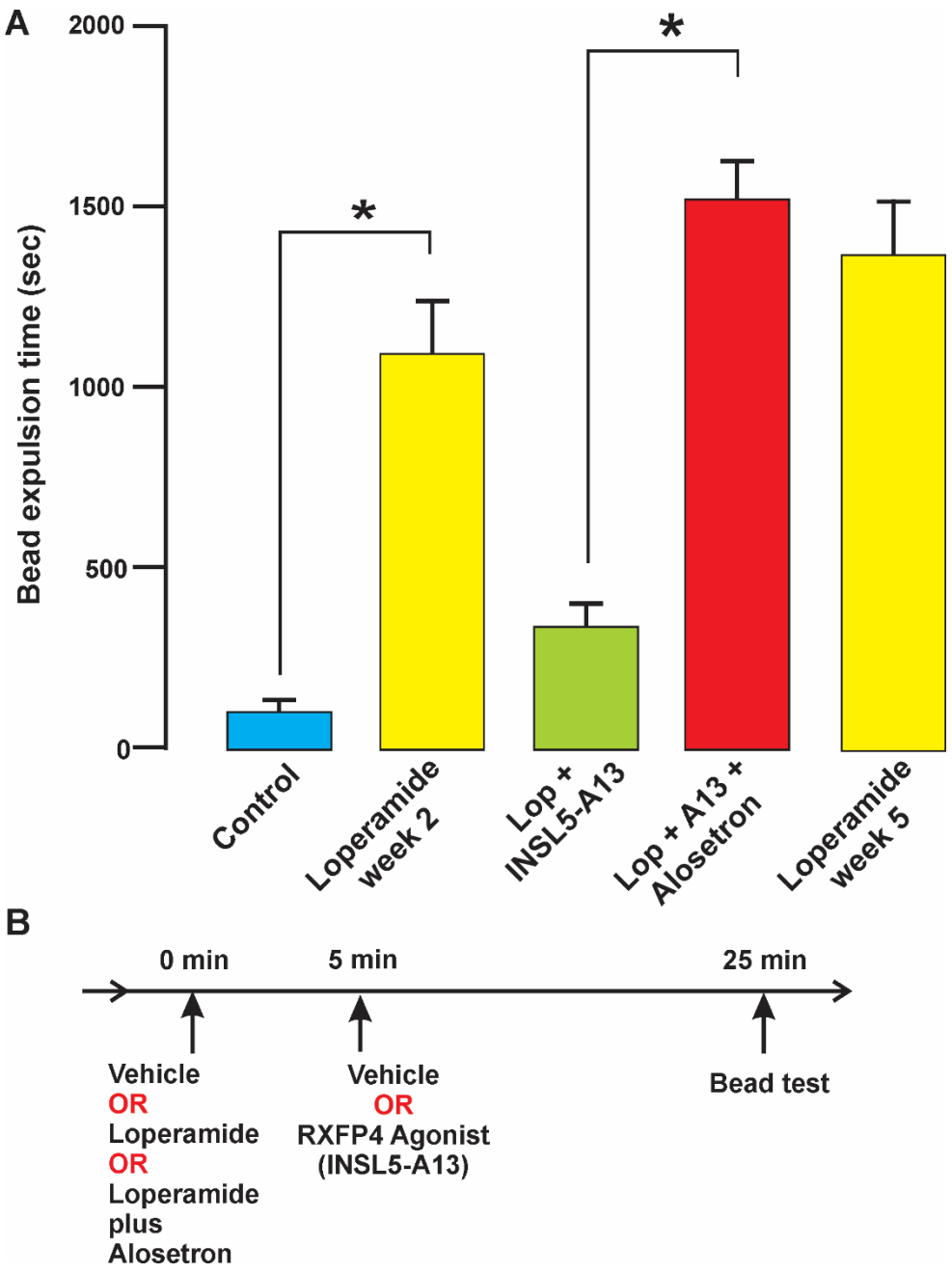

Fig. 5 Reversal of the stimulation of colorectal propulsion with the RXFP4 agonist, INSL5-A13, by the 5-HT_3 receptor antagonist, alosetron. A: Bead expulsion times recorded from conscious mice. The same mice were treated with no drug in week 1 (blue column marked control), loperamide alone in week 2, loperamide plus INSL5-A13 in week 3, loperamide plus INSL5-A13 and alosetron in week 4, and loperamide alone in week 5. Loperamide significantly delayed bead expulsion compared to control ($p < 0.05$). This effect was substantially reversed by INSL5-A13 (green column). Alosetron significantly delayed bead expulsion that had been accelerated by INSL5-A13 (Loperamide/INSL5-A13 compared with loperamide + INSL5-A13 and alosetron, $*p < 0.05$). B: Timing of drug administration to mice. Lop = Loperamide. Plotted are mean ± SEM, n = 9 mice.

Figure 1

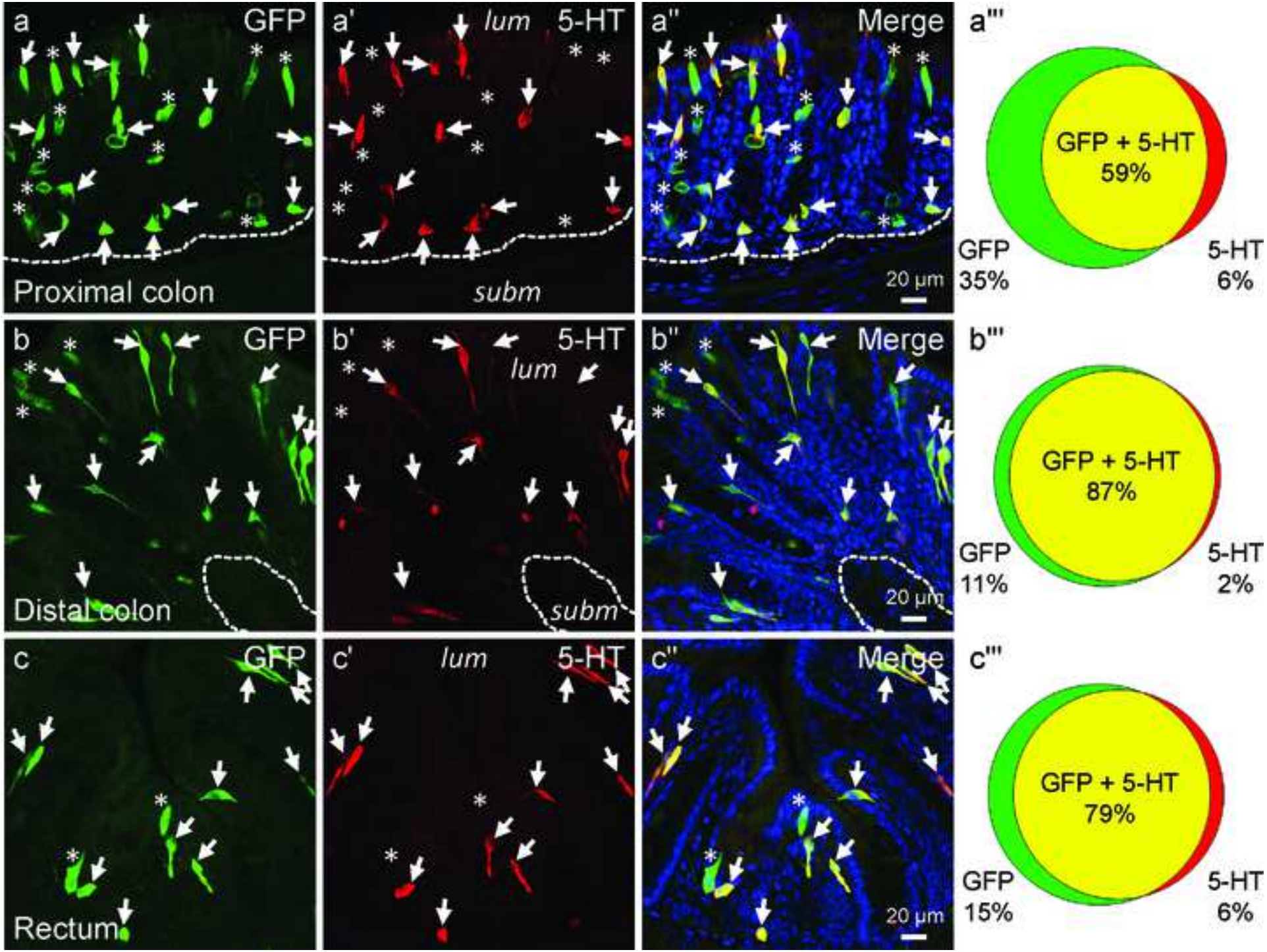

Figure 2

Click here to access/download;Figure;Fig 2 RXFP4 5HT OXM.tif

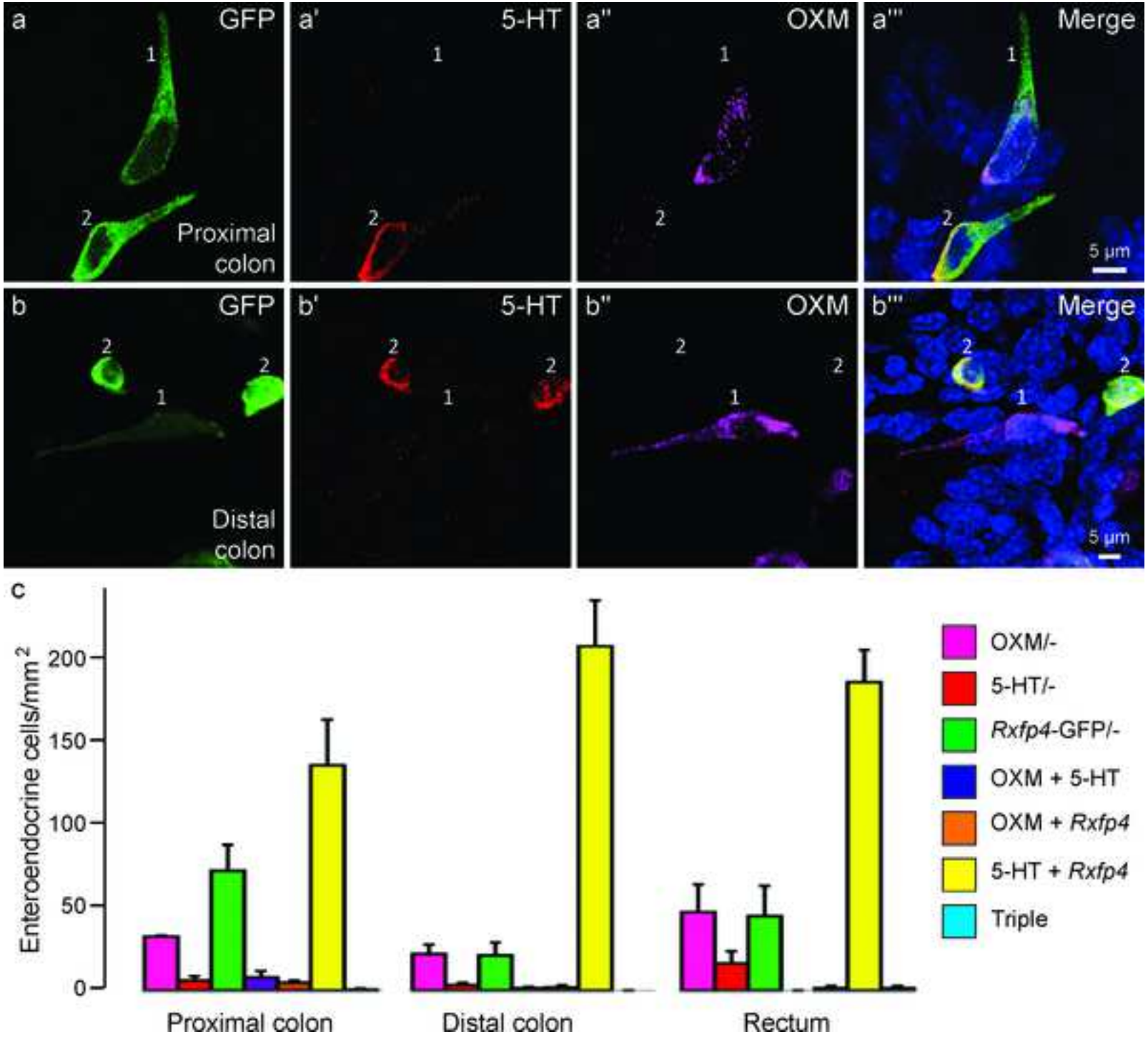

Figure 3

Click here to access/download;Figure;Fig 3 RXFP4 nerve fibres.tif

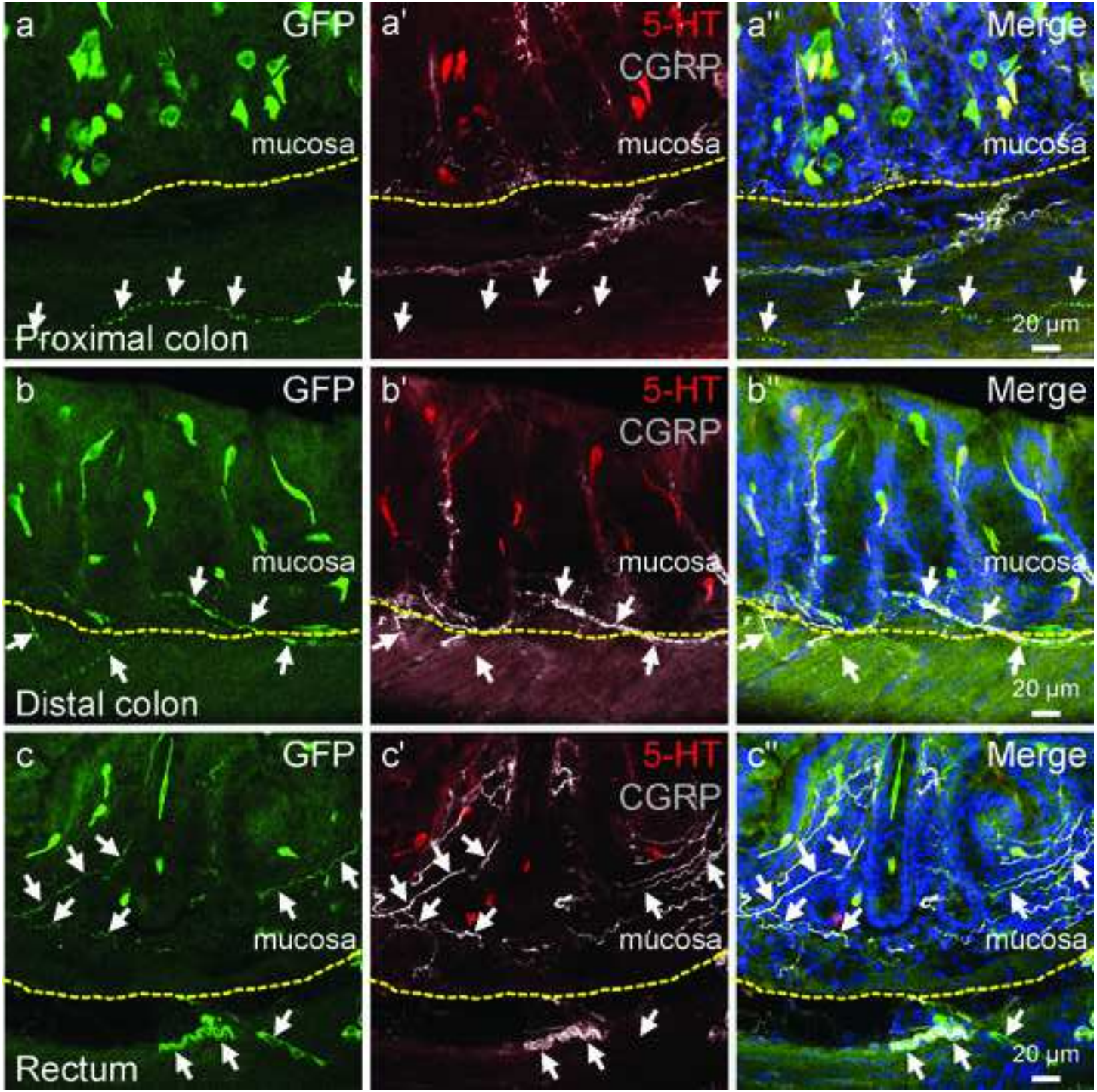

Figure 4

Click here to access/download;Figure;Fig 4 Coloc Varicosities.tif

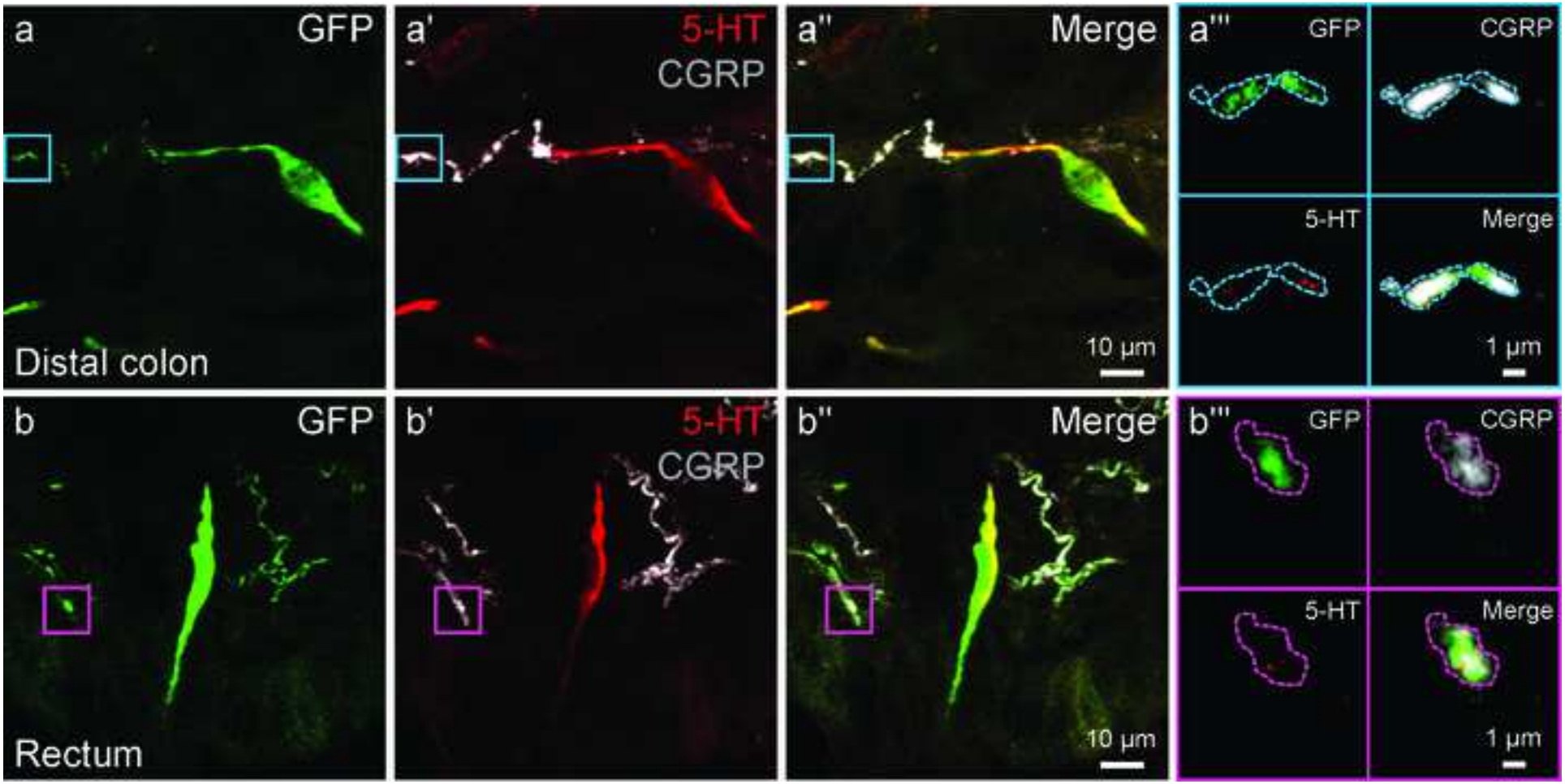

Figure 5