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Luminal 5-HT$_4$ Receptors – A Successful Target for Prokinetic Actions

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Serotonin and the Enteric Nervous System

Intestinal propulsion is primarily controlled by the myenteric plexus of the enteric nervous system (ENS) contained within the gut wall (Figure 1). It is also heavily dependent on serotoninergic signalling with pharmacological agents targeting serotonin (5-HT) receptor subtypes used clinically for decades to treat functional bowel disorders causing diarrhea and constipation [1-3]. Shokrollahi et al. (2019) in the current issue of *Neurogastroenterology and Motility*, examined the effects of luminal prucalopride, a highly specific 5-HT_4 receptor (5-HT_4R) agonist used clinically to treat various forms of constipation in humans, on colonic propulsion in the rabbit [4]. In this mini-review we discuss evidence for luminal 5-HT_4 Rs as valuable targets for prokinetic agents in facilitating propulsive motor patterns.

The actions and roles of serotonin (5-Hydroxytryptamine, 5-HT) within the gastrointestinal tract (GIT) have been extensively studied over decades and yet 5-HT signalling is so complex that many questions remain unanswered. This is due to the many different 5-HT receptors and their subtypes found in the gut, their diverse locations and the fact that there are two distinct sources of 5-HT, mucosal and neuronal [1,5,6]. It is well known that 95% of the body's 5-HT is found in the GIT where approximately 90% is synthesized and released from specialised mucosal enteroendocrine cells (enterochromaffin, EC) cells. Up to 5% is found in myenteric serotonergic neurons which, although a small population, have widespread projections suggesting they play a significant role in modulating gut motility [7,8]. The two primary sources of serotonin have different synthetic pathways. In EC cells 5-HT synthesis from L-tryptophan is mediated by the rate-limiting enzyme tryptophan hydroxylase 1 (TPH1), whereas neuronal 5-HT synthesis is mediated TPH2 [9-12]. This has conveniently

allowed the relative roles of mucosal and neuronal 5-HT in modulating gastrointestinal motility to be investigated, but to this day remains an area surrounded by much controversy. For example, there is continuing debate over the role of 5-HT_3 receptors in the generation of mouse colonic migrating motor complexes (CMMCs) *in vitro* where some studies report mucosal 5-HT plays an important role [9,13-15] and others conclude it is unimportant [16-21].

5-HT_4Rs and Prokinetic agents

There are 7 different types of 5-HT receptor (5-HT_1-5-HT_7) some with multiple subtypes [22]. Those identified in the gastrointestinal tract include 5-HT_{1A}, 5-HT_{1P}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_3, 5-HT_4 and 5-HT_7 receptors [5]. The G-protein coupled 5-HT_4 receptor (5-HT_4R) is widely studied, primarily because agonists at these receptors have prokinetic effects on intestinal motility. In the human colon 5-HT_4Rs are a therapeutic target for treatment of functional bowel disorders including chronic constipation (CC) and constipation predominant irritable bowel syndrome (IBS-C)[23-28] whilst in the upper gut 5-HT_4R agonists promote gastric emptying [29,30] and have been used clinically to treat gastroparesis [31].

Despite the clinical success of 5-HT_4R agonists, their mechanism(s) of action is still being debated. The locations of 5-HT_4Rs (Figure 1) within the GIT are widespread, including various types of enteric neurons and epithelial cells including 5-HT containing EC cells, plus smooth muscle and interstitial cells of Cajal (ICC). Additional complexity derives from differential 5-HT_4R expression between anatomical regions and between different species. Furthermore, several carboxy-terminal splice variants have been identified which include (a), (b), and (e) isoforms in rats [32] (a), (b), (e), and (f) isoforms in mice [32,33] and (a), (b), (c), (d), (e), (g), (i) and (n) isoforms in humans (Table 1). Another variant has a 14 residue insertion in the second extracellular loop and is found in human brain tissue (5-$HT_{4(h)}$, Table 1) [34].

Notwithstanding these complexities, clinical studies in humans and many pharmacological studies in isolated preparations in small animals reveal a common theme: 5-HT_4Rs initiate and/or enhance peristaltic reflexes and/or propulsion. This has been demonstrated in guinea pig ileum [35-37] and colon [38-41], mouse [42], rat [38,39,43-45] and dog colon [46] and human jejunum [39,47] and colon [48-51]. Use of early 5-HT_4R agonists in humans including cisapride, mosapride and tegaserod was limited by their adverse secondary effects on other systems

following their systemic absorption. For example, cisapride inhibits the human ether-à-go-go related gene (hERG) potassium channel at therapeutic concentrations leading to adverse cardiac electrophysiological events including QT prolongation, ventricular tachycardia and ventricular fibrillation [25,52]. Highly selective 5-HT_4R agonists with improved safety have since been developed. In particular, prucalopride, used by Shokrollahi et al. (2019) to demonstrate prokinetic effects on propulsive motor patterns in rabbit proximal colon, has emerged as a popular and effective therapeutic agent for promoting gut motility. Prucalopride displays high affinity binding to human 5-$HT_{4(a)}$ and 5-$HT_{4(b)}$ receptor isoforms [53,54] which is consistent with its clinical success [23,49,55-57] since these isoforms are expressed in the human colon (Table 1). Other high affinity agonists have also been developed including naronapride (ATI-7505), velusetrag (TD-5108) and YH12852. YH12852 [58] and naronapride [59] have high affinity for human 5-$HT_{4(a)}$ and 5-$HT_{4(b)}$ isoforms respectively and velusetrag (TD-5108) is highly potent at the 5-$HT_{4(c)}$ receptor splice variant [60]. While these drugs are still in development, like prucalopride, they appear to be clinically effective in patients with chronic constipation, with no apparent side effects [25,50,58,60,61] and they target 5-HT_4R isoforms expressed in the human intestine (Table 1).

Prokinetic effects on propulsive motor patterns

It is clear from Shokrollahi et al. (2019) that prokinetic effects on colonic propulsive motor patterns in the rabbit can be achieved by luminal application of prucalopride. A key feature of this paper is the insight it provides into how prucalopride augments human colonic motor patterns.

Neurally mediated propagating contractions in the large bowel have been studied in mammals from small rodents to humans and appear to be generated via similar mechanisms [62]. However, several difficulties arise when comparing these motor patterns across different species, including humans. The first relates to the large range of nomenclature used to describe these motor patterns and their myoelectric correlates. These include, but are not limited to, colonic migrating motor complexes (CMMCs, mice), giant migrating contractions (GMCs, dogs, rats), migrating spike bursts (cats), long distance contractions (LDCs, rabbits) peristaltic contractions (guinea-pigs), high amplitude propagating contractions (HAPCs, humans), and repetitive propagating pressure sequences (PPSs, humans). To address this issue it was decided at a consensus meeting to use the term

"colonic motor complex" to refer to neurogenic repetitive peaks of pressure and/or electrical activity in the colon (eg CMMCs) and "neural peristalsis" to refer to anterograde propulsive movements triggered by distension with liquids or solids in animals (eg peristaltic contractions, LDCs) (See Table 4 [63]). It was also determined at the consensus meeting that HAPCs in humans likely correspond to neural peristalsis and repetitive PPSs likely equate to CMCs in animal colon (See Table 5 [63]). Secondly, the relative contribution of myogenic and neurogenic activity to the propulsive motor patterns in different species needs to be considered. For example, the rabbit colon displays distinct myogenic and neurogenic motor patterns that interact so that the neurogenic colonic migrating motor complex is superimposed on the underlying myogenic rhythmic contractions. The myogenic activity manifests as single fast propagating contractions (FPCs) but formation of clusters of FPCs relies on neural activity. The neurogenic long distance contraction (LDC) is the most forceful circumferential propulsive contraction in rabbit colon [64-66]. Shokrollahi et al. (2019) report that both FPC clusters and LDCs are components of the colonic motor complex at different levels of excitation and both are enhanced by intraluminal prucalopride. Similar interactions between myogenic and neurogenic activity have been described in the human colon [67,68]. In contrast, propulsive colonic motor patterns in guinea-pigs, rats and mice are predominantly neurogenic [62]. Further, there are distinct structural differences in the longitudinal muscle between mice, rats, cats and dogs and rabbit and human colon (Figure 2). The rabbit has a 3-taeniated region in the proximal colon that resembles the structure of the 3-taeniated human colon. The different regions display distinct motor patterns. For example, haustral boundary contractions are specific to the 3-taeniated region of rabbit proximal colon and have also been described in humans. These are slowly propagating ring contractions of the circular muscle occurring at a regular frequency (0.5 cpm) that divide the colon into pockets or haustra (Figure 2). They predominantly propagate anally and are neurally mediated although myogenic activity is also involved [65-67,69].

High resolution manometry (HRM) has emerged as a tool to more accurately define human colonic motor patterns and as a method to diagnose colonic motor dysfunction [70]. High amplitude pressure waves (HAPWs) measured using standard manometry techniques and further characterized using HRM are now termed HAPCs [63]. Although relatively infrequent, these are the dominant propulsive motor pattern in human colon and are probably necessary for defecation. Absence of spontaneous or evoked HAPCs may identify abnormal

colonic function contributing to constipation [71,72]. Importantly, HRM has identified more frequently occurring simultaneous pressure waves (SPWs) which are difficult to discriminate with standard manometry. SPWs have been shown to contribute to propulsion of luminal contents and gas expulsion and may become of diagnostic value in patients with colonic dysmotility [67,71]. Quan et al. (2017) showed that LDCs and FPCs detected in rabbit colon with HRM are equivalent to human colonic HAPCs and SPWs, respectively [66,67,71,73]. Shokrollahi et al. (2019) showed prucalopride enhances both FPCs and LDCs in 3-taeniated rabbit proximal colon. Similarly prucalopride induces HAPCs in humans [49]. HRM has also identified haustral boundary transients in humans that may be the pressure pattern caused by haustral contractions, and equivalent to haustral boundary contractions and associated pressure patterns of rabbit colon [65,67,71,74,75]. These similarities suggest the rabbit colon is a highly translatable model of colonic propulsion in humans, especially when combined with the wealth of knowledge gained from studies of colonic propulsion in mice, rats and guinea pigs.

Mechanisms underlying prokinetic actions of 5-HT_4R agonists

Understanding how prokinetics achieve their effects depends on identifying their site(s) of action. It is generally accepted that one mechanism involves activation of presynaptic 5-HT_4Rs located on myenteric neurons, the primary neurons controlling intestinal motility. These receptors facilitate neurotransmitter release and enhance peristaltic reflexes augmenting propulsion in guinea-pigs, rats and mice [76-80]. In addition, 5-HT_4Rs mediate slow depolarizations in some myenteric neurons in guinea pig ileum [81]. Both are consistent with 5-HT_4Rs being coupled to Gs, leading to adenylate cyclase stimulation and increased cAMP production, which depolarizes enteric neurons and facilitates transmitter release. But Shokrollahi et al., and another key study by Hoffman et al. (2012), show luminal 5-HT_4Rs are an alternative and effective target for serotonergic prokinetic agents [4,40]. There are multiple mechanisms by which this might occur including activation of neural pathways controlling motility and also neural and non-neural pathways promoting secretion.

Hoffman et al. (2012) identified 5-HT_4Rs in the colonic epithelium of mouse, rat, guinea pig, and humans, where they are expressed by 5-HT-containing EC and goblet cells (Figure 1). They found that a 5-HT_4R agonist applied to the colonic mucosa in mice causes a TTX-insensitive increase of 5-HT release that was blocked by a 5-HT_4R antagonist. They also

showed that direct activation of 5-HT_4Rs on other epithelial cells, including mucous secreting goblet cells, leads to enhanced secretion. These observations are consistent with the intracellular signalling mechanism that follows 5-HT_4R activation and coupling to Gs as increased cAMP in enterocytes and EC cells causes secretion and sensory mediator release. Release of mucosal 5-HT activates mucosal terminals of intrinsic (and extrinsic) primary afferent neurons to excite peristaltic and secretory reflex pathways. Other studies showing enhanced peristaltic reflexes and propulsion via luminal, but not serosal, application of pharmacological agents including 5-HT_4R agonists, further support this mechanism. These include Shokrollahi et al. (2019), and studies of human [39], guinea pig [40,41] and rat [44] colon showing luminal application of 5-HT_4R agonists increases propulsive activity and peristaltic reflexes.

Despite the tendency to treat the myenteric and submucosal plexuses as separate entities controlling motility and secretion respectively, circuits controlling motility and secretion are interconnected and integrated [82-84]. Activation of neural pathways leading to enhanced propulsion probably also excites neural pathways controlling secretion to increase stool softening and facilitate transit. Furthermore, enhanced secretion leading to distension of the gut wall would in turn activate peristaltic reflexes promoting motility. Thus, increased secretion is another way luminal 5-HT_4Rs might lead to prokinetic effects on colonic motor patterns. 5-HT induces fluid secretion in isolated small and large intestine in small animals and humans. Neuronal and non-neuronal mechanisms are involved and 5-HT_4Rs are implicated in both mechanisms. In rat distal colon, 5-HT elicits secretion via activation of 5-HT_4Rs on colonic epithelial cells and a neurally mediated response to 5-HT occurs via 5-HT_3Rs [85,86]. A similar pattern is seen in guinea pig ileum [87,88]. In contrast, neural 5-HT induced secretion occurs via 5-HT_4Rs in pigs [89] and humans [90]. In the upper gut, activation of 5-HT_4Rs on mucosal epithelial cells in rat and mouse duodenum mediates 5-HT evoked protective bicarbonate secretion [91,92]. Further, 5-HT release caused by mucosal stroking activates 5-HT_4 receptors on enteric sensory neurons, evoking a neuronal reflex that stimulates chloride secretion in human jejunum [93,94]. Importantly, 5-HT also causes secretion via a direct activation of mucosal 5-HT_4Rs on human colonic epithelial cells [40,95]. Thus there are two ways in which luminal 5-HT_4R agonists can target mucosal 5-HT_4Rs to enhance secretion contributing to prokinetic effects on colonic transit: a direct action on colonic epithelial cells

and an indirect action via release of 5-HT from EC cells which excites neural secretory reflex pathways thereby enhancing chloride secretion. Both mechanisms would complement the 5-HT_4R mediated activation of myenteric neural circuits controlling motility in producing prokinetic effects on intestinal propulsion (Figure 1).

There are few definitive receptor localization studies of 5-HT_4Rs in the gut but their findings show multiple potential targets for 5-HT_4R agonists in achieving prokinetic effects (Figure 1). An early study used *in vitro* receptor autoradiography to identify 5-HT_4R in the myenteric plexus of human and guinea pig colon [96]. More recently, Poole et al. (2006) identified 5-HT_4 receptor immunoreactivity in subpopulations of enteric neurons in guinea-pig small intestine and rat and mouse colon, in particular on myenteric and submucosal intrinsic primary afferent neurons (IPANs) in guinea-pig small intestine [97]. In situ hybridization studies found a similar pattern of 5-HT_4R expression in mouse colon [77] and expression of 5-$HT_{4(a)}$ 5-$HT_{4(b)}$, 5-$HT_{4(e)}$ and 5-$HT_{4(f)}$ isoforms in mouse gut. The 5-$HT_{4(e)}$ and 5-$HT_{4(f)}$ isoforms were myenteric plexus specific. Ray et al. (2009) found immunoreactivity (IR) for rat 5-$HT_{4(a)}$, 5-$HT_{4(b)}$ and 5-$HT_{4(c1)}$ isoforms throughout the GIT with highest density in the colon [98]. They also found abundant IR for (a) and (b) on enteric neurons in both small and large intestine. Table 1 summarizes molecular studies investigating expression of different 5-HT_4R splice variants in the human GIT and elsewhere. It is clear there are multiple commonly expressed 5-HT_4R isoforms: (a), (b), (c), (g), (i) and (n) are found in the GIT and also in the heart and brain. This has important implications for readily absorbable agents whose actions are designed to target the gut (see below). Thus, the 5-HT_4R localisation studies are consistent with a mechanism whereby activation of presynaptic 5-HT_4Rs on myenteric neurons facilitates transmitter release and also with 5-HT_4R mediated depolarisation of enteric neurons that excites the circuitry. However, the findings of Hoffman et al. (2012) described above, convincingly highlight luminal 5-HT_4Rs on multiple mucosal cell types as other targets for 5-HT_4R agonists in promoting colonic motility (Figure 1).

5-HT_4R expression is also reported on smooth muscle in guinea-pigs, mice, rabbits, dogs and humans [46,77,97,99,100] (Figure 1). In human colon, activation of 5-HT_4Rs causes relaxation possibly due to smooth muscle receptors [101,102]. Activation of these receptors leads to increased cAMP, decreasing intracellular Ca^{2+} levels causing relaxation. In rabbit, this occurs via modulation of a plasma membrane Ca^{2+} ATP-ase pump that removes intracellular

calcium [99]. It is unclear how smooth muscle 5-HT_4Rs would enhance motility since it is difficult to reconcile widespread smooth muscle relaxation with increased propulsion of luminal content. But the *in vivo* effects of prucalopride on canine colon are region specific where smooth muscle relaxation occurs distally, but not proximally, and the stimulatory effect via neuronal 5-HT_4Rs proximally induces GMCs. In this scenario 5-HT_4R mediated smooth muscle relaxation distally might facilitate transit [46].

Future Directions

The variable affinities of 5-HT_4R agonists and antagonists at individual receptor isoforms and their differential expression in particular organs and tissues (Table 1) complicates pharmacological studies investigating functional roles of 5-HT_4Rs. This is a significant consideration for design of future pharmacological agents. 5-HT_4R agonists, including prucalopride, show preferential affinity for particular isoforms [53,54] (Table 1). This is ideal when designing experimental drugs targeting tissues predominantly expressing a particular splice variant, but whether agents are readily absorbed systemically becomes a key consideration when many 5-HT_4R isoforms are commonly expressed in other tissues. Prucalopride is rapidly absorbed and slowly metabolized in the liver leaving 5% in the faeces, while 60-65% of the original dose is eliminated unchanged in the urine. Thus, only a small proportion of any dose reaches the colonic mucosa [103]. This has important mechanistic implications for the prokinetic actions of 5-HT_4 agonists in humans: via synaptic transmission within the enteric neural circuitry by systemically absorbed drug and/or by luminal actions targeting the colonic mucosal epithelium. Although there is no evidence prucalopride causes serious adverse side effects at off target 5-HT_4Rs in humans, there are many *potential* sites of action outside the gut where the same receptor isoforms are located, including the CNS (for reviews see [104,105]) (Table 1). Indeed, a small proportion of patients receiving prucalopride suffer from headaches in the short-term following commencement of treatment [103]. Thus, development of 5-HT_4R agonists formulated to target and remain in the colonic mucosa, or more rapidly inactivated in the liver, is justified to provide a safer method of delivery.

Conclusions

Luminal 5-HT_4Rs are a probable and effective target for 5-HT_4R agonists in achieving prokinetic effects on GI motility as demonstrated by Shokrollahi et al. in the current issue.

Nonetheless prokinetic effects are probably achieved via activation of both luminal 5-HT_4Rs and 5-HT_4Rs within the myenteric circuitry with the relative contribution of each depending on the mode of administration and the pharmacokinetics of individual pharmacological agents.

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Author Contributions

RMG reviewed the literature and wrote the manuscript.

JCB provided advice and performed editorial revisions.

Both authors approved the final version.

Disclosures

The authors do not have any conflicts of interest to disclose.

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Figure 1. Schematic diagram showing the layers of the intestinal wall (colon) in cross-section demonstrating known locations of 5-HT_4Rs (yellow triangles) from studies performed in mice, rats, guinea-pigs and humans.

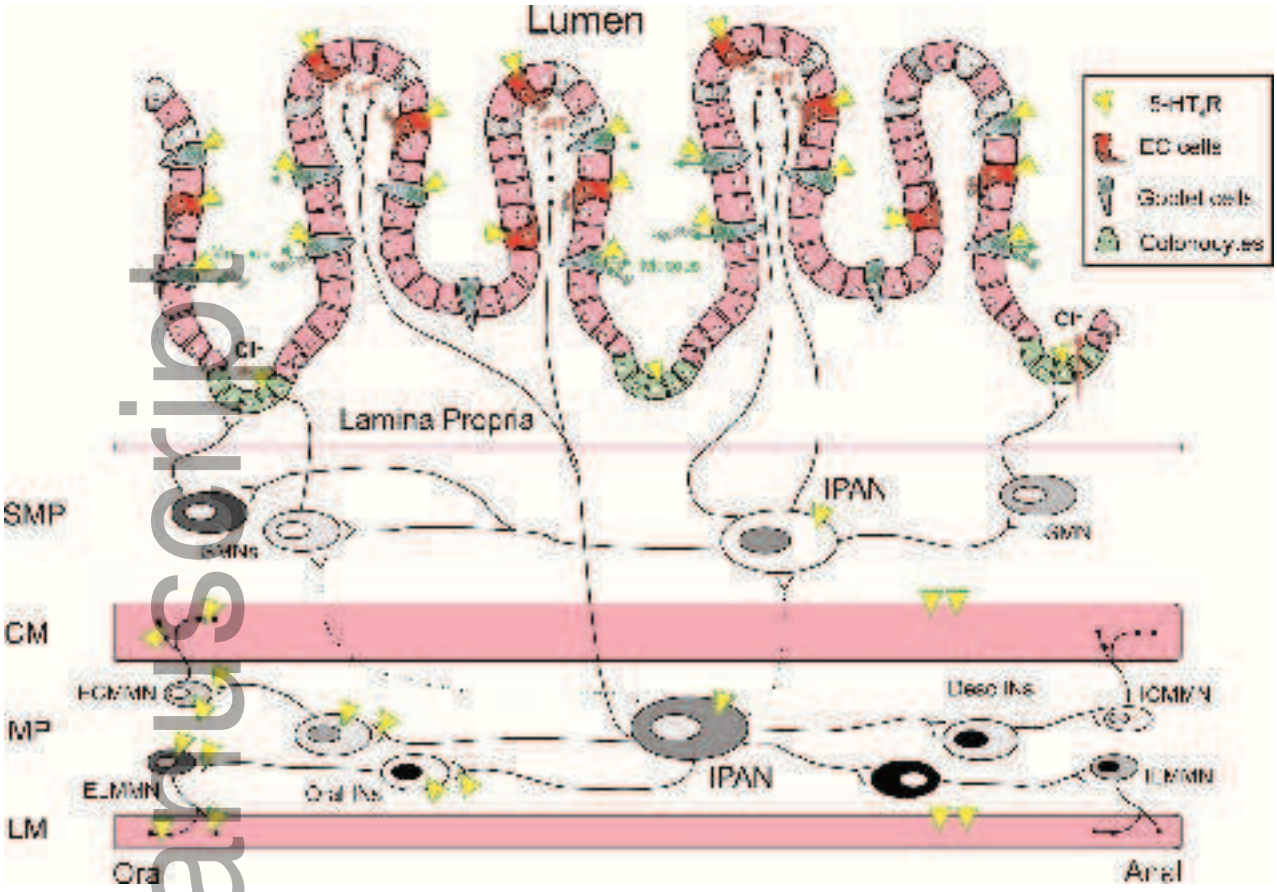
Luminal targets for prokinetic agents targeting 5-HT_4Rs include EC cells (red), colonic enterocytes (green) and goblet cells (pale blue)(see legend). Activation of 5-HT_4Rs on EC

cells leads to basal 5-HT release exciting mucosal terminals of myenteric and submucosal intrinsic primary afferent neurons which then activate peristaltic and secretory reflex pathways promoting motility. 5-HT_4R activation on enterocytes and goblet cells leads to enhanced luminal Cl^- and mucous secretion respectively, softening stool composition and facilitating transit. Activation of presynaptic 5-HT_4Rs on myenteric neurons facilitates neurotransmitter release in ascending excitatory pathways and at the neuromuscular junction enhancing peristaltic reflexes and propulsion. Direct activation of 5-HT_4Rs on enteric neurons causes neuron depolarisation and further excitation within the circuitry. Due to a lack of definitive data on locations of 5-HT_4Rs in descending pathways no labelling is shown.

SMP –submucosal plexus, **CM**-circular muscle layer, **MP**-myenteric plexus, **LM**-longitudinal muscle layer, **IPAN**-intrinsic primary afferent neuron, **IN**-interneuron, **SMN**-secretomotor neuron, **CMMN**-circular muscle motor neuron, **LMMN**-longitudinal muscle motor neuron, **E**-excitatory, **I**-inhibitory

Figure 2. Differences in anatomical structure of the colon in mice, rabbits and humans.

The structure of the longitudinal muscle coat in rabbit and human colon (middle and bottom panels) is different from that in mice (top panel) and other species. The rabbit has a 3-taeniated region in the proximal colon that resembles the structure of the 3-taeniated human colon. Haustral boundary contractions specific to this 3-taeniated region divide the colon into pockets or “haustra” and have also been described in humans. The mouse colon is un-taeniated and resembles the distal colon of the rabbit.



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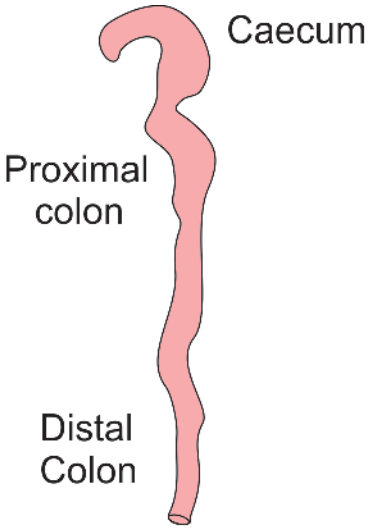

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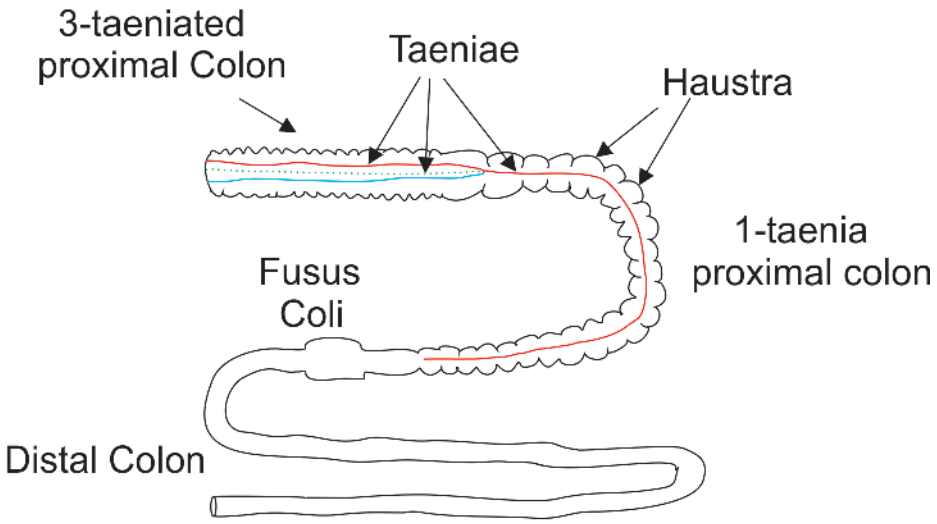

HUMAN