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Serotonin Signaling in the Gastrointestinal Tract::

Functions, dysfunctions, and therapeutic targets

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Abstract

Serotonin (5-HT) has been recognized for decades as an important signaling molecule in the gut, but it is still revealing its secrets. We continue to discover novel gastrointestinal (GI) functions of 5-HT, as well as actions of gut-derived 5-HT outside of the gut, and we are learning how 5-HT signaling is altered in GI disorders. Furthermore, new therapeutic targets related to 5-HT signaling are being identified that can hopefully be exploited to alleviate the symptoms of functional GI disorders. Conventional functions of 5-HT in the gut involving intrinsic reflexes include stimulation of propulsive and segmentation motility patterns, epithelial secretion, and vasodilation. Activation of extrinsic vagal and spinal afferent fibers results in slowed gastric emptying, pancreatic secretion, satiation, pain and discomfort, as well as nausea and vomiting. Within the gut, 5-HT also exerts non-conventional actions that include serving as a pro-inflammatory signaling molecule and as a trophic factor to promote the development and maintenance of neurons and interstitial cells of Cajal. Platelet 5-HT, which comes from the gut, can promote hemostasis, influence bone development, and contribute to allergic airway inflammation. 5-HT_3 receptor antagonists and 5-HT_4 receptor agonists have been used to treat functional disorders with diarrhea or constipation, respectively. More recently, the synthetic enzyme, tryptophan hydroxylase has been targeted, and there are recent findings suggesting that epithelial 5-HT_4 receptors could be targeted to provide a safe and effective treatment for constipation. Here we provide an overview of these serotonergic actions and treatment strategies.

Introductory remarks

One could argue that we should be using the term *enteramine*, rather than serotonin, when referring to 5-hydroxytryptamine (5-HT). After all, this substance was first extracted from rabbit gastric mucosa in 1937 by the Italian pharmacologist, Vittorio Erspamer, who named the bioactive amine that he discovered enteramine[1]. About a decade later, Maurice Rapport, Arda Green and Irvine Page of the Cleveland Clinic reported they had isolated a compound

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from bovine serum that they called serotonin for its vasoconstrictive properties[2]. Within a few years, it was determined that the structure of both of these compounds is 5-HT, and it appears that the second name stuck because it was made available to researchers by Upjohn Pharmaceuticals, who referred to the compound as serotonin[3]. Early on, Erspamer postulated that 5-HT must play an important role in gut function because he detected it in the gastrointestinal (GI) tracts of every vertebrate that he studied, from fish to frogs to pigeons to primates, and because he found that the vast majority of the serotonin in the body is synthesized and contained within the GI tract. Nevertheless, it is doubtful that Erspamer could have envisioned the variety and broad distribution of 5-HT receptors that are expressed in GI tissues, the array of functions that 5-HT serves in the gut, the actions of gut-derived serotonin outside of the GI tract, or the number of serotonergic targets that are in use or in development for the treatment of GI disorders. These are the areas of focus of this review.

Serotonin signaling in the gut

Mucosal 5-HT signaling in the gut

The majority of 5-HT in the body is synthesized, stored, and released from a subset of enteroendocrine cells called enterochromaffin (EC) cells in the intestinal mucosa[4, 5] (Fig 1a,c). Because the intrinsic and extrinsic afferent nerves of the gut do not reach into the lumen where they could sample for nutrients, toxins or other cues to initiate reflex responses, EC cells function as sensory transducers that respond to chemical and mechanical stimuli[6]. Enterochromaffin cells use the rate limiting enzyme tryptophan hydroxylase 1 (TpH1) to synthesize serotonin[7, 8]. As is the case for other types of enteroendocrine cells, the secretory granules of EC cells are predominantly located at the basal surface, and this is thought to represent the major site of 5-HT release. However, there is morphological evidence for 5-HT release at the apical surface of EC cells[9], and this is consistent with the ability to detect 5-HT release at the mucosal surface with electrochemistry techniques[10–12]. As described in greater detail below, 5-HT released from EC cells mediates many GI functions, including peristalsis, secretion, vasodilation and perception of pain or nausea, through activation of a diverse family of 5-HT receptors on intrinsic and extrinsic afferent nerve fibers that are located in the lamina propria.

An important property of intercellular signaling is the termination of the signal by local enzymatic degradation, or by uptake into nearby cells or nerve terminals for recycling or degradation. In the case of 5-HT, there are no intercellular degradative enzymes, and since it is a highly charged molecule at physiological pH, it requires a specialized transport mechanism to cross the plasma membrane where it can be degraded by intracellular enzymes such as monoamine oxidase[13, 14]. The serotonin-selective reuptake transporter (SERT), which is also responsible for 5-HT uptake in the brain, is expressed by essentially all epithelial cells of the intestinal mucosa[13–16] (Fig. 1b,c). The serotonin transporter is a member of the neurotransmitter:sodium symporter (NSS) family, TC 2.A.22, which includes the monoamine transporters DAT (dopamine) and NAT (noradrenaline), and internalizes 5-HT via a sodium- and chloride-dependent mechanism[17, 18]. Since all of the epithelial cells in the lining of the intestines appear to express SERT, it serves as a selective sponge to remove

5-HT from the interstitial space following release by EC cells. By functioning to inactivate 5-HT through rapid reuptake into epithelial cells of the intestinal mucosa, SERT serves as a critical molecule in the local regulation of 5-HT availability and actions in the intestines. Studies of the intestine during early postnatal development, and in adult intestine, have demonstrated that decreased SERT function, via lower expression levels of SERT[19, 20] or pharmacological blockade[20], lead to higher levels of extracellular 5-HT. Serotonin that is not taken up into the cells of the epithelial lining enters the circulation via the dense capillary bed of the lamina propria, where it is taken up into platelets expressing SERT (Fig. 1c).

It is worth noting that regulation of SERT transcription in the intestinal epithelium differs from that for neuronal SERT. In intestinal epithelial cells, transcription starts downstream from the start site used in neurons, and there is a region of intron 1 located between these start sites that can regulate epithelial SERT expression, but does not affect neuronal SERT expression [21, 22]. If the gut-specific signaling pathways were identified that could influence SERT expression and function, it would be possible to differentially regulate gut 5-HT signaling with drugs that target the gut epithelium.

The lamina propria is rich in mast cells, and murine mast cells are capable of synthesizing and releasing 5-HT. However, under basal conditions, human mast cells in the mucosal layer of the gut do not appear to synthesize 5-HT. Figure 1 illustrates 5-HT-immunoreactivity in the human colon, and while EC cells are obvious, mast cells are not observed. This is consistent with the findings of Buhner and Schemann, who also failed to detect 5-HT immunoreactivity in human mast cells[23]. On the other hand, there is evidence that human enteric mast cells express TpH[24]. Also, in some individuals with mastocytosis, 5-HT levels are increased in a manner that does not correlate with platelet numbers[25]. Interestingly, individuals with mastocytosis who have low levels of circulating 5-HT are more likely to exhibit GI and neurological complaints[26]. Regardless, the fact that mast cells in mice express 5-HT indicates that mast cells must be considered as a source of 5-HT in murine studies, and it is also possible that activated mast cells synthesize and release 5-HT under pathological conditions in humans, contributing to conditions such as hypersensitivity.

Neuronal 5-HT signaling in the gut

While the major depository of the body's 5-HT is the mucosa of the GI tract, enteric neurons also express 5-HT. Like neurons in the brain, synthesis of 5-HT by enteric neurons is dependent on TpH2[7, 8]. It is estimated that approximately 2% of the neurons in the myenteric plexus are serotonergic [27], and they synapse preferentially, but not exclusively, on other serotonergic neurons, forming a chain of serotonergic interneurons that project downstream along the gut tube [28, 29].

Studies of stimulus-evoked synaptic responses in the myenteric plexus demonstrate that 5-HT released from these neurons can contribute to both fast and slow excitatory postsynaptic potentials (EPSPs). Virtually all evoked fast EPSPs in the enteric nervous system (ENS) are mediated predominantly by acetylcholine acting at nicotinic receptors; however, roughly 10% of fast EPSPs also include a component sensitive to 5-HT_3 receptor antagonists[30]. The small proportion of fast synaptic events including a serotonergic component corresponds

with the small percentage of neurons in the myenteric plexus that express 5-HT, and the small proportion of neurons that appear to be surrounded by 5-HT-immunoreactive nerve terminals. It is reasonable to presume that these represent synaptic events involved in descending projections. This is consistent with the finding that descending reflex pathways that ultimately affect circular muscle activity are sensitive to 5-HT_3 receptor inhibition[31]. On the other hand, the projection patterns of enteric 5-HT neurons are difficult to reconcile with data showing that inhibition of 5-HT_3 receptors can interrupt ascending contractile reflexes in response to mucosal stimulation[32]. The physiological role of 5-HT-mediated slow EPSPs is less clear, but antagonists of 5-HT_{1P}[33, 34] and 5-HT_7[35] receptors can inhibit them.

Alterations in mucosal 5-HT signaling in pathophysiological conditions

Because of its importance as a signaling molecule in the gut, numerous studies have investigated various elements of mucosal 5-HT signaling in inflamed intestinal mucosa from human biopsies and in animal models of intestinal inflammation. Most studies have reported changes in one aspect or another, and here we aim to highlight the common findings in the context of potential mechanisms underlying the relationship between altered mucosal 5-HT signaling and altered GI function and sensation.

Inflammation

Human studies have been conducted with mucosal biopsies from a number of inflammatory conditions, including Crohn's disease[36, 37], ulcerative colitis [16, 36, 37], diverticulitis[38] and active celiac disease[39, 40]. In patients with ulcerative colitis, there is a decrease in 5-HT content and a trend towards fewer EC cells in the mucosa, although mucosal 5-HT release does not appear to be altered[16]. In celiac disease, elevations in 5-HT content, EC cell numbers, and circulating 5-HT have been reported, whereas in diverticulitis, no changes in 5-HT content or EC cell numbers have been detected. While changes at the upstream end of the mucosal 5-HT signaling pathway vary amongst these inflammatory disorders, all of the conditions described above are associated with decreased epithelial SERT expression levels. It is worth noting that a decrease in SERT has not been detected in inflamed pediatric samples[41].

Serotonin signaling has also been investigated in a number of animal models of intestinal inflammation, including TNBS colitis[19] and ileitis[42] in guinea pig, and TNBS colitis[21], DSS colitis[43], *Citrobacter rodentium* enteritis[44], and *Trichinella spiralis* enteritis[45] in mouse. In all of these conditions, 5-HT levels and EC cell numbers are increased, and where studied, 5-HT release is increased as well. Another consistent feature amongst these models is a decrease in epithelial SERT expression, and it has been demonstrated that this decrease in SERT can lead to increased 5-HT availability under both basal and stimulated conditions[19], supporting the concept that 5-HT signaling is affected by both release and reuptake events.

The findings that mucosal 5-HT content and EC cell numbers are elevated in the animal models and in celiac disease, whereas they are decreased in inflammatory bowel disease (IBD), may reflect the duration of the inflammatory condition, with animal models and active celiac disease representing shorter time points than chronic IBD. With regard to

SERT, it is likely that expression is down-regulated by the inflammatory response. In the Caco-2 human epithelial cell line, SERT mRNA and protein levels, and 5-HT uptake are decreased in an additive manner by treatment with interferon gamma and tumor necrosis factor alpha, or by conditioned medium from activated lymphocytes[46]. Co-culture of Caco-2 cells with *E. coli* also down-regulates SERT expression[47] and epithelial SERT expression and function in the intestine is decreased in mice with an enteropathogenic *E. coli* infection[47]. Thus, SERT expression and function are down regulated in inflammatory conditions leading to elevated 5-HT availability locally, and in the circulation.

Functional GI disorders

Many aspects of functional GI disorders are confusing and controversial, and the role of mucosal 5-HT signaling is no exception. Irritable Bowel Syndrome (IBS) is characterized by chronic abdominal pain or discomfort, associated with altered stool frequency or form, in the absence of organic disease that would be likely to explain the symptoms[48]. IBS patients also typically exhibit a reduced threshold for pain, or visceral hypersensitivity. Due to an absence of clear pathological markers, as in intestinal inflammation, the diagnosis is made based on presence of symptoms and further classified by the nature of the predominant stool pattern; IBS-C (constipation), IBS-D (diarrhea), or IBS-M (mixed or alternating) [48, 49]. These subgroups also have physiological relevance as stool consistency (hard to watery) reflects the amount of water absorption as a function of intestinal transit time [49]. Mucosal 5-HT signaling has been investigated in IBS-D and IBS-C, but not in IBS-M.

Possibly the first molecular alteration reported in the GI tracts of individuals with IBS was a decrease in SERT expression in specimens from individuals with IBS-D or IBS-C[16]. These findings led to much discussion and follow-up studies by several groups because it was intuitively difficult to fathom how a decrease in SERT could be associated with the divergent symptoms of diarrhea and constipation.

In IBS-D, no differences in basal or stimulated mucosal 5-HT release are detected relative to healthy controls, despite the finding that both mucosal 5-HT content and TpH1 mRNA levels are decreased[16]. While Camilleri and colleagues did not detect a change in mucosal SERT mRNA in their population of IBS-D patients[50], decreased SERT expression in IBS-D has been reported by others in adults[40, 51] as well as children[41]. In patients with IBS-C, no change was detected in the study by Camilleri[50].

As described above, circulating 5-HT comes primarily from the gut, and represents the 5-HT that is not captured by SERT in the cells of the epithelial lining. Therefore, circulating 5-HT is often studied in GI disorders as a reflection of 5-HT availability in the mucosa. Postprandial 5-HT levels are elevated in platelet-poor plasma samples obtained from IBS-D[52–54] or post-infectious IBS[54] patients, but they have been reported to be reduced[54] or unchanged[53] in IBS-C. In contrast, platelet 5-HT levels are reduced in IBS-D[55], but they are doubled as compared to healthy controls in IBS-C[53]. Collectively, these results are consistent with a decrease in 5-HT uptake by the intestinal epithelium in both of these forms of IBS since it appears that more 5-HT is ending up in the circulation. The ability to detect elevated postprandial 5-HT levels in platelet-poor blood in IBS-D, but not IBS-C, may reflect differences in platelet SERT function in these disorders. Uptake of 5-HT into

platelets appears to be disrupted in individuals with IBS-D[40, 55, 56]. This could account for the elevated 5-HT in platelet poor plasma samples from patients with IBS-D, given that 5-HT is normally difficulty to detect in such samples, even postprandially. Further studies will be required to determine whether SERT function is altered in the mucosa and/or platelets of individuals with IBS-C.

The pattern of mucosal 5-HT signaling changes that have been detected in chronic constipation differ from those reported in IBS-C or IBS-D, but as in these forms of IBS, the net result appears to be an increase in 5-HT availability. In chronic constipation, SERT expression is not altered [57], but 5-HT content, EC cell numbers, and 5-HT release are increased [57, 58]. The differences in 5-HT fingerprints of these disorders suggest that they represent different entities.

While mounting evidence strongly supports the concept that mucosal 5-HT signaling is altered in functional GI disorders, the cause and effect relationship has not been unequivocally resolved. It is clear that increasing mucosal 5-HT availability can alter intestinal function. Chronic treatment of mice with the selective serotonin reuptake inhibitor (SSRI) paroxetine decreased stool output and delayed upper GI transit[59], and mice lacking SERT exhibit alternating bouts of diarrhea and constipation[15], similar to individuals who suffer from IBS-M. In humans, chronic exposure to SSRIs enhances orocecal transit[60], but also can induce constipation by reducing peristaltic activity [61]. On the other hand, acute SSRI administration increases colonic motility and high-amplitude propagated contractions associated with abdominal cramping[62]. These findings suggest that altered mucosal 5-HT signaling could contribute to the symptoms of IBS. Furthermore, changes in gut function do not necessarily lead to changes in the elements of 5-HT signaling as demonstrated in a study of mucosal biopsies from individuals with opiate-induced constipation[57]. In this study, no differences were detected in 5-HT content or release, EC cell numbers, TpH1 mRNA or SERT mRNA levels in individuals with opiate-induced constipation as compared to healthy controls.

The cause of decreased SERT expression in IBS has not been determined. One possibility is that decreased SERT expression could involve increased permeability and a related low level of inflammation as is thought to exist in IBS[63]. Another possibility is that individuals with IBS have a genetic predisposition for decreased SERT expression. There is a polymorphic region in the human SERT gene (SERT-LPR), upstream of exon 1a, that comprises long (l) and short (s) variants depending on variable numbers of tandem repeats[64]. Interest in this polymorphism was heightened by the finding that, when expressed in lymphocytes, the s/s and l/s genotypes are associated with lower levels of SERT expression and function, as compared to the l/l genotype[64]. A possible relationship exists between SERT-LPR alleles and psychiatric disorders, anxiety-related personality traits, suicide, alcoholism, and responsiveness to antidepressants[65]. Studies of the SERT-LPR in humans with IBS have demonstrated trends in some populations, but a meta-analysis of these findings failed to detect a clear relationship between the SERT-LPR and IBS[66], although SERT expression level in the gut does appear to be influenced by the SERT-LPR genotype [67]. Furthermore, there appears to be a relationship between the SERT-LPR genotype and depressive symptoms with IBS, with IBS sufferers with the short allele being

more likely to have a history of depression [68]. A study of the single nucleotide polymorphism, rs25531, demonstrated the carriers of the rare G allele are three times as likely to have IBS than healthy controls [69].

Functions of 5-HT in the gastrointestinal tract

Conventional actions of 5-HT in the gut

One of the unique features of the ENS is the existence of intrinsic reflex circuitry that mediates the basic regulated activities of tissues in the GI tract necessary for digestion and the elimination of waste products. These regulated activities include (1) motility - generation of motor patterns via coordinated contractions and relaxations of the muscularis externa, which are required for mixing and propelling the luminal contents, (2) epithelial secretion, which is involved in diluting and dissolving luminal contents for digestion, and (3) vasodilation, which facilitates the active processes of digestion and absorption. The various intrinsic reflex circuits that control these fundamental activities can be mediated by the release of 5-HT from EC cells. In addition 5-HT released from EC cells can also activate receptors on extrinsic afferent fibers to send signals to the central nervous system.

Motility—The concept that 5-HT synthesized and released by EC cells in the intestinal mucosa can play a role in initiating peristalsis was originally proposed by Edith Bülbring and her colleagues in the late 1950's[70–73]. Amongst their observations, they found that 5-HT is released by the EC cells in an intraluminal pressure-dependent manner[72], and that intraluminal infusion of 5-HT restored peristalsis when it had been halted pharmacologically[70]. Since that time, it has been demonstrated in a number of experimental paradigms, including human preparations, that stroking of the mucosal surface results in 5-HT release[19, 74–76]. Serotonin release can also be induced by mechanical stimulation of BON cells, a human cell line model of EC cells[77]. It is clear from the elegant studies of groups led by Jack Grider[78], Terry Smith[79], and Joel Bornstein[31, 80] that 5-HT released in response to mucosal stimulation can activate the ascending contractile and descending relaxant limbs of the peristaltic reflex. Additional support for a role of 5-HT release in propulsive motility comes from *ex vivo*[19, 78, 81] and *in vivo* [82, 83] studies demonstrating that antagonists of 5-HT_3 and/or 5-HT_4 receptors slow intestinal motility, as well as a study demonstrating that corticotropin-releasing hormone-induced defecation is reversed by peripheral actions of the 5-HT_3 receptor antagonist, ramosetron[84]. Furthermore, in isolated guinea pig colon preparations, propulsive motility is disrupted by desensitization of 5-HT_4 receptors[85], or high concentrations of the SERT inhibitor fluoxetine[13], which presumably leads to 5-HT receptor desensitization by increasing 5-HT concentrations in the interstitial space.

In summarizing one of her papers, Edith Bülbring concluded, "Whether 5-HT is of primary importance for the initiation of the peristaltic reflex could not be decided as the amount of 5-HT was never reduced to zero…"[71]. New studies once again raise the question of the primary importance of 5-HT in the initiation of peristalsis. Recently, Gershon and colleagues demonstrated that intestinal transit and colonic bead expulsion, which presumably involve peristalsis, are unchanged in TpH1 deficient mice[86]. Furthermore, Spencer and colleagues removed the mucosa from mouse and guinea pig colonic

preparations and were still able to demonstrate peristalsis activated by stretch of the tissue[87, 88]. Based on our current knowledge, it is probably best to conclude that 5-HT released from EC cells is sufficient, but not necessary, to initiate the peristaltic reflex.

Another motor pattern that is generated by enteric nerves is segmentation, which consists of alternating contractions of the muscularis in a given region without forward propulsion of the luminal contents. It serves to mix the contents of the small intestine and maximize their exposure to digestive enzymes during the digestive process. Experimentally, segmentation can be activated by luminal infusion of fatty acids, and a recent study demonstrates that mucosal 5-HT is likely involved in the production of this motor pattern. In an *ex vivo* guinea pig small intestine assay, segmentation was induced by addition of the SERT inhibitor, fluoxetine, and segmentation patterns initiated by fatty acids or fluoxetine are inhibited by antagonists of the 5-HT_3 or 5-HT_4 receptor, as well as CCK receptor antagonists[89]. The authors therefore concluded that both 5-HT and CCK are involved in the regulation of segmentation motor patterns.

Secretion—In the intestinal epithelium, active secretion can be initiated by intrinsic enteric reflexes that are comprised of primary afferent (sensory) and secretomotor neurons with cell bodies located in nearby submucosal ganglia [90]. The afferent neurons receive signals from projections to the lamina propria, and when stimulated, they synaptically activate secretomotor neurons, which in turn, release neurotransmitters such as acetylcholine and vasoactive intestinal peptide (VIP) to activate secretion from the epithelial cells. Serotonin released from EC cells is an important activator of these reflex-mediated secretory responses[77, 91, 92]. Mechanical stimulation of the mucosal surface leads to 5-HT release, which activates neurally mediated Cl^- and bicarbonate secretion. The neurogenic secretory responses appear to be mediated primarily by 5-HT_3 and 5-HT_4 receptors, but 5-HT_{1P} receptors may also be involved[90, 91, 93–96 97]. Serotonin released from EC cells can also stimulate secretion through a direct paracrine action on nearby enterocytes, and this response is mediated by the activation of 5-HT_2 receptors[94]. Serotonin-initiated secretion contributes to the dilution and neutralization of luminal contents, but it is also involved in the protective response to eliminate luminal pathogens[98–102].

Vasodilation—Vascular tone is regulated solely by the sympathetic division of the autonomic nervous system throughout most of the body. A unique feature of the gut is the ability of intrinsic reflex circuitry to locally regulate blood vessel diameter. The vasodilatory motor neurons are located in submucosal ganglia, but two types of reflex circuits appear to exist: local circuits (≤ 2mm) consisting of afferent/sensory and motor neurons in the submucosal plexus, and a farther reaching circuit (up to 18 mm) that includes submucosal afferent neurons, projections to the myenteric plexus where signals are passed up and down stream along the gut tube, and submucosal vasodilator motor neurons[103–107]. These reflexes likely involve the same afferent neurons that activate secretomotor responses, and vasodilatory responses that are activated by mucosal stimulation are blocked by inhibition of 5-HT_3 and 5-HT_4 receptors[96].

Activation of extrinsic afferent nerves—Early efforts to record from extrinsic afferent fibers innervating the GI tract were hampered by the fact that animals were fasted prior to

the experiments, and the afferents were quiescent in the absence of the chemical and mechanical stimuli that normally lead to their activation[108]. The first electrophysiological recordings of vagal afferent fibers innervating the gut were accomplished by Autar Singh Paintal[109, 110]. He demonstrated in the 1950's that quiescent sensory fibers innervating the stomach and intestine could be awakened by vascular infusion of phenyldiguanide. Phenyldiguanide, also known as phenylbiguanide, was subsequently shown to be a potent 5-HT_3 receptor agonist.

Vagal afferent fibers arising in the upper GI tract send messages that result in a variety of responses upon activation by 5-HT. Pharmacological and immunohistochemical studies have demonstrated that vagal afferent nerve endings in the mucosal layer of the gut express 5-HT_3 receptors[111–114]. Furthermore, infusion of hyperosmotic solutions or carbohydrate breakdown products into the lumen of the duodenum evokes release of 5-HT from EC cells which acts on 5-HT_3 receptors to stimulate vagal afferent neurons[115]. Glucose in the lumen also causes 5-HT release from EC cells, and activates a vago-vagal reflex that inhibits gastric emptying[116]. These stimuli also result in pancreatic secretion[117], and both the gastric inhibitory response and the pancreatic secretory response are sensitive to 5-HT_3 receptor antagonists[117]. Satiety signals arising from the gut also involve 5-HT release since lipid-induced suppression of food intake is mediated entirely by simultaneous activation of 5-HT_3 and CCK_1 receptors[118]. In humans, inhibition of 5-HT_3 receptors increases the volume of a liquid meal that is ingested [119], supporting a role for 5-HT receptors and vagal afferent signaling in the regulation of satiety and hunger in humans.

In addition to activation of digestive and homeostatic reflexes described above, 5-HT released from EC cells in the intestine can lead to feelings of intense discomfort. Cytotoxic chemotherapeutic agents such as cisplatin cause a robust release of 5-HT and activation of 5-HT_3 receptors on vagal afferent fibers resulting in nausea and emesis [120–122]. It is hard to imagine how a simple signaling molecule such as 5-HT, acting on one class of receptor, present on the same set of afferent fibers, can lead to such a multitude of reflex responses. One possible factor in sorting and processing these signals could involve simultaneous activation of vagal afferent nerves that express CCK_1 receptors, which are distinct from those that express the 5-HT_3 receptor[123]. Another contributing factor may be that the amount of 5-HT released in response to chemotherapeutic agents is far greater than that released in response to nutrients and osmotic signals.

While less is known about 5-HT-induced activation of spinal afferent fibers innervating the gut, as compared to 5-HT and vagal afferents, it is clear from *in vivo*[124] and *ex vivo*[125] studies of colonic afferent nerves that spinal afferents express 5-HT_3 receptors on their peripheral endings. In studies of noxious colorectal distension in the rat, the 5-HT_3 antagonist, alosetron inhibited reflex responses and c-fos immunoreativity in the dorsal horn[124]. Furthermore, the sensitivity of spinal afferents to 5-HT is enhanced during inflammation, both in terms of the proportion of afferent fibers responding to 5-HT and the intensity of the response[126]. This is consistent with the recent finding that the number of nerve fibers expressing 5-HT_3 receptors is increased in the mucosal layer of the DSS-inflamed colon[127]. Interestingly, the hypersensitivity to 5-HT persists following recovery

from inflammation[126], suggesting that the analgesic effect of 5-HT_3 agonists in IBS could involve attenuation of 5-HT's ability to stimulate of spinal afferent terminals in the gut.

Non-conventional actions of 5-HT in the gut

In addition to the 5-HT-mediated activities described above, recent studies have revealed that this simple in structure, but complex in actions, signaling molecule can also serve as a trophic factor as well as a pro-inflammatory mediator in the GI tract.

Neuroprotective and trophic factor actions of 5-HT—Since enteric serotonergic neurons depend on TpH2 for the synthesis of 5-HT, TpH2 deficient mice have been used to investigate the roles of neuronal 5-HT in the bowel. An interesting finding of these studies is that 5-HT appears to be an important factor in the development and survival of enteric neurons. Mice lacking the TpH2 gene exhibit a lower density of myenteric neurons, particularly dopaminergic and GABAergic nerve cells[128]. These actions of 5-HT may involve the 5-HT_{2B} receptor, which has been shown to influence enteric neuronal expansion, particularly in the developing gut. Stimulation of 5-HT_{2B} receptors enhances the differentiation of enteric neurons in cultures from both dissociated cultures of mixed fetal gut cells and in cultures of neural crest-derived cells isolated from the gut[129], indicating that 5-HT may influence the fate of developing enteric neurons through actions on 5-HT_{2B} receptors.

The 5-HT_{2B} receptor is also expressed by interstitial cells of Cajal (ICC) and is important for the integrity of the ICC network. 5-HT promotes the survival of ICC cells in culture via actions on 5-HT_{2B} receptors[130], and the density of ICC is decreased in 5-HT_{2B} receptor deficient mice. Modeling of these changes suggests that alterations of the ICC network would impair slow wave propagation and ultimately motility and transit in the intestines[131].

The 5-HT_4 receptor also enhances the development and survival of enteric neurons. Studies from 5-HT_4 knockout mice indicate that the density of neurons is not altered at birth, but 5-HT_4 receptor deficiency leads to a postnatal reduction in enteric neurons, and treatment of adult mice with a 5-HT_4 agonists promotes the generation of new enteric neurons[132, 133]. Furthermore, studies of cultured enteric neurons show that stimulation of 5-HT_4 receptors increases the proliferation and/or survival of enteric neurons and enhances neurite outgrowth[132, 133]. The results of these investigations are supported by the studies of Miyako Takaki and colleagues demonstrating that 5-HT_4 receptor stimulation enhances the formation of neural networks in an embryoid body culture system[134]. They also show significant improvement in regeneration of enteric reflexes and recovery of a defecation reflex that are disrupted by colonic resection[135, 136].

TpH2 deficient mice exhibit disrupted intestinal transit[86]. Given these trophic/protective influences of 5-HT on the integrity of the myenteric plexus it is not clear whether the dysmotility findings from the TpH2 knockout mouse experiments reflect an alteration in serotonergic neurotransmission and/or changes in the circuitry due to loss of neurons.

Pro-inflammatory actions of 5-HT in the gut—As described above, 5-HT availability is increased in various animal models of intestinal inflammation, as well as human

inflammatory conditions. Recent evidence strongly suggests that 5-HT can act as a pro-inflammatory signaling molecule in the mucosal layer of the gut. On one hand, inflammation is intensified in the IL-10 knockout and TNBS models of colitis when 5-HT availability is increased by lack of the 5-HT transporter in SERT deficient mice [137, 138]. On the other hand, the extent of inflammation in DSS and DNBS colitis models is reduced when mucosal 5-HT availability is reduced by deletion of the gene that encodes TpH1 or by reduction of 5-HT synthesis with parachlorophenylalanine[139]. Bypassing TpH1 in TpH1-deficient mice by treatment with the 5-HT precursor, 5-hydroxytryptophan increased the severity of colitis in these animals. The pro-inflammatory actions of 5-HT in the mucosa involves a chain of events that are likely initiated by activation of 5-HT receptors on dendritic cells in the lamina propria[140].

Functions of gut-derived 5-HT outside of the GI tract

It has long been generally accepted that “virtually all” peripheral 5-HT arises from the gut. This notion is based on the early studies of Erspamer and Bertaccini who demonstrated that total gastroenterectomy in rats or dogs caused a disappearance of the metabolite 5-Hydroxyindoleacetic acid (5-HIAA) from the urine, and dramatically lowered 5-HT levels in the blood, spleen and lungs[141, 142]. They also demonstrated that 5-HT levels in portal venous blood are much higher than those of vena cava blood. Based on these findings, and the ability of SERT-expressing platelets to distribute 5-HT throughout the body, it has been presumed that non-cerebrospinal actions of 5-HT outside of the gut involve 5-HT arising from enteric EC cells.

Many tissues express 5-HT receptors, and actions of 5-HT outside of the gut include vasoconstriction or vasodilation (depending on the vascular bed), platelet aggregation, hematopoiesis, regulation of bone density, bronchoconstriction, itching sensation and nociception[143]. Furthermore, 5-HT appears to be involved in heart and mammary development, as well as oocyte maturation. The roles of 5-HT in decreased bone density and allergic airway inflammation (AAI) are of particular interest in the context of this review because TpH inhibitors have been tested as a therapeutic strategy for these conditions.

Low Density Lipoprotein Receptor-Related Protein-5 (Lrp5) is a Wnt coreceptor that is important in the control of bone density, and there is evidence that it affects bone indirectly through regulation of 5-HT synthesis by EC cells [144]. In Lrp5 deficient mice, TpH1 expression and mucosal 5-HT concentrations are elevated. Increased serotonin levels decrease bone formation by interacting with 5-HT_{1B} receptors to inhibit osteoblast proliferation. Inhibition of TpH1 has been shown to have protective action in an ovariectomy model of osteoporosis, further supporting a role for 5-HT as a regulator of bone growth and density[145].

A role for 5-HT in the manifestation of allergic airway inflammation has been well established, yet the source of the 5-HT was not clearly defined until recently, and it was not known whether TpH1 was involved in synthesis of that 5-HT. A recent study by Idzko and colleagues demonstrated that platelets provide the 5-HT that contributes to asthma pathogenesis, and that symptoms in a mouse model of allergic airway inflammation are

greatly reduced in TpH1 deficient mice or mice treated with a TpH1 inhibitor[146]. These findings indicate that the 5-HT that interacts with airway tissues during asthma attacks originates in the gut, and that TpH1 inhibitors acting in the gut could provide a novel therapeutic approach for the treatment of asthma.

While it is likely that most, if not all, platelet serum arises in the gut, recent evidence indicates that TpH1 expression can be activated locally in a variety of tissues[147]. For example, there is considerable controversy as to whether it is gut-derived 5-HT, and/or 5-HT from local TpH1-expressing osteoclasts, that regulates bone remodeling by affecting both oseaclast and osteoblast development[148]. Furthermore, mouse mammary epithelium expresses mRNA for TpH1 as well as and aromatic amine decarboxylase when stimulated by prolactin[149], and cells expressing TpH1 in the placenta give rise to 5-HT that is critical for brain development[150]. Regardless of the source of 5-HT regulating these systems under normal circumstances, pharmacological interventions and pathological conditions that affect gut 5-HT signaling, and ultimately circulating 5-HT levels, have the potential to impact many other tissues.

Serotonergic targets for the treatment of GI disorders

5-HT receptors in the GI tract

Serotonin receptors are widely expressed within the GI tract, and five of the seven known families, 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, and 5-HT_7 receptors, are expressed in the gut and can affect gut functions[151] (Fig. 3). The 5-HT_3 and 5-HT_4 receptor subtypes have been most extensively studied in the gut, and have been targeted for the treatment of diarrhea and constipation, respectively.

5-HT_3 receptors—Because of their value as a therapeutic target in the gut, 5-HT_3 receptors have been extensively investigated. They are expressed on the excitable cells of the gut, including intrinsic and extrinsic afferent nerves that extend into the mucosa, interneurons, inhibitory and excitatory motor neurons, ICCs, smooth muscle and enterocytes (Fig. 3). As described above, 5-HT_3 receptors are involved in the activation of intrinsic and extrinsic afferent nerves by 5-HT released from EC cells, and they contribute to a small subset of EPSPs. In an elegant and technically difficult investigation, Bertrand and colleagues demonstrated that AH neurons in the myenteric plexus, which serve as primary afferent neurons and project to the mucosa, are directly activated by 5-HT applied to the mucosa, and that these responses are exclusively mediated by 5-HT_3 receptors[152].

Initial therapeutic use of 5-HT_3 receptor antagonists arose from the landmark discovery in 1986 that specific 5-HT_3 receptor antagonists could inhibit cisplatin-induced emesis[153, 154]. 5-HT_3 receptor antagonists likely exert their anti-nausea effects via at least two sites of action, vagal afferents in the gut and in the area postrema, and they are particularly effective in treating the acute phases of chemotherapy- and radiation therapy-induced nausea and emesis[121, 122]. A common adverse side effect of 5-HT_3 receptor antagonist being used for anti-nausea and anti-emetic purposes is constipation, and this action has been exploited for the treatment of IBS-D[83, 155].

5-HT_3 antagonists have been shown to be effective in treating both the diarrhea and abdominal discomfort symptoms of IBS-D[156, 157]. While the precise mechanisms of action of these compounds have not been definitively determined, it is likely that they inhibit 5-HT_3 receptors located on intrinsic and extrinsic afferent nerve fibers in the mucosa that are activated by 5-HT released from EC cells. They also inhibit the stimulation of 5-HT_3 receptors on interneurons and motor neurons that contribute to fast EPSPs. Collectively, this would decrease propulsive motility and secretion locally within the gut, thus alleviating diarrhea, as well as decreased signaling from spinal afferent nerves that deliver signals of pain and discomfort to the CNS. As described above, the 5-HT_3 antagonist, alosetron, inhibits activation of dorsal horn neurons in response to noxious distention of the colon[124], indicating that 5-HT_3 receptors are involved in the activation of pain signals from the gut. However, the viscero-analgesic benefits of 5-HT_3 antagonists may also involve central mechanisms of action[158].

With the knowledge that 5-HT_3 receptors can participate in the activation of propulsive motility and secretory responses in the gut, 5-HT_3 agonists have been developed and tested for the treatment of constipation. Emphasis has been concentrated on partial agonists because 5-HT_3 receptors desensitize rapidly, and as outlined above, stimulation of 5-HT_3 receptors on vagal and spinal afferent fibers can lead to nausea and abdominal discomfort, respectively. One such compound is pumosetrag (DDP733/MKC733), which is being developed for the treatment of IBS-C and has also been shown to reduce reflux events in individuals with gastroesophageal reflux disease[159, 160]. Another, somewhat counterintuitive strategy, is to use a high affinity, low intrinsic activity 5-HT_3 receptor partial agonists for the treatment of IBS-D[160]. The concept being proposed is that these compounds would interrupt activation of 5-HT_3 receptors by excessive background levels of 5-HT, such as those that are thought to exist in IBS-D, while allowing the receptors to be activated during reflex responses, thus reducing the chance of developing constipation.

5-HT_4 receptors—Agonists of the 5-HT_4 receptor alleviate constipation in IBS-C and in chronic constipation, they provide pain relief in IBS-C, and they can accelerate the rate of gastric emptying[161–164]. It is interesting to note that benzamides, such as metoclopramide, cisapride and renzapride, were recognized as prokinetic agents in the gut even before the existence of 5-HT_4 receptors was discovered, and because they enhanced acetylcholine release from myenteric neurons[165, 166], they were initially considered cholinergic compounds. Soon after the discovery of 5-HT_4 receptors, it was determined that these compounds are 5-HT_4 receptor agonists, and efforts were made to determine the mechanisms by which they promote motility. Considerable physiological data suggest that 5-HT_4 receptor stimulation accelerates the peristaltic reflex[11, 167–169]. Furthermore, transgenic mice that lack 5-HT_4 receptors exhibit slowed colonic motility[133], suggesting that 5-HT_4 receptors are physiologically activated and contribute to the regulation of propulsive motility, and/or they promote normal motility through their trophic actions mentioned above. Within the muscularis externa, 5-HT_4 agonists act presynaptically on nerve terminals to enhance the release of acetylcholine[169–173]. By acting in this manner, they are thought to enhance naturally occurring reflex activity rather than to generate neurotransmission. This is an important distinction because efficient propulsive motility is a complex and carefully

orchestrated process, and simply activating transmitter release by neurons throughout the gut tube actually inhibits propulsive motility[174].

Unfortunately, while shown to be effective, the 5-HT_4 agonists that were initially made available for the treatment of constipation, cisapride and tegaserod, were ultimately removed or restricted because of potential cardiovascular side effects. It is thought that the adverse effects of the early 5-HT_4 compounds are related to their relative lack of selectivity, and resultant actions on other targets, including hERG potassium channels, dopamine receptors, 5-HT_1 and 5-HT_2 receptors [175, 176]. Therefore, more selective agonists such as prucalopride, naranopride (ATI-7505), mosapride, and velusetrag (TD-5108) are becoming available or are being developed to fill this gap.

We have recently published the results of a study that reveals a novel target for 5-HT_4 agonists that could provide clinical benefits while minimizing the risk of adverse side effects[11]. In *ex vivo* motility and peristaltic reflex assays, we and others found that 5-HT_4 agonists accelerate propulsive motility when they are infused into the lumen or applied to the mucosal surface[11, 78, 168, 177]; however addition of agonists to the bathing solution where they would be in position to access the nerve terminals of the peristaltic reflex circuitry did not enhance motility[11]. These results suggested the existence of 5-HT_4 receptors in the mucosa. Evaluation of fluorescence in a strain of mice in which the promoter of the 5-HT_4 gene drives the expression of enhanced green fluorescent protein revealed that essentially all of the cells of the colonic mucosa express the 5-HT_4 receptor (Fig. 4). Additional studies demonstrated the presence of transcript for this receptor in the colonic mucosa of mouse, rat and human . Furthermore, mucosal application of 5-HT_4 agonists activated 5-HT release from EC cells, mucus discharge by goblet cells and Cl^- secretion by enterocytes, and 5-HT_4 receptor antagonists blocked these actions. Any or all of these actions could alleviate constipation, and formulating 5-HT_4 agonists such that they target the colon without being absorbed could result in a safe and effective treatment of IBS-C and chronic constipation.

A welcome, but puzzling, action of 5-HT_4 receptor agonists is that they attenuate visceral pain arising from the colon. In humans, oral administration of the 5-HT_4 receptor agonist tegaserod reduced rectal sensitivity to distension in individuals with IBS[178] Furthermore, intraperitoneal or intraluminally administered tegaserod attenuated nociceptive responses, in a visceral hypersensitivity model involving awake, freely moving rats, [11, 179]. The reason this is puzzling is that 5-HT_4 receptor stimulation leads to activation of the cyclic AMP-protein kinase A pathway, which has an excitatory effect on neuronal activity, as is seen with the presynaptic facilitory actions of 5-HT_4 agonists. It has been proposed that the anti-nociceptive actions of these compounds involve their non-selective inhibitory action on 5-HT_{2B} receptors, but the actions of tegaserod in these studies are blocked by 5-HT_4 receptor antagonists[11, 180]. Furthermore, intraluminal administration of the selective 5-HT_4 receptor antagonist ATI-7505 inhibits visceral hypersensitivity[11]. One possible mechanism by which 5-HT_4 receptors could attenuate visceral nociception is through an inhibitory action on intramural mechanoreceptor activation. In a rectal distension study in cats, tegaserod decreased afferent discharge at nociceptive intraluminal pressures without altering compliance[181]. However, even if mechanoreception is inhibited by 5-HT_4 agonists, the precise mechanism of this inhibition has not been clearly determined.

Tryptophan hydroxylase

There is substantial evidence that 5-HT released from EC cells can activate enteric secretory and motor reflexes, as well as send signals to the CNS. Therefore, it stands to reason that suppressing this signaling apparatus by decreasing 5-HT release could possibly attenuate the symptoms of IBS-D. Accordingly, efforts have been made to develop TpH inhibitors that decrease GI 5-HT levels without affecting serotonergic signaling in the brain by limiting their ability to cross the blood-brain barrier[182–184]. Results of a double-blind, placebo-controlled study of one of these compounds, LX-1031, indicate that this approach shows promise for treating the symptoms of non-constipating IBS[183]. In this 4 week trial, LX-1031 resulted in significant relief of pain and discomfort, and in improvement of stool consistency. Treatment with the TpH inhibitor decreased peripheral 5-HT levels, as reflected by dose-dependent decreases in urine levels of the 5-HT metabolite, 5-HIAA, and interestingly, there was a correlation between symptom improvement and reduction in 5-HIAA.

Concluding remarks

Despite our best efforts over the past 6 decades to understand 5-HT signaling in the gut, many unanswered questions remain, and new questions are still arising. It is generally accepted that 5-HT plays a role in many GI functions and in sending signals from the gut to the CNS. Also, it is clear that drugs that target 5-HT signaling molecules are effective at alleviating the symptoms of functional GI disorders. Figuring out how to identify subpopulations of individuals with a given type of IBS that respond best to these treatments is an important goal. One aspect of 5-HT biology not extensively addressed in this review is the interrelationship between genetic polymorphisms in the genes of 5-HT signaling molecules and various forms of IBS. This is because no clear answers have yet emerged, but it is important to keep trying. Another important goal is to understand more precisely the distributions of 5-HT receptors and to determine which receptors in what locations are physiologically relevant. Much of our information is from whole organ or *in vivo* assays with which it is impossible to resolve the precise locations of receptors involved in a given reflex response. Gaining the resolution to answer these questions will require returning to the labor intensive, low throughput approaches such as those used by Bertrand and colleagues to demonstrate that 5-HT_3 receptors are located on mucosal projections from myenteric primary afferent neurons. Another emerging feature of 5-HT signaling is that most of the targets of therapies for FGIDs are expressed by epithelial cells or within 20 μm of the epithelial surface. These include Tph1, as well as 5-HT_3 and 5-HT_4 receptors. Attempting to reach these receptors directly, rather than via the systemic circulation, could greatly improve the benefit to risk ratio.

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Key Points

- Serotonin is an important gastrointestinal signaling molecule that conveys signals from the lumen of the gut to intrinsic and extrinsic sensory neurons, and contributes to synaptic signals in the enteric nervous system.
- Fundamental properties of mucosal serotonin signaling are altered in response to inflammation and in functional GI disorders.
- Actions of serotonin released from mucosal enterochromaffin cells include activation of intrinsic reflexes such as peristalsis, segmentation, secretion and vasodilation. Serotonin also can activate signals sent to the CNS that stimulate digestive reflexes and can cause abdominal pain and discomfort, satiety, or nausea.
- Recent investigations have demonstrated that mucosal serotonin can promote intestinal inflammation, and serotonin in the muscularis propria can promote neuron and interstitial cell of Cajal survival, and promote neural regeneration. Furthermore, gut-derived serotonin can have extra-alimentary actions such as influencing bone densitity and contributing to allergic airway inflammation.
- Because of its importance as a signaling molecule in the gut, compounds that affect serotonin signaling in the gut have been developed, including drugs that target 5-HT_3 receptors, 5-HT_4 receptors, and tryptophan hydroxylase.
- Because most targets related to serotonin signaling in the gut are within or close to the epithelial surface, emphasis should be placed on compounds that can mediated their actions without enteric the circulation, thus minimizing adverse actions while maximizing efficacy.

Review criteria

In the process of preparing this Review we used our collective 35 years of experience studying serotonin signaling and serotonin receptors in the gastrointestinal tract, and we conducted a number of literature searches. PubMed database searches involved combinations of terms that included serotonin, SERT, tryptophan hydroxylase, receptor, inflammation, IBS, motility, secretion, vagal afferent, spinal afferent, intrinsic afferent, AH neuron, gastrointestinal, intestine, stomach, colon, and enteric nervous system.

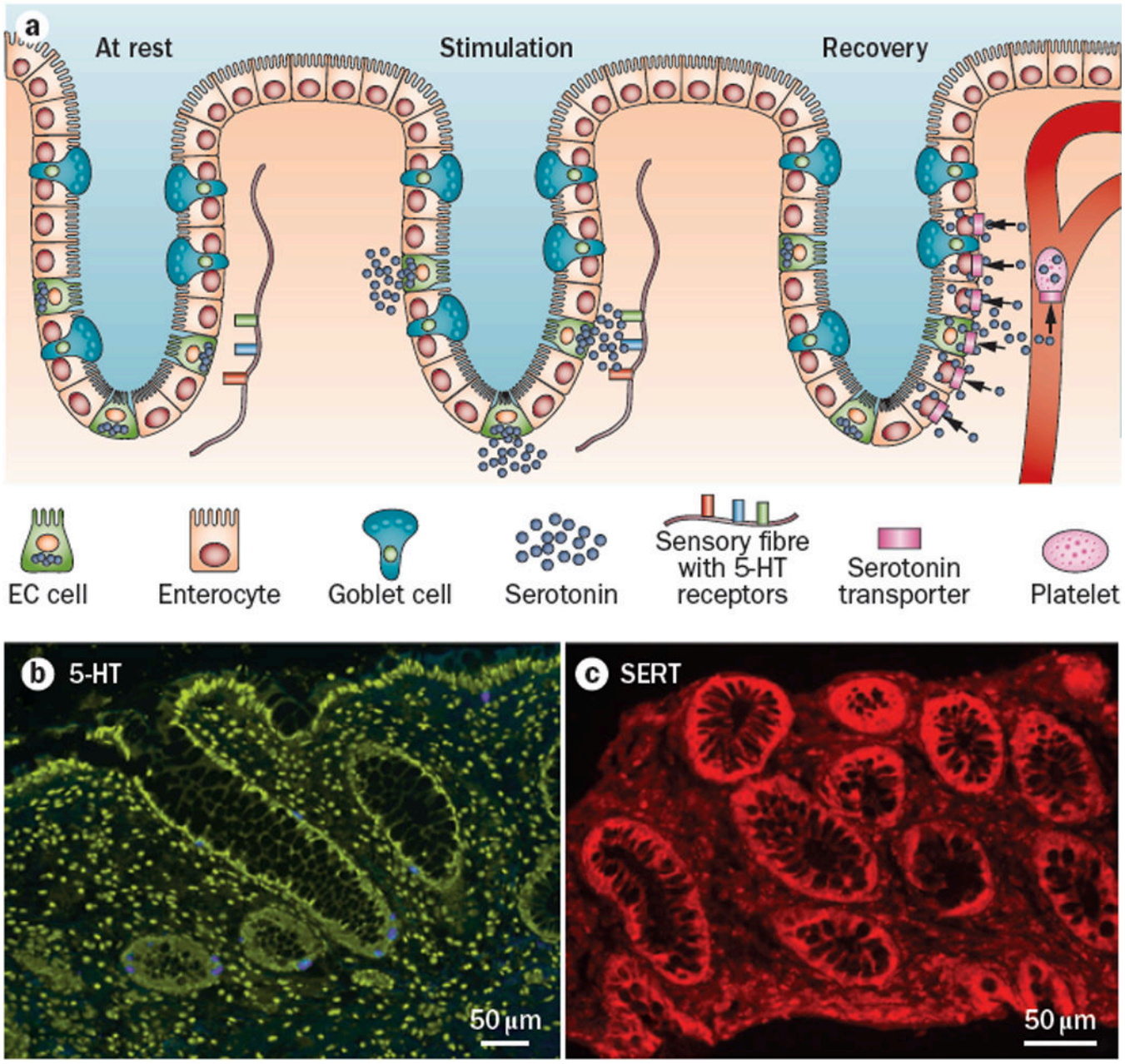

Figure 1.

In the intestinal epithelium, 5-HT is synthesized and released by a small subset of cells called enterochromaffin (EC) cells, and the 5-HT is then removed from the interstitial space by the serotonin-selective reuptake transporter (SERT), which is expressed by essentially all epithelial cells. a. Immunstaining for 5-HT in a biopsy from the transverse colon of one of the authors (GMM). 5-HT is stained blue, and the section is counterstained with the nucleic acid stain, yoyo. Note that there are no mast cells stained for 5-HT in this micrograph; 5-HT-immunoreactive mast cells are common in mouse and rat, but not in human. b. Immunostaining for SERT in a human rectal biopsy specimen demonstrating that essentially all cells in the colonic glands are SERT-immunoreactive. c. Schematic diagram illustrating

the sequence of events involved in 5-HT signaling in the gut. At rest, 5-HT is synthesized by EC cell. Upon mechanical or chemical stimulation, 5-HT is released into the interstitial space of the lamina propria and binds to receptors on nearby nerve fibers. The 5-HT signaling is terminated during the recovery phase. 5-HT is transported by SERT into epithelial cells where it is enzymatically degraded, or it enters the blood stream where it is transported into platelets where it is stored for future use.

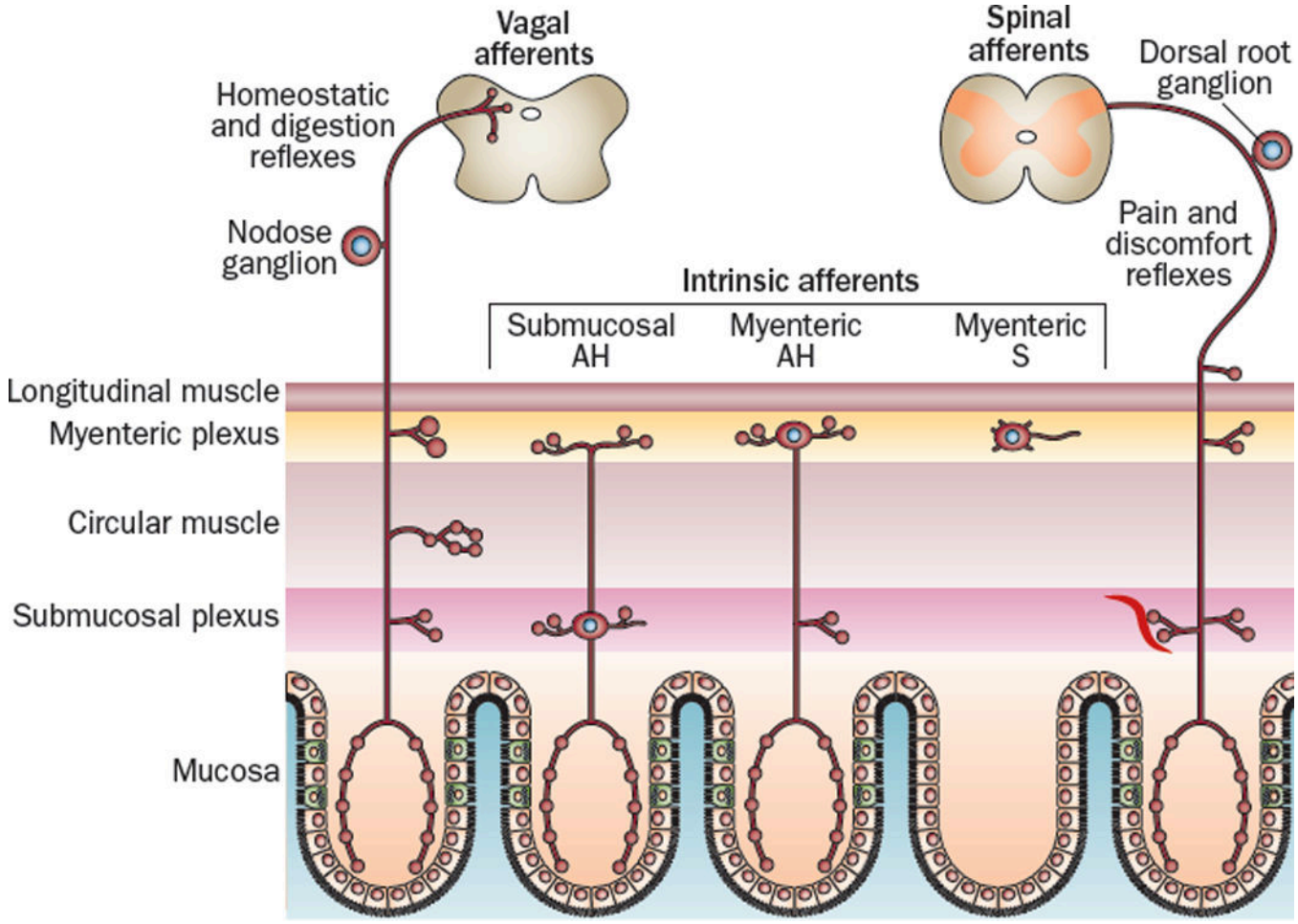

Figure 2.

Most of the intrinsic and extrinsic primary afferent neurons that innervate the gut extend processes into the lamina propria of the mucosal layer where they can become exposed to 5-HT released by EC cells[185]. These include vagal afferent fibers arising from the nodose ganglion, spinal afferent fibers arising from dorsal root ganglia, and intrinsic AH neurons located in submucosal and myenteric ganglia. There is also a class of mechanosensative S neurons in the myenteric ganglia that do not project to the mucosal layer.

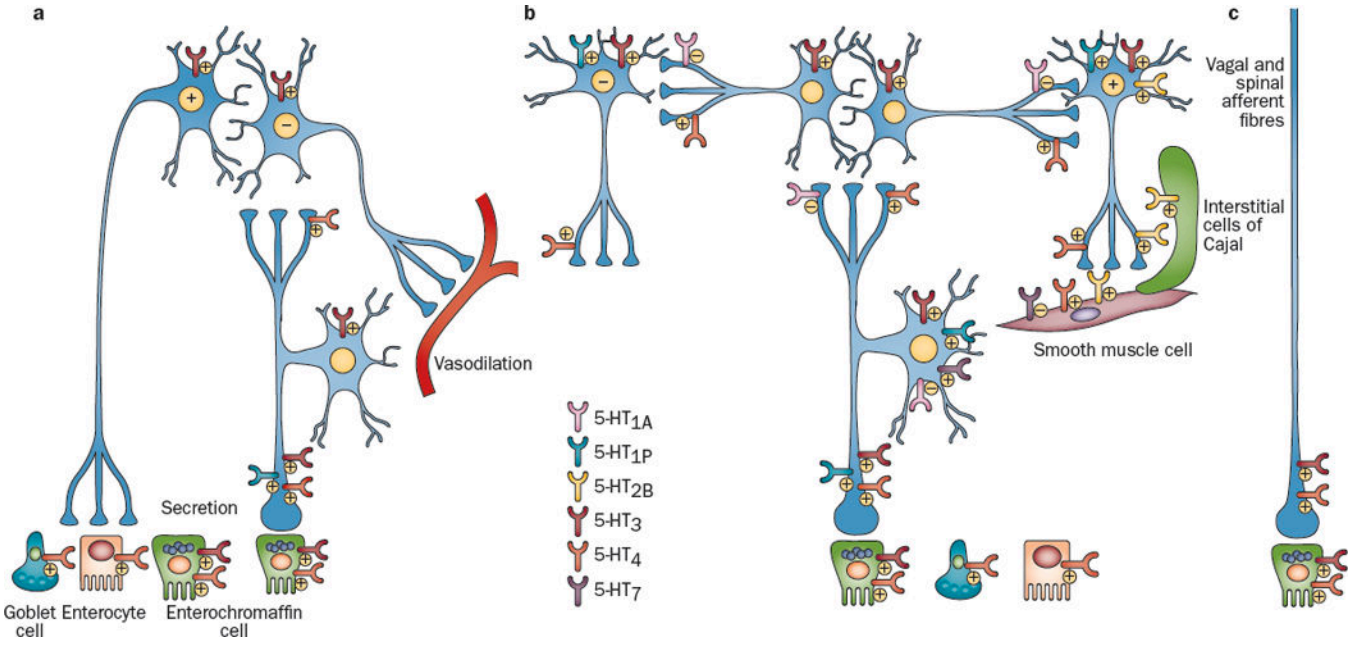

Figure 3.

Distribution of 5-HT receptors on enteric neurons, extrinsic nerve fibers and other excitable cells in the gut. At least 6 subtypes of 5-HT receptors are expressed in the wall of the gut, and they can exert excitatory and/or inhibitory influences depending on their location and on the target cell type. a. intrinsic circuits for epithelial secretion and vasodilation. The motor neuron may be the same neuron, but it promotes epithelial secretion and relaxes vascular smooth muscle. b. Intrinsic circuit for propulsive motility. c. extrinsic vagal and spinal afferent fibers. The "+" and "-" symbols indicate excitatory and inhibitory actions, respectively. References: 5-HT_{1A}[171, 186–188]; 5-HT_{1P}[33, 74, 92, 97, 186, 189–191]; 5-HT_2[129, 130, 192–194]; 5-HT_3[33, 74, 81, 106, 112, 116, 117, 119, 123, 124, 152, 186]; 5-HT_4[11, 74, 133, 168, 170, 171, 173, 186, 195]; 5-HT_7[35, 196].

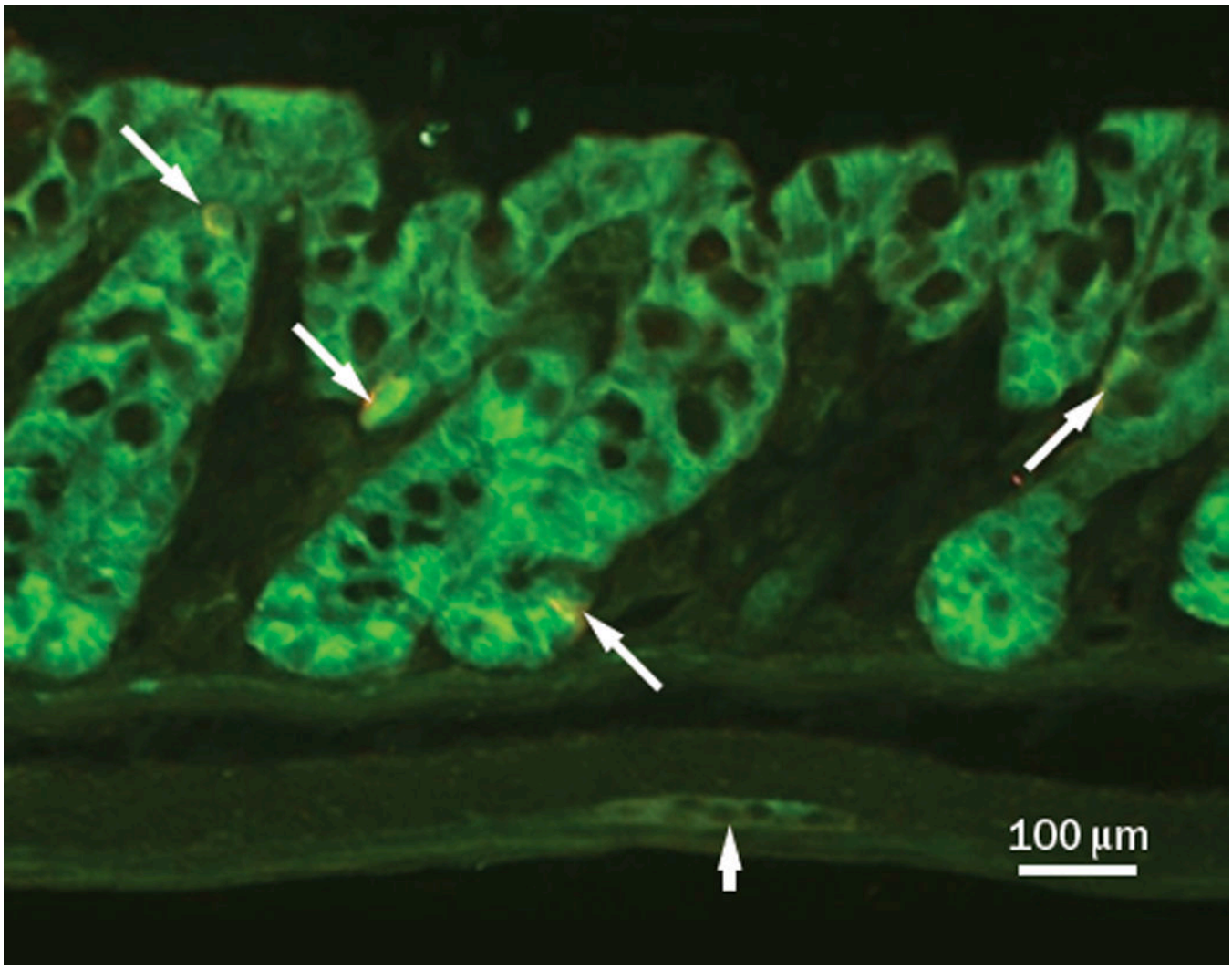

Figure 4.

The 5-HT_4 receptor is expressed by virtually all epithelial cells, including EC cells, in the murine colon[11]. This is a photomicrograph from a section of the distal colon of a 5-HT_4R(BAC)-eGFP mouse in which cells that express the 5-HT_4 receptor fluoresce green. Green fluorescence is present in all of the epithelial cells, as well as a layer of cells in the muscularis mucosa, and in a myenteric ganglion (block arrow). This section was also processed for 5-HT-immunoreactivity to label EC cells, shown in red (arrows).