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Author/s:

Koo, A;Fothergill, LJ;Kuramoto, H;Furness, JB

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5-HT containing enteroendocrine cells characterised by morphologies, patterns of hormone co-expression, and relationships with nerve fibres in the mouse gastrointestinal tract

Ada Koo¹ · Linda J. Fothergill^{1,2} · Hirofumi Kuramoto³ · John B. Furness^{1,2}

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Abstract

5-HT containing enteroendocrine cells (EEC), the most abundant type of EEC in the gut, regulate many functions including motility, secretion and inflammatory responses. We examined the morphologies of 5-HT cells from stomach to rectum, patterns of hormone co-expression in the stomach and colon, and the relationship of 5-HT cells with nerve fibres. We also reviewed some of the relevant literature. The morphologies of 5-HT cells were distinct, depending on their location in the gut. A noticeable feature of some 5-HT cells in the antrum and colon was their long basal processes, which resembled processes of neurons, whereas 5-HT cells in the small intestinal mucosa lacked basal processes. In the stomach, numerous 5-HT cells, including cells with basal processes, were identified as enterochromaffin-like cells by their expression of histidine decarboxylase. In the colon, we observed a small number of 5-HT cells that were in close contact with, but distinct from, oxyntomodulin (OXM) and PYY immunoreactive EEC. We did not find specific relationships between nerve fibres and the processes of colonic 5-HT cells. We conclude that five major features, i.e., gut region, morphology, hormone content, receptor repertoire and cell lineage, can be used to define 5-HT cells.

Keywords 5-HT · Enteroendocrine cells · Gut hormones · Nerve fibres

Introduction

Enterochromaffin (EC) cells, which are defined by their content of 5-hydroxytryptamine (5-HT, also known as serotonin), are found throughout the gastrointestinal (GI) tract from the stomach to rectum and are the largest population of EEC (Sjölund et al. 1983). EC cells were first histologically observed based on their reaction with chrome salts (Heidenhain 1870), now known to be due to the presence of 5-HT which reacted with the chrome salts to form a coloured deposit. The majority of the body's 5-HT is produced by EC cells in the gut (Gershon and Tack 2007). For a long time, it

was suggested that EC cells are a morphologically uniform, flask-shaped cell type, distinct from other EEC, that had the main or only function to initiate peristaltic reflexes in the small intestine and colon (Bülbring and Crema 1958; Vialli and Erspamer 1933). However, it has now been shown that intestinal 5-HT has diverse effects including the exacerbation of intestinal inflammation, vasodilation, enteric neurogenesis, suppression of osteoblast proliferation, promotion of hepatic regeneration and pancreatic enzyme release, stimulation of bicarbonate secretion, and inhibition of insulin secretion (Jones et al. 2020). Gut-derived 5-HT is also the source of 5-HT for platelet storage (Li et al. 1997; Pletscher 1987).

Sub-populations of 5-HT containing cells also contain a variety of other hormones, including CCK, motilin, PYY, secretin, and tachykinins, in different regions of the gut (Cetin 1990; Fothergill et al. 2017; Lukinius et al. 1986; Pearse et al. 1974; Reynaud et al. 2016; Roth and Gordon 1990; Usellini et al. 1990) and thus they cannot be considered a cell type that is distinct from other EEC. Differences in receptor expression by 5-HT containing EEC in different gut regions (Lund et al. 2018; Martin et al. 2017) also

✉ John B. Furness
j.furness@unimelb.edu.au

¹ Department of Anatomy and Neuroscience, University of Melbourne, Parkville, VIC 3010, Australia

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

³ Department of Applied Biology, Kyoto Institute of Technology, Kyoto 606-8585, Japan

suggest that they belong to different functional subtypes. In the large intestine, 5-HT cells also differ in morphology, notably 5-HT cells with long basal processes occur in the colon (Kuramoto et al. 2007, 2021).

Here we utilize thick sections to compare 5-HT containing EEC by morphology, position and colocalization of peptide hormones throughout the mouse GI tract. We relate our observations to published literature, to better define 5-HT cell types.

Materials and methods

Animal and tissue preparation

All procedures were conducted according to the National Health and Medical Research Council of Australia guidelines and were approved by the University of Melbourne Animal Experimentation Ethics Committee. Five male C57BL/6 mice, age 8–10 weeks old, were used in this study. Animals received standard laboratory chow and water ad libitum. Animals were deeply anesthetised with isoflurane and euthanized by cervical dislocation. The GI tract from the stomach to rectum was excised and different segments were isolated. The stomach was opened along the greater curvature and the intestinal segments were opened along the mesenteric border and pinned on balsa wood with mucosa facing up. Segments were washed with phosphate-buffered saline (PBS; 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH7.2) and fixed overnight at 4 °C in Zamboni's fixative (2% w/v formaldehyde and 0.2% w/v picric acid in 0.1 M sodium phosphate buffer, pH7.2). Tissues were washed three times with dimethyl sulfoxide followed by three washes in PBS

prior to PBS-sucrose-azide (0.1% w/v sodium azide and 30% w/v sucrose in PBS) storage at 4 °C overnight. Tissues were transferred to a 1:1 ratio of PBS-sucrose-azide and OCT compound, then embedded in 100% OCT compound and frozen in isopentane cooled with liquid nitrogen.

Immunohistochemistry of free-floating sections

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 h 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 min each, followed by incubation with a mixture of secondary antibodies overnight at 4 °C. Sections were washed twice with PBS, 10 min each, and quenched with quenching buffer (5 mM copper sulfate and 50 mM ammonium acetate in distilled water, pH5.0) for 1 h at room temperature. Sections were washed once with PBS and twice with distilled water, 5 min each, followed by an incubation with Hoechst 33,258 (10 µg/mL in distilled water; Sigma-Aldrich, Sydney, NSW, Australia) for 45 min at room temperature. Sections were washed 3 times with distilled water, 5 min each, and then mounted on microscope slides in non-fluorescent mounting medium (Dako, Carpinteria, CA, USA).

Images acquisition and analysis

Z-stack images were captured by super-resolution confocal microscope (LSM880 Airyscan Fast, Carl Zeiss, Sydney, NSW, Australia) using a ×20 air objective and selected cells were imaged using ×63 oil objective in Zeiss Zen (black

Table 1 Primary and secondary antibodies used in this study and dilutions

	Target	Catalogue	Source	Species	Dilution	RRID
Primary antibodies	5-HT	20079	ImmunoStar	Goat	1:5000	AB_572262
	HDC	16045	Progen	Rabbit	1:1000	AB_1541512
	Gastrin	8007	Gift from Dr. Jens Rehfeld	Rabbit	1:2500	AB_2762851
	Somatostatin	S895	Buchan et al. (1990)	Mouse	1:500	AB_2783535
	Oxyntomodulin	AB-323-AO010	Ansh Labs	Mouse	1:1000	–
	PYY	HPA010973	Sigma-Aldrich	Rabbit	1:100	AB_1855194
	CGRP	1780	Arnel Products	Goat	1:1000	AB_2783523
	Tachykinins	SK-SP1	Eskay	Rabbit	1:800	AB_2814842
	VIP	V31ASC	Accili et al. (1995)	Mouse	1:1000	–
	PGP9.5	AB1761-1	Merck	Rabbit	1:500	AB_11213577
Secondary antibodies	Goat IgG	Ab150133, Alexa Fluor® 488	Abcam	Donkey	1:500	AB_2832252
	Rabbit IgG	A21206, Alexa Fluor® 488	Molecular Probes	Donkey	1:800	AB_2535792
	Rabbit IgG	Ab150070, Alexa Fluor® 555	Abcam	Donkey	1:1000	AB_2783636
	Sheep IgG	A11016, Alexa Fluor® 594	Molecular Probes	Donkey	1:1000	AB_10562537
	Mouse IgG	A31571, Alexa Fluor® 647	Molecular Probes	Donkey	1:2000	AB_162542

edition) software. Cells were manually selected based on 5-HT immunoreactivity. Images were deconvoluted using Airyscan Processing in Zen black and brightness and contrast were adjusted using Fiji Image J (<https://imagej.nih.gov/ij/>) then converted to RGB colour before exporting as TIFF files. Images were imported into Imaris (Bitplane AG, Zurich, Switzerland) for three-dimensional analysis. 3D rendering was done by generating surface recognition based on fluorescent labelled objects.

Data is available by contacting the first author.

Results

Morphology of 5-HT cells throughout the GI tract

For this study we used thick sections (60 μm), which are better than conventional thin sections to reveal the existence of long processes of EEC. Moreover, because the full extents of cells are included, whether or not the cells have processes could be determined. EEC displayed the two classical EEC morphologies, open and closed; open cells generally had a flask shape with the thin apical end exposed to the lumen and the base of the flask facing toward the lamina propria (Fig. 1a, a'). Closed cells did not have an apical end exposed to the lumen (Fig. 1b, b'). Some of both the open and closed cells had basal process, which are thought to facilitate interactions with nearby epithelial cells, other EEC and nerve endings (Bohorquez et al. 2015; Chandra et al. 2010; Larsson et al. 1979) (Fig. 1c, c', d, d'). Furthermore, there was a distinguishing morphology of a type of 5-HT cell which displayed long basal processes resembling axons (Fig. 1e, e', f, f'). These have been previously reported in rat and mouse large intestine (Kuramoto et al. 2007, 2021). The processes ran along the bases of epithelial cells and some extended more than 100 μm from the cell body. Some of these 5-HT cells had processes at both ends of the cell body (Fig. 1e').

In the stomach, 5-HT cells were rare in the corpus where they were primarily round closed cells without basal process (Fig. 2a). 5-HT cells were more numerous in the antrum than the corpus. Both open and closed cell types were observed, and some antral 5-HT cells had long basal processes (Fig. 2b). Similar observations were made in the rat stomach in which fewer 5-HT cells, primarily closed cells, were also found in the corpus, compared to antrum (Hunne et al. 2019). In human, three morphologies of 5-HT containing EEC were observed in the oxyntic mucosa: round and closed cells, typical flask-shaped open cells, and cells with multiple basal processes (Fakhry et al. 2019). In the current study, we observed that the long process of antral 5-HT cells ran along the base of the epithelial lining and exhibited swollen bulges that were similar to nerve varicosities in projection

views (Fig. 3a) and by 3D reconstruction. These bulges in the processes of colonic EC cells were shown by electron microscopy to contain accumulations of storage vesicles (Kuramoto et al. 2007). It has been shown that similar bulges (varicosities) of neuron processes release neurotransmitter that acts on neighbouring cells, suggesting 5-HT may also be released from varicosities of 5-HT cell processes. There were also open cells that had two processes (Fig. 3b).

In the thick (60 μm) sections that were used in the current study, almost all 5-HT cells in the small intestine displayed the typical open flask-shaped morphology, and none were observed to have basal processes (Fig. 2c–f). In the large intestine, 5-HT cells were abundant and some had basal processes of various lengths. 5-HT cell processes in the caecum and proximal colon were generally shorter than those in the distal colon and rectum (Fig. 2g–j).

Location and colocalisation patterns

Stomach

In the corpus, there were very few 5-HT cells, and these were generally found in the outer two-thirds of the mucosa (Fig. 4a). 5-HT cells were abundant in the mouse antrum and the majority of these were located in the inner third of the mucosa (Fig. 4b). Antral 5-HT cells with long processes were primarily found in the middle third of the mucosa (Fig. 4b). Enterochromaffin-like (ECL) cells, a cell type that is numerous in the stomach, were identified by immunoreactivity for histidine decarboxylase (HDC) and were also predominantly present in the inner third of the mucosa (Fig. 4a', b'). The majority of the antral 5-HT cells, including many cells with long processes, were ECL cells (Fig. 4b'''). Colocalisation of HDC and 5-HT also occurs in EEC of the rat stomach (Hunne et al. 2019) and in pig stomach, where approximately 90% of 5-HT cells were HDC positive in the antrum and 70% in the corpus (Fothergill et al. 2019). Colocalisation of 5-HT and pancreastatin (another marker for ECL cells) was found in ECL cells with processes in the human corpus (Fakhry et al. 2019). We observed that many antral cells with processes expressed both 5-HT and HDC (Fig. 4c–c'''). Some of the 5-HT/pancreastatin cells in the human antrum also possess processes (Fakhry et al. 2019).

There was no overlap of 5-HT with either gastrin or somatostatin in cells of the mouse stomach (Fig. 5). A similar observation was made in the rat antrum and corpus except there was a very small number of antral cells that expressed both 5-HT and gastrin; fewer than 1% of gastrin cells expressed 5-HT (Hunne et al. 2019). ECL-derived histamine stimulates gastric acid production, but 5-HT has

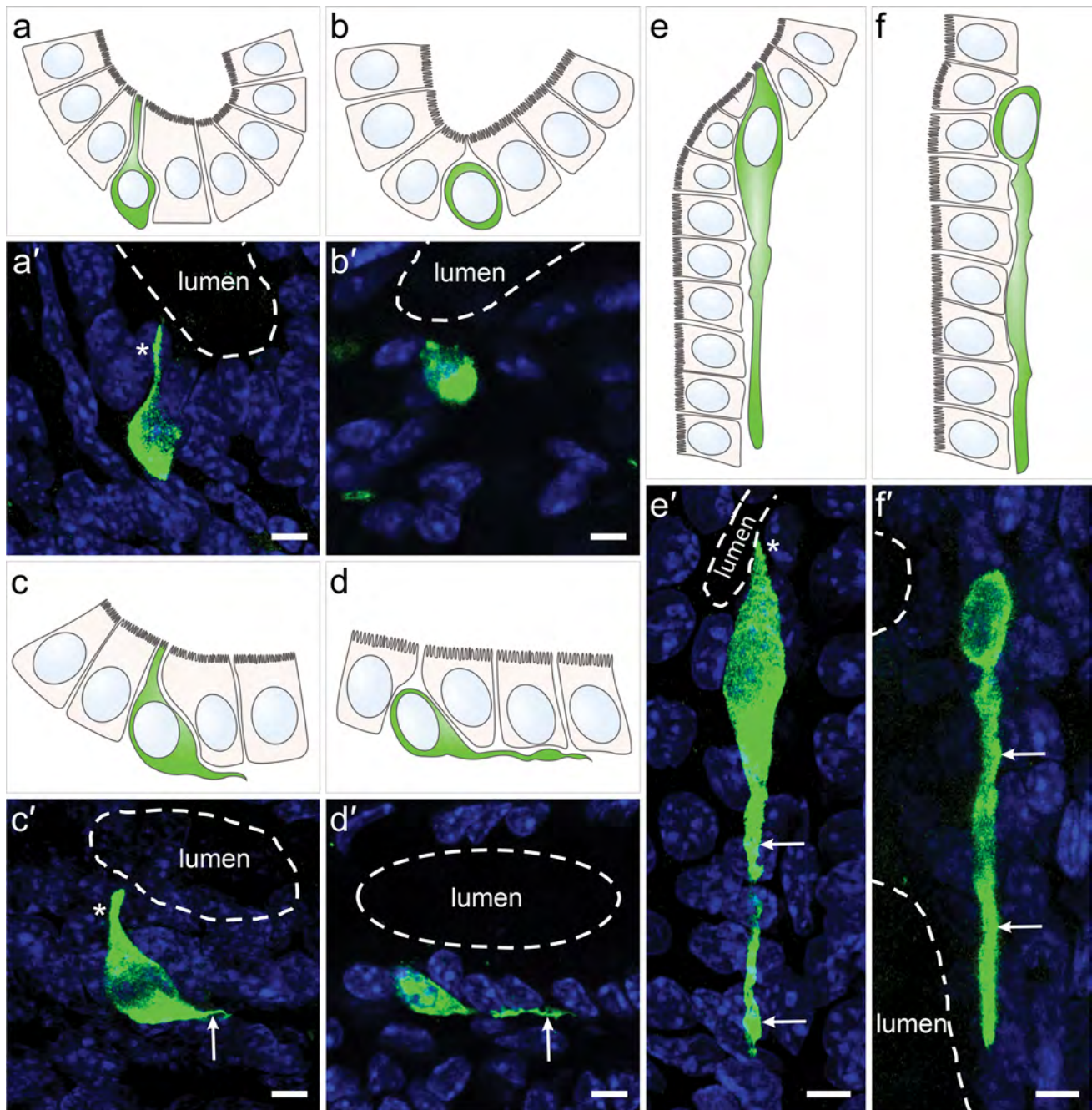


Fig. 1 Morphology of EC cells. Drawings (a–f) and confocal images (a'–f') of 5-HT containing EEC. Images are maximum intensity z stacks. Corresponding images show an open cell (a, a'), a closed cell (b, b'), an open cell with basal process (c, c'), a closed cell with basal process (d, d'), an open cell with long basal process (e, e'), and

a closed cell with long basal process (f, f'). Dotted lines represent the boundaries between the epithelial surface and the lumen. Basal processes are indicated by arrows and apical processes of open cells by asterisks. a–e 3.2 μm z-projection, f 9.6 μm z-projection. Scale bars: 5 μm (for all micrographs)

been reported to inhibit gastric acid secretion (Canfield and Spencer 1983; LePard and Stephens 1994). The role of antral cells that express both 5-HT and HDC, and that are distant from the acid secreting cells, is unclear, given their opposite effects, will require further investigation (see “Discussion” section).

Small intestine

5-HT cells were numerous in both villi and crypts throughout the small intestine. Many co-express peptide hormones with 5-HT. Secretin and cholecystokinin (CCK) are commonly co-expressed with 5-HT in mouse small intestine

Fig. 2 5-HT immunoreactive cells in the mucosa throughout the mouse GI tract. Long basal process (closed triangles) are observed pointing away from lumen in the antrum (**b**), distal colon (**i**), and rectum (**j**). Cells in the duodenum, jejunum, proximal and distal ileum were flask-like without any detectable basal processes (**c–f**). Some cells in the caecum had basal processes (**g**). Inserts of panels **a–b** and **g–j** are high resolution $\times 63$ images, insert scale bars $2\ \mu\text{m}$. Inserts of panels **c–f** are zoomed $\times 20$ images, insert scale bars $5\ \mu\text{m}$. Lower power micrographs, scale bars: $50\ \mu\text{m}$

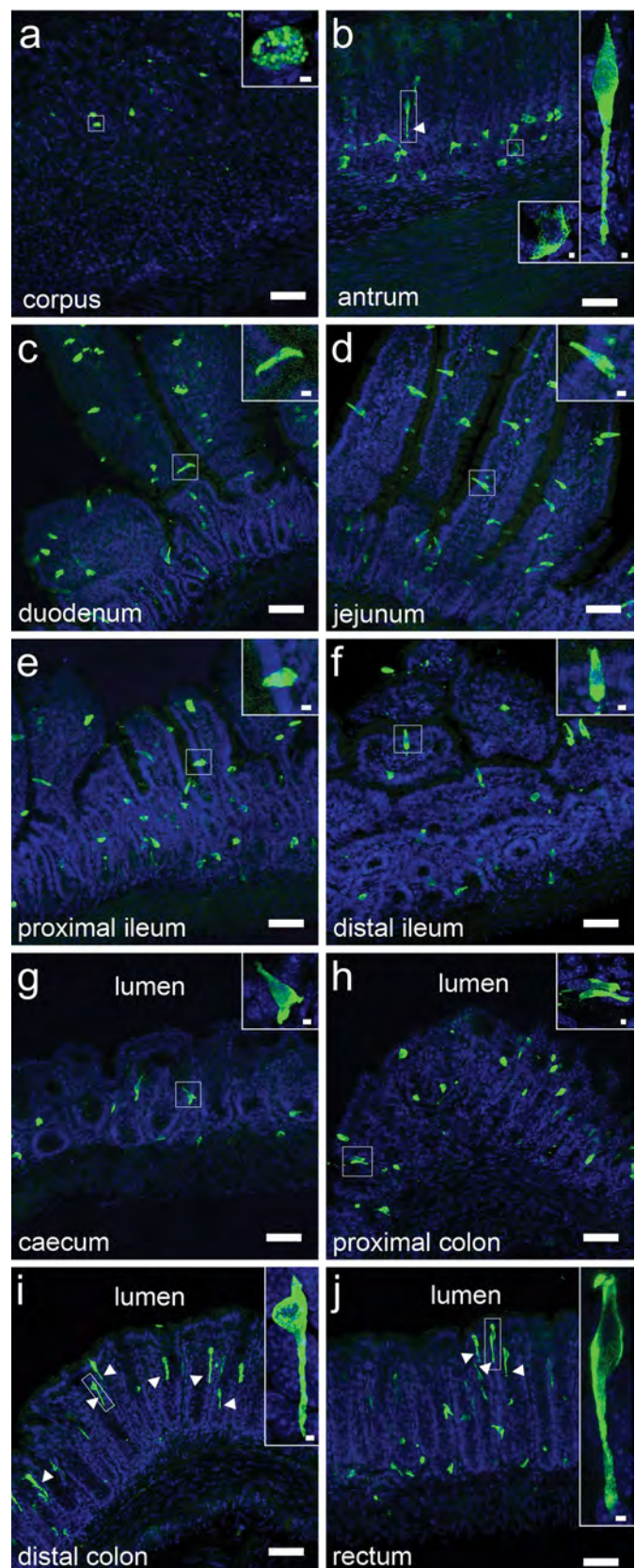
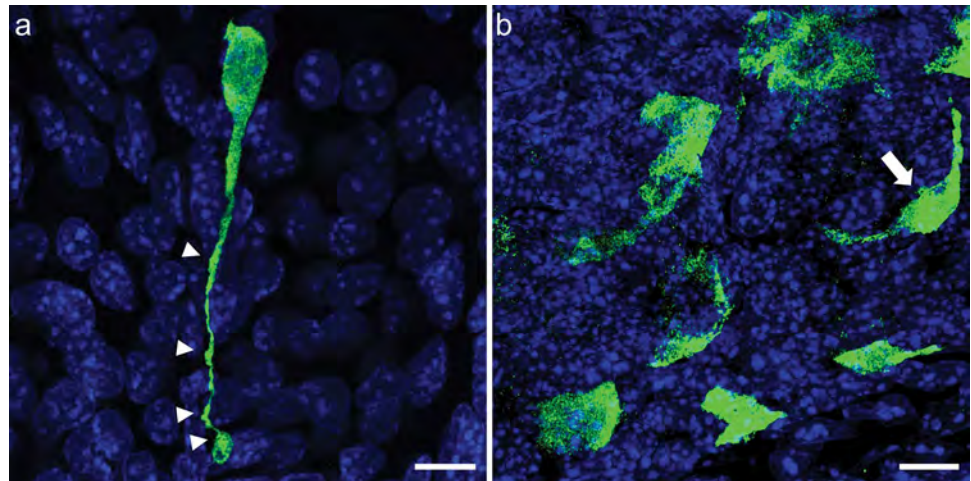


Fig. 3 A closed 5-HT cell in the mouse antrum with a long process displaying swollen regions that are reminiscent of axon varicosities (closed triangles) (a) and a bipolar open 5-HT cell (arrow) amongst other 5-HT cells in the antrum (b). Scale bars: 10 μ m



(Aiken et al. 1994; Aiken and Roth 1992; Cho et al. 2014; Diwakarla et al. 2017; Fothergill et al. 2017; Reynaud et al. 2016). 5-HT and secretin colocalisation are found in some 5-HT cells in the villi but not in the crypts, while some 5-HT cells in the crypt were found to be immunoreactive for tachykinins. Recent studies using a time-resolved Neurog3 reporter mouse, which allows high temporal resolution of cell lineages confirm earlier suggestions that the 5-HT/TK cells become 5-HT/secretin cells as they migrate from the crypts to the villi (Gehart et al. 2019).

The overlap of 5-HT and CCK was also found with other peptide hormones including ghrelin, secretin, oxyntomodulin (OXM), neurotensin, glucose-dependent insulinotropic peptide (GIP), and peptide YY (PYY) (Egerod et al. 2012; Reynaud et al. 2016; Sykaras et al. 2014). In mouse duodenum, 5-HT co-localises with either CCK or secretin, and it also co-localises with both CCK and secretin (Diwakarla et al. 2017).

Large intestine

Colocalisation of preproglucagon products, such as glucagon-like peptide-1 (GLP-1) and OXM, with PYY is common in EEC, commonly referred to as L-cells. These cells are found from duodenum to rectum, increasing in frequency from the proximal to distal GI tract (Gunawardene et al. 2011). We investigated whether 5-HT is colocalised with preproglucagon products and PYY in the mouse colonic EEC using triple labelling. We utilised a monoclonal anti-OXM antibody to mark cells that produce proglucagon products. We observed many cells that were both OXM and PYY immunoreactive (Fig. 6a''') and initially, based on low magnification ($\times 20$) images, it appeared to us that there were a small number of OXM/PYY cells that also co-express 5-HT (Fig. 6a''' arrow). However, closer examination using super-resolution imaging at high power ($\times 63$) and 3D rendering revealed that 5-HT did not colocalise with OXM and PYY,

but rather the two cell types occasionally intertwined with one another (Fig. 6b-b''').

Lund et al. (2018) showed that colonic 5-HT cells express GLP-1 and PYY Y1 receptors suggesting that some 5-HT cells may respond to the release of GLP-1 and PYY from nearby cells. It was demonstrated that 5-HT release could be induced by a GLP-1 receptor agonist (Lund et al. 2018). Other subpopulations of colonic 5-HT cells that did not come in close contact with GLP-1 and PYY cells may respond to other stimuli such as metabolites from gut microbiota and mechanical distortion. Gut microbiota modulates 5-HT biosynthesis (Yano et al. 2015) and it has been shown that short-chain fatty acids produced by the gut microbes promote 5-HT secretion (Bhattarai et al. 2017; Reigstad et al. 2015; Vincent et al. 2018).

Relationship of 5-HT processes with nerve fibres

EEC are intermediaries in signalling the state of the gut to the brain. For example, CCK cells detect nutrients and release CCK that acts on vagal nerve endings to provide a satiety signal (Raybould and Lloyd 1994). In some cases, the afferent nerve endings are closely apposed to basal processes of EEC, where a synapse-like relationship is observed (Bohorquez et al. 2015). We observed that 5-HT cells in the upper small intestine were flask-shaped open cells without basal processes. Physiological connections of 5-HT cells include those with the endings of vagal afferent fibres that detect potentially injurious substances in the upper small intestine and evoke nausea and vomiting (Andrews and Sanger 2002). In humans, the nausea that originates from noxae in the upper gut is inhibited by 5-HT₃ receptor antagonists such as ondanseron and granisetron. This signalling occurs without there being basal processes of the 5-HT cells.

Due to the resemblance of 5-HT cell processes to neuronal processes, we investigated whether there may be contact points between the 5-HT process and nerve fibres in

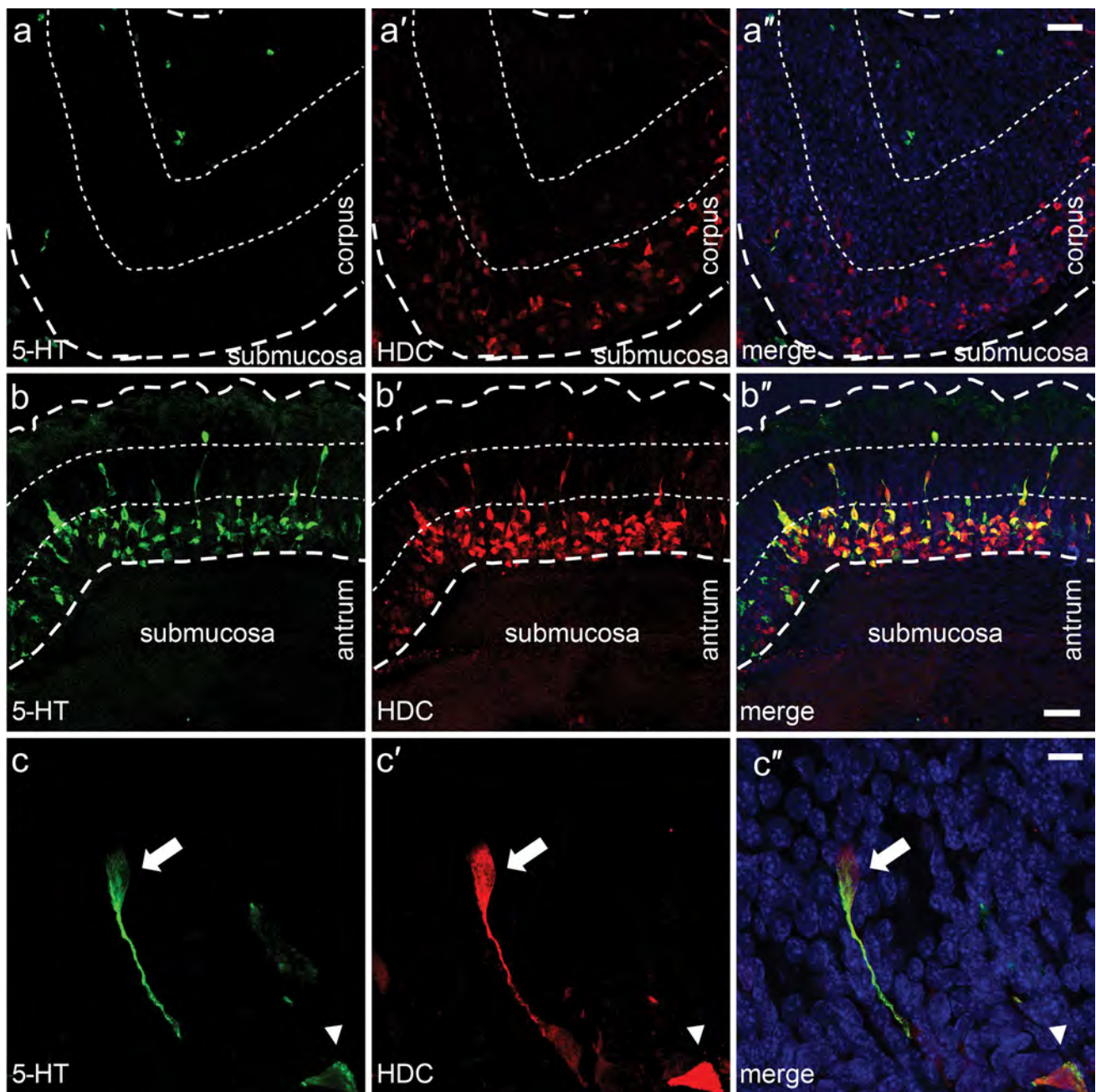


Fig. 4 Relationship of 5-HT and HDC cells in the mouse corpus (**a–a''**) and antrum (**b–b''**). 5-HT cells are rare in corpus (**b**) and do not overlay with histamine-producing ECL cells (**b''**). Colocalisation of 5-HT and HDC is common in the antrum (**b–b''**) including cells with and without processes. **b–b''**: high magnification of 5-HT and

HDC colocalisation in antral cells with (arrow) or without a process (closed triangle). The lines indicate the outer, middle, and inner parts of the mucosa. **c–c''** ECL cell with a long process is immunoreactive for 5-HT. Scale bars: **a–a''** and **b–b''**, 50 μ m; **c–c''**, 10 μ m

the mouse gastric and colonic mucosa. We investigated the relationships of colonic 5-HT cells with CGRP and TK containing nerve fibres, because of the presence of these peptides in afferents supplying the gut and VIP fibres because this is one of the most numerous fibre types in the mucosa (Fig. 7). However, we did not observe any consistent close relationship between 5-HT cells and nerve fibres. However,

we did observe some EEC containing both 5-HT and TK in the proximal colon that were in close proximity to TK nerve fibres (Fig. 7d–d'''). The selective TK expression in 5-HT cells was observed in the proximal but not the distal colon. The tachykinin gene (*Tac1*) codes for multiple peptides: substance P, neurokinin A, neurokinin B, and neuropeptide K, which all share similar N-terminal sequences and

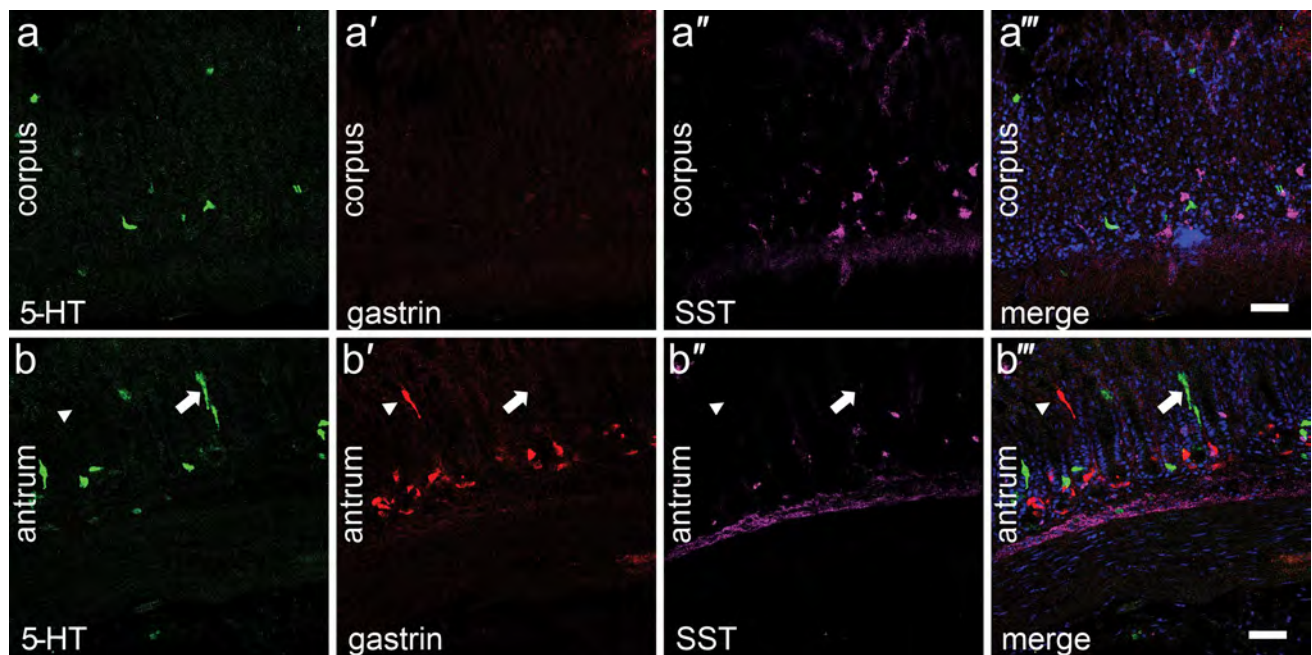


Fig. 5 Relationship of 5-HT, gastrin, somatostatin (SST) cells in mouse corpus (**a–a'''**) and antrum (**b–b'''**). Gastrin immunoreactive EEC were only observed in the antrum (**b'**) and not in the corpus

(**a'**). Some gastrin cells possess basal processes (closed triangle) (**b'**). 5-HT did not colocalise with gastrin or somatostatin. A 5-HT cell with a long process indicated by the arrow (**b**, **b'''**). Scale bars: 50 μ m

stimulatory effects on tachykinin receptors. Further study is needed to find out which tachykinin products are produced by EEC to better understand its possible roles and relationship with TK containing nerve fibres.

Because there are abundant 5-HT cells, it is inevitable that some nerve fibres may come into close proximity to them, making it difficult to quantify and evaluate whether 5-HT cells truly have direct contact with nerve fibres. However, the long processes of 5-HT cells lie between the basal lamina and the epithelium, whereas nerve fibres are generally on the other side of the basal lamina, suggesting the functional relationship of 5-HT cell processes in the colon is with the epithelium (Kuramoto et al. 2007).

We also used antibodies against the pan-neuronal marker, PGP9.5, to further elucidate the relationship of mucosal nerve fibres to 5-HT cells in antrum, jejunum and distal colon. However, this investigation was confounded by the presence of PGP9.5 in EEC (Wilson et al. 1988), some of which were strongly immunoreactive for PGP9.5.

Subcellular localization of 5-HT and tachykinins

One of the features of 5-HT cells in the small intestine, mainly the crypts, and in the glands of the large intestine (Fig. 7) is that they are commonly immunoreactive for tachykinins and they express the preprotachykinin gene (*Tac1*) (Aiken and Roth 1992; Lund et al. 2018; Gehart et al. 2019). We used super-resolution microscopy to examine the

subcellular localization of 5-HT and tachykinins (Fig. 8). In both the small and large intestine, 5-HT and tachykinin immunoreactivities were localized to different storage vesicles. It was common that TK and 5-HT vesicles were clumped together (e.g., Fig. 8b''). It would be informative to also examine the subcellular storage of 5-HT and histamine in gastric EEC cells. However, HDC, which was used to locate these histaminergic cells (Fig. 4), is a cytoplasmic enzyme and we have been unable to obtain reliable anti-histamine antibodies to locate the storage vesicles.

Discussion

This study demonstrates a diversity of 5-HT cell morphologies throughout the mouse GI tract and extends investigations of different morphologies in colonic EC cells (Kuramoto et al. 2007, 2021) to other GI regions. Overall, we observed six morphologies of 5-HT cells in different regions of the gut (Fig. 1). EEC differentiate from pluripotent stem cells in the bases of crypt and migrate towards the lumen as they mature. Gehart et al. (2019) showed that during this migration, 5-HT/tachykinin cells in the crypt transform to 5-HT/secretin cells. We observed that 5-HT cells with basal processes were positioned in the middle-to-outer region of the mucosa, and the longer the process, the closer the cell body was to the luminal side of the mucosa. This suggests that the formation of basal processes may be part of the

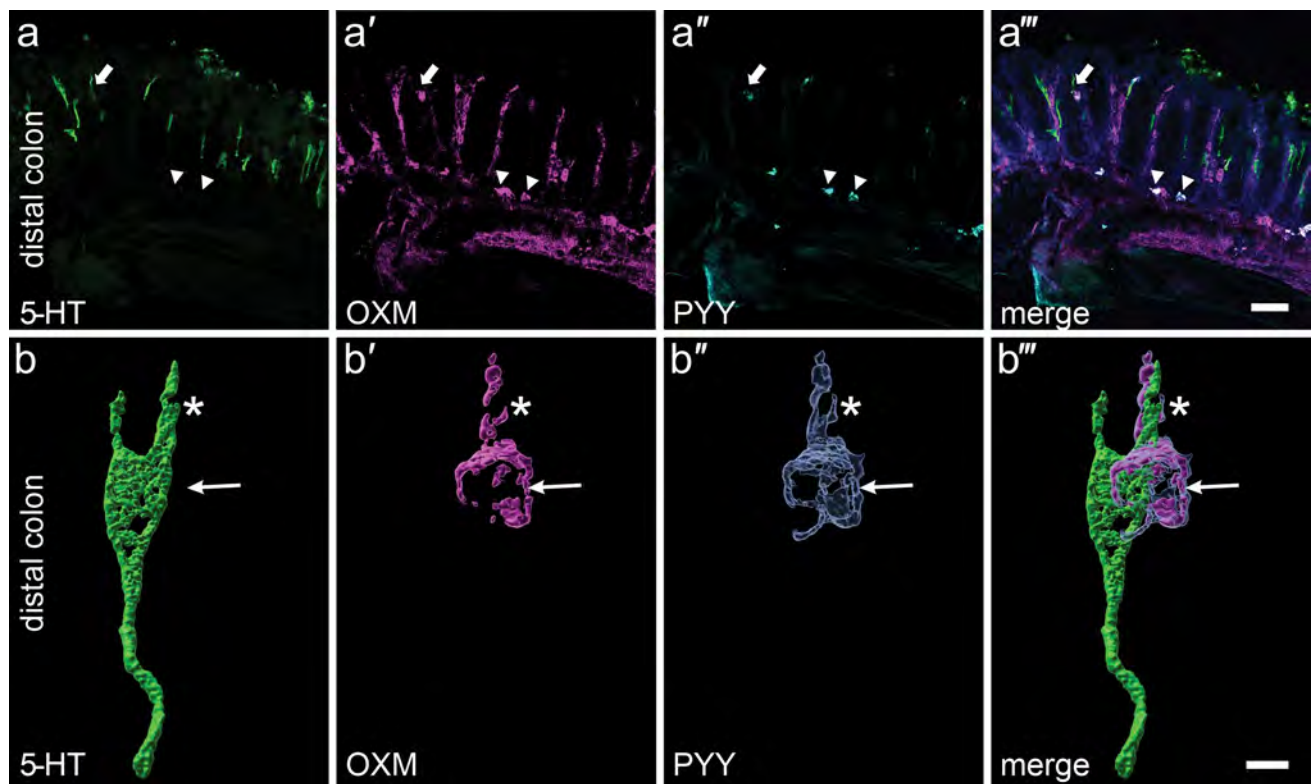


Fig. 6 Triple labelling of 5-HT, OXM, and PYY in mouse distal colon. OXM and PYY colocalisation was observed in L-type EEC of both the proximal (data not shown) and distal colon (closed triangles) (**a–a'''**). No overlap of 5-HT with OXM and PYY in the same cell occurred. However, a few 5-HT cells were in very close proximity to cells that were immunoreactive for OXM and PYY (arrow)

(**a'''**, **b–b'''**). **b–b'''** high magnification 3D rendering of the closely related 5-HT and OXM **b'''**/PYY cells that are indicated by the arrow in panel **a**. In this view, the cell body of the L cell is in front of the 5-HT cell (arrows), whereas the apical process of the L cell is behind the apical process of the 5-HT cell. Scale bars: **a–a'''**, 50 μ m; **b–b'''**, 5 μ m

developmental program of a subset of 5-HT cells, these cells extending basal processes as they migrate from the base of the mucosa toward the lumen. 5-HT cells with long processes were found in antrum, distal colon and rectum (Fig. 2). Electron microscope investigation shows that the long processes of EC cells in the large intestine contain clumps of vesicles resembling synaptic vesicles, they closely approach the bases of the epithelial cells and do not receive innervation (Kuramoto et al. 2007). We found that the processes were strongly immunoreactive for 5-HT and that the intensity of 5-HT immunoreactivity in the processes often appeared to exceed that in the cell bodies (e.g. Fig. 4c). Like Kuramoto et al. (2007), we did not find a close innervation of the processes. It seems likely from the structural studies that the processes in the colon are output processes of the EC cells. 5-HT acts on enterocytes and on enteric neurons to stimulate water and electrolyte secretion in the colon and EC-derived 5-HT may contribute to stress or bacterially induced diarrhea (Cooke 2000; Dong et al. 2019).

The diversity of co-expression of hormones is also likely to be related to differences in physiological functions of subgroups of 5-HT cells. For example, co-expression of 5-HT

and secretin in a subset of EEC could protect the duodenum from acidification, via the stimulation of bicarbonate release by both hormones (Säfsten et al. 2006). 5-HT and CCK, which are colocalised in some EEC, may both be involved in nutrient-sensing and inducing satiety (Hayes and Covasa 2005; Raybould 2010; Savastano and Covasa 2007). However, the role of tachykinins in EEC is unknown. Tachykinins, including SP, are found in enteric neurons and TK immunoreactive nerve fibres are numerous in the enteric nervous system. Tachykinins released by enteric neurons induces smooth muscle contraction (Holzer and Holzer Petsche 1997) and it is also considered a proinflammatory mediator in the GI tract (El-Salhy et al. 2017). 5-HT and SP co-released from rat caecal mucosa was reported, and interestingly the release of SP was inhibited by 5-HT (Simon et al. 1992).

Receptors on 5-HT cells

Transcriptome analysis reveals that 5-HT cells in the mouse gastrointestinal tract express numerous receptors that allow

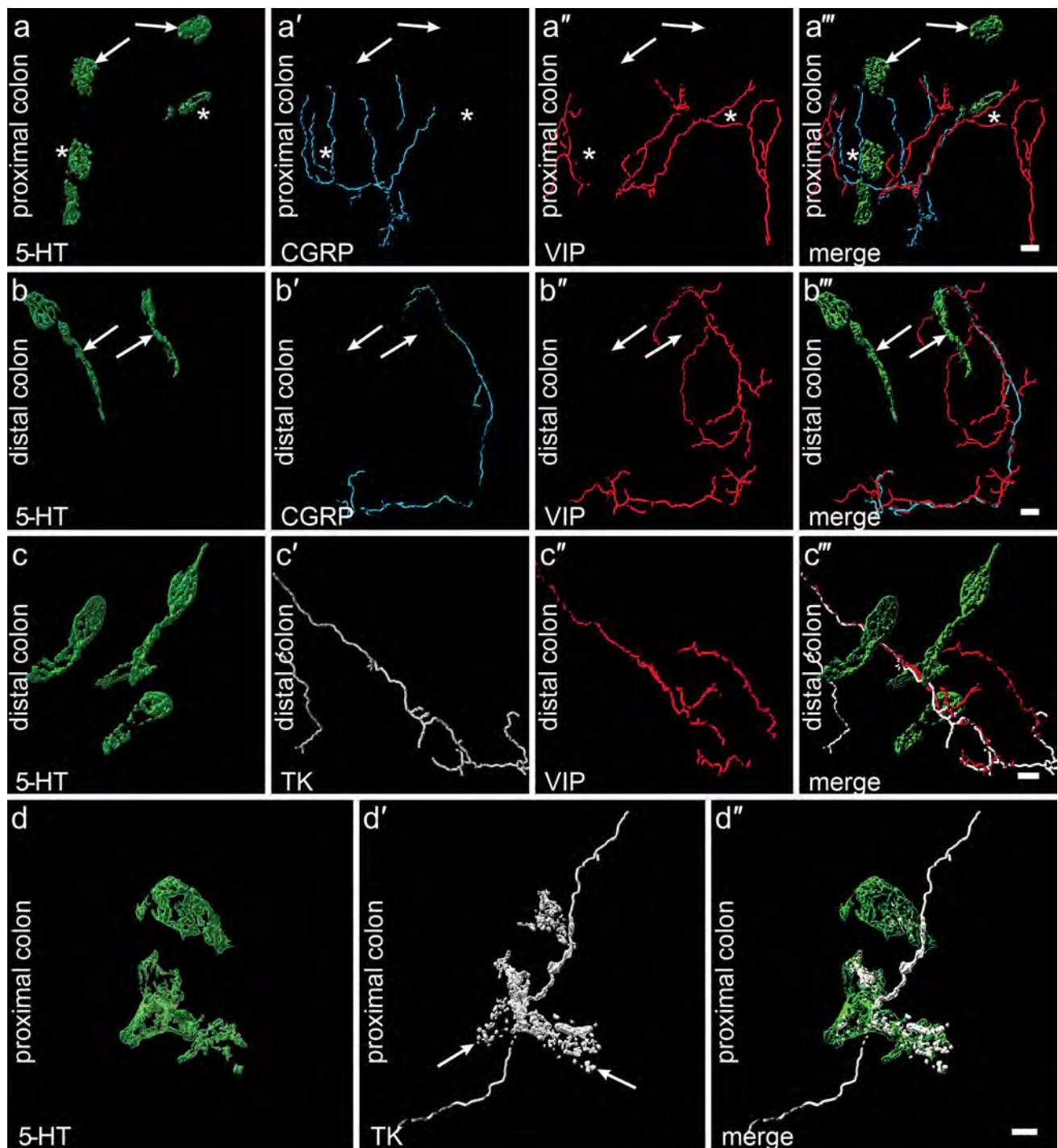


Fig. 7 Triple labelling and 3D rendering of 5-HT cells and nerve fibres in high-power images shown as maximum intensity projections (30 μm Z thickness). **a–a'''** and **b–b'''** relationships between 5-HT cells and CGRP and VIP fibres. The 5-HT cells indicated by the arrows in **a–a'''** have no close nerve fibres. Asterisks indicate close approaches to other 5-HT cells. The arrows in **b–b'''** indicate the processes of 5-HT cells; these do not have close nerve fibre approaches.

c–c''' three 5-HT cells have tachykinin (TK) and VIP fibres running past them, but they do not appear to be innervated. **b–b'''** TK immunoreactivity was observed in the cytoplasm of 5-HT cells in the proximal colon (but not in the distal colon). The cytoplasmic TK immunoreactivity appears to be in synaptic vesicles (arrows in **d''**) as further demonstrated in Fig. 8. Scale bars: 5 μm

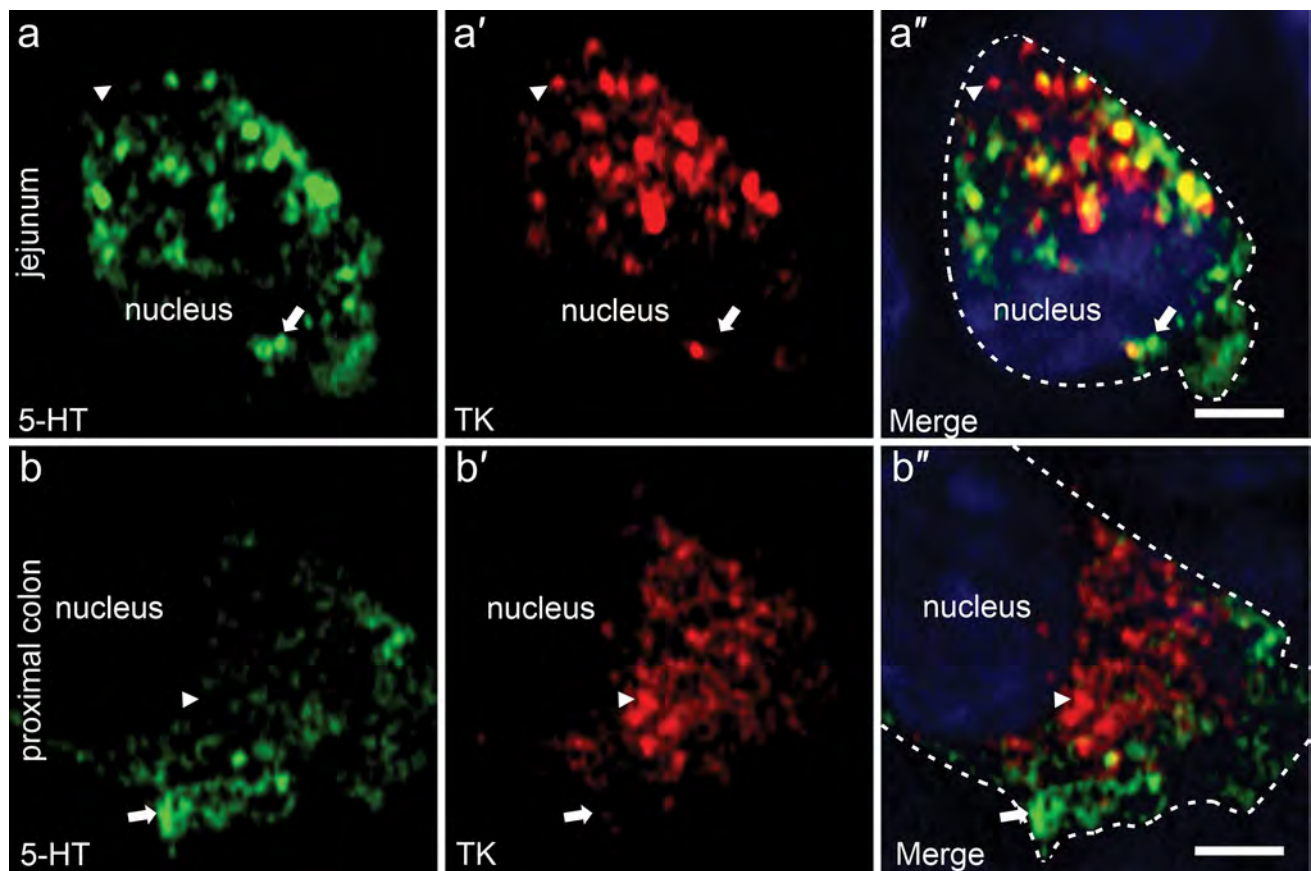


Fig. 8 Localization of 5-HT and tachykinins (TK) in EEC of the jejunum (a–a'') and proximal colon (b–b''). In each region, the storage was in separate vesicles; examples of vesicles immunoreactive only for 5-HT are indicated by arrows and vesicles immunoreactive only

for tachykinins by arrowheads. It was common for vesicles of each type to be in separate clumps (e.g., b''). The outlines of the cells are shown in the merged panels, and in these panels the blue nuclear stain is shown. Scale bars: 2 μ m

5-HT cells to sense and respond to the gut environment (Billing et al. 2019; Lund et al. 2018).

The phytochemical receptor, TRPA1, has been reported to regulate GI motility via the release of 5-HT (Doihara et al. 2009; Nozawa et al. 2009). TRPA1 occurs in the GI tract of human, mouse and rat, and is enriched in 5-HT cells (Cho et al. 2014; Nozawa et al. 2009). AITC, an agonist of TRPA1 found in mustard, radish, horseradish and wasabi, directly activated 5-HT cells in small intestine organoids to release 5-HT (Bellono et al. 2017), suggesting a role of open 5-HT cells in detecting pungent oils in the gut. TRPA1 has also been shown to be a mechanosensor and may work with Piezo2, another mechanosensor, in response to mechanical stimuli in the gut (Linan-Rico et al. 2016). Piezo2 is expressed in human and mouse small intestine and colon, is enriched in 5-HT cells, and the activation of Piezo2 by mechanical forces leads to 5-HT release (Alcaino et al. 2018; Dickson 2018; Linan-Rico et al. 2016; Wang et al. 2017). Furthermore, cells with long processes were observed in the antrum and distal colon and these processes may assist them in detecting distortion. However, TRPA1 and Piezo2

have not yet been specifically localised to the EC with long processes.

Some 5-HT cells express microbial metabolite receptors including *Ffar2*, *Olfir558*, and *Olfir78* in the colon and they thus sense metabolites produced by gut microbiota (Billing et al. 2019; Lund et al. 2018). These receptors are expressed on the basolateral surface of the cells (Gribble and Reimann 2019). We have observed strong 5-HT immunoreactivity in the apical ends of open cells in the proximal colon and it has been reported that 5-HT is released into the gut lumen upon stimulation (Fujimiya et al. 1995; Hata et al. 2017), which implies that 5-HT could be released via the apical surface when cells are stimulated by microbial metabolites through basolateral receptors.

Toll-like receptors (TLR) play an important role in the innate immune system. Immunoreactive TLR2 was present in human and mouse colonic 5-HT cells (Bogunovic et al. 2007). In disease states, such as coeliac disease, inflammatory bowel disorders, and some cases of gastrointestinal dysmotility, there is an increase in circulating 5-HT (Coleman et al. 2006; Diwakarla et al. 2017; Enerbäck et al. 1983;

Mawe and Hoffman 2013). When specific-pathogen free mice were treated with antibiotics, TLR2 expression, numbers of 5-HT cells, and level of secreted 5-HT were reduced (Wang et al. 2019). These observations indicate that 5-HT cells with TLR are involved in recognising pathogens and toxins and mediate inflammatory responses. We have confirmed the common observation that duodenal villus 5-HT cells are open type cells, which accords with the observation that cells that express 5-HT/TLR2 are open cells (Bogunovic et al. 2007).

5-HT and ECL cells

Most 5-HT immunoreactive cells in the gastric antrum were also positive for the ECL marker, HDC; however, the physiological role of the 5-HT of ECL cells is unclear. The primary hormone produced by ECL cells is histamine which stimulates parietal cells to produce acid, while 5-HT inhibits gastric acid secretion (Canfield and Spencer 1983; LePard and Stephens 1994). ECL cells express aromatic amino acid decarboxylase (AADC) and vesicular monoamine transporter (VMAT) and can convert 5-hydroxytryptophan (5-HTP) to 5-HT (Chen et al. 1998), which is consistent with their content of 5-HT. Histamine reduces 5-HT secretion in rat stomach and duodenum and pig small intestine through the H₃ receptor (Kleinrok et al. 1984; Schwörer et al. 1992). Therefore, ECL cell histamine may regulate 5-HT release in the stomach. Interestingly, we observed a high intensity of 5-HT immunoreactivity in the basolateral regions of ECL cells, including in basal process, while HDC was more evenly distributed in the cells (Fig. 4c) suggesting 5-HT may be released from the basolateral sides of the cells.

Relationship between OXM/PYY cells and 5-HT cells

We did not observe colocalisation of 5-HT, OXM, and PYY in colonic EEC, but we observed a small number of 5-HT cells entwined with OXM/PYY cells. A single-cell transcriptomic profiling of murine colonic EEC detected two separate major clusters, one enriched for *Gcg* and *Pyy* that express either *Nts* or *Insl5* and a cluster of 5-HT cells (identified by high levels of *Tph1*) that co-expressed *Tac*, *Piezo2*, and *Sct* (Billing et al. 2019). Billing et al. (2019) found that the *Gcg/Pyy/Nts* cells were located in the proximal colon while *Gcg/Pyy/Insl5* cells occurred in the distal colon and rectum. Furthermore, mRNA expression of RXFP4, the INSL5 receptor, was found in colonic 5-HT cells (Lewis et al. 2020) implying the release of INSL5 from *Gcg/Pyy/Insl5* could stimulate 5-HT release via RXFP4. Thus, both our observation of the cell relationships and the location of the INSL5 receptor to 5-HT cells suggest a paracrine relationship. Both INSL5 and 5-HT have been reported to stimulate colorectal propulsion (Diwakarla et al. 2020; Kendig and Grider 2015)

and they may work together to respond to the gut environment and to coordinate colonic contraction. Further investigation is needed to examine whether the OXM/PYY cells that were close to 5-HT cells also express INSL5.

Conclusion

5-HT cells in the corpus were round closed cells and were not immunoreactive for other hormones that we examined. In the antrum, both open and closed cells were observed, and a small number of 5-HT cells had long basal process. Colocalisation of 5-HT and HDC was common in the antrum, but there was no overlap of 5-HT with gastrin or somatostatin. In the small intestine, open flask-shaped cells without basal processes were the dominant 5-HT cell morphology. In the colon, all morphologies that we have described were observed, but long processes were most common in the distal colon and rectum. Colonic 5-HT cells were distinct from OXM and PYY cells, however, a few 5-HT cells were observed in close contact with OXM and PYY cells, suggesting potential direct interaction between these cell types. Examination of 5-HT cell relationships with nerve fibres were difficult to assess since there was no consistent quantifiable relationship, but specific relationships were not found. Morphology is an important feature that should be included in the suite of properties used to classify 5-HT containing enteroendocrine cells.

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Compliance with ethical standards

Conflict of interest Authors declare no conflict of interests.

Ethical approval The research was conducted in accordance with the National Health and Medical Research Council of Australia guidelines and were approved by the University of Melbourne Animal Experimentation Ethics Committee (Approval 1814569).

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