Critical role of 5-HT_{1A}, 5-HT_{3}, and 5-HT_{7} receptor subtypes in the initiation, generation, and propagation of the murine colonic migrating motor complex

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Submitted 9 December 2009; accepted in final form 20 April 2010

Dickson EJ, Heredia DJ, Smith TK. Critical role of 5-HT_{1A}, 5-HT_{3}, and 5-HT_{7} receptor subtypes in the initiation, generation, and propagation of the murine colonic migrating motor complex. Am J Physiol Gastrointest Liver Physiol 299: G144–G157, 2010. First published April 22, 2010; doi:10.1152/ajpgi.00496.2009.—The colonic migrating motor complex (CMMC) is necessary for fecal pellet propulsion in the murine colon. We have previously shown that 5-hydroxytryptamine (5-HT) released from enterochromaffin cells activates 5-HT_{3} receptors on the mucosal processes of myenteric Dogiel type II neurons to initiate the events underlying the CMMC. Our aims were to further investigate the roles of 5-HT_{1A}, 5-HT_{3}, and 5-HT_{7} receptor subtypes in generating and propagating the CMMC using intracellular microelectrodes or tension recordings from the circular muscle (CM) in preparations with and without the mucosa. Spontaneous CMMCs were recorded from the CM in isolated murine colons but not in preparations without the mucosa. In mucosaless preparations, ondansetron (3 μM; 5-HT_{3} antagonist) plus hexamethonium (100 μM) completely blocked spontaneous inhibitory junction potentials, depolarized the CM. Ondansetron blocked the preceding hyperpolarization associated with a CMMC. Spontaneous CMMCs and CMMCs evoked by spraying 5-HT (10 and 100 μM) or nerve stimulation in preparations without the mucosa were blocked by SB 258719 or SB 269970 (1–5 μM; 5-HT_{7} antagonists). Both NAN-190 and (S)-WAY100135 (1–5 μM; 5-HT_{1A} antagonists) blocked spontaneous CMMCs and neurally evoked CMMCs in preparations without the mucosa. Both NAN-190 and (S)-WAY100135 caused an atropine-sensitive depolarization of the CM. The precursor of 5-HT, 5-hydroxytryptophan (5-HTP) (10 μM), and 5-carboxamidotryptamine (5-CT) (5 μM; 5-HT_{3/5/7} agonist) increased the frequency of spontaneous CMMCs. 5-HTP and 5-CT also induced CMMCs in preparations with and without the mucosa, which were blocked by SB 258719. 5-HT_{1A}, 5-HT_{3}, and 5-HT_{7} receptors, most likely on Dogiel Type II/AH neurons, are important in initiating, generating, and propagating the CMMC. Tonic inhibition of the CM appears to be driven by ongoing activity in descending serotonergic interneurons; by activating 5-HT_{7} receptors on AH neurons these interneurons also contribute to the generation of the CMMC.

5-hydroxytryptamine; AH neurons; cholinergic transmission; circular muscle; enterochromaffin cells; mucosa; serotonergic transmission; smooth muscle

OVER 95% OF THE SEROTONIN IN THE BODY, also called 5-hydroxytryptamine (5-HT), resides in the gastrointestinal tract, where it plays an important role in regulating gut motility, secretion, sensation, and bone metabolism (6, 15, 16, 45, 46). Most of this 5-HT is in enterochromaffin (EC) cells in the mucosa although it is also present in a small population (~1%) of descending myenteric interneurons that project to the submucus, suggesting they may modulate secretomotor reflexes. Secretomotor reflexes and motility reflexes can be activated by 5-HT release from EC cells (5, 6, 16, 18, 37), which appear to stimulate the endings of Dogiel Type II neurons in the submucus and myenteric plexus via 5-HT_{1P} and 5-HT_{3} receptors, respectively (1, 2, 5, 22). A number of gut inflammatory and constipatory conditions involve altered levels of serotonin and signaling, in part brought about by altered numbers of EC cells and 5-HT-containing neurons (15, 16). There are numerous 5-HT receptors in the gut, and there is much interest in developing antagonists and agonists to these receptors, especially 5-HT_{3} and 5-HT_{4} receptors, to treat a variety of gastrointestinal conditions (10, 16, 19, 27, 31, 33).

The circular muscle (CM) of the large bowel is under constant tonic neural inhibition, which keeps the muscle in a hyperpolarized state (1, 4, 8, 26, 38). Tonic inhibition appears to be necessary for normal pellet transit and stool formation in the murine colon (8). Spontaneous inhibitory junction potentials (IJPs) in the CM contribute to tonic inhibition of the CM in the murine large bowel. These IJPs are blocked by atropine, a blocker of the small-conductance calcium-activated K^{+} channels, suggesting that they are generated by the release of purines from inhibitory motor neurons (8, 34).

Superimposed on the ongoing inhibition is an important neurally mediated, contractile motor pattern called the colonic migrating motor complex (CMMC) that slowly propagates along the large bowel (4, 8). Rather than a just a simple peristaltic reflex arc, the CMMC is likely generated and propagated by the activation of myenteric AH sensory neurons along the length of the colon (1). Activity in myenteric AH neurons appears to be initiated by the release of 5-HT from EC cells in the mucosa, which excite 5-HT_{3} receptors on the mucosal endings of these neurons, and by ascending and descending interneurons (1, 8, 18). Electrically, the CMMC consists of a brief hyperpolarization followed by fast oscillations with action potentials superimposed on a slow depolarization of the CM (4, 8). The preceding inhibition is generated by activation of descending inhibitory nervous pathways, whereas the fast oscillations and the slow depolarization were thought to be generated largely by the that release acetylcholine (ACh) and disinhibition, respectively (4, 26, 38). More recent studies suggest that both phases of the CMMC are largely generated by the release of ACh and tachykinins from excitatory motor neuron activation activated by ascending excitatory nervous pathways (8). Although the physiological role of the CMMC is unclear in large mammals (35), including humans (9), it drives fecal pellet propulsion in the murine colon (16).

In the present study, we have attempted to determine the possible role of 5-HT_{1A}, 5-HT_{3}, and 5-HT_{7} receptors in initiating, generating, or propagating the CMMC. These particular...
5-HT receptors were chosen because their effects on the membrane properties and synaptic events have been studied fairly extensively using intracellular recordings from myenteric neurons, at least in the guinea pig small intestine (13, 14, 22, 27, 29, 30).

Our findings suggest that 5-HT1A, 5-HT3, and 5-HT7 receptors have distinct roles and are all critically involved in the generation of the CMMC. Also, ongoing activity in descending serotonergic neurons is essential for tonic inhibition of the CM.

MATERIALS AND METHODS

Male C57BL/6 mice (28–42 days old) were humanely killed by inhalation of anesthetic (Isofluorane; Baxter, Deerfield, IL) and cervical dislocation, in accordance with the approval of the animal ethics committee of the University of Nevada School of Medicine, and the whole colon was removed (8, 18).

Preparation 1: mechanical activity of the CM during the CMMC. The isolated murine large bowel was attached to the floor of the organ bath by pinning the mesentery. Suture silk was used to connect two or three force transducers (model TST125C; Biopac Systems, Santa Barbara, CA) along the whole colon, while another transducer was attached to an epoxy-coated pelleted that was held in the center of the preparation, so as to regulate CMMCs (18). Resting tension was initially set at 8 mN and monitored using an MP100 interface and recorded on a PC running Acknowledge software 3.2.6 (BIOPAC Systems, Santa Barbara, CA).

Preparation 2: mechanical activity of the CM during the CMMC in sock preparations. Previously we have shown that, when the mucosa is removed from the colon, no spontaneous CMMCs are observed (1, 8, 18). However, we wanted to investigate whether preserving circumferential integrity of the myenteric plexus following removal of the mucosa still resulted in a lack of spontaneous CMMC activity. Firstly, the mesenteric attachment was dissected away from the entire length of colon. Secondly, the muscle layers and mucosa/submucosa were carefully separated from one another at the oral end, and the mucosa was pinned to the dissection dish. The muscle layers were then gently pulled back away from the mucosa, to avoid damaging the myenteric plexus, resulting in an intact tube consisting of both muscle layers and the myenteric plexus that we have referred to as a “sock” preparation.

Preparation 3: intracellular microelectrode recordings from preparations with and without the mucosa. In other experiments, a 20-mm incision was made along the mesenteric border in the middle of the colon and pinned circumferentially with the serosa uppermost. Impalements were made into CM cells from the opened segment of murine colon. Platinum transmural stimulating wires (diameter 0.2 mm), which were connected to a Grass Instruments SD stimulator (Quincy, MA), were placed above and below the preparation (1 mm apart and 2–4 mm oral of the recording site).

All dissections were performed in 4°C Krebs solution so as to minimize the uptake of 5-HT released from the mucosa into descending serotonergic interneurons (28).

Analysis of data and statistical methods. Microelectrode electrophysiology and tension recordings were analyzed using in-house written algorithms as described previously (18). The frequency, duration, and amplitude of contractile complexes was measured using AcqKnowledge 3.2.6 software (BIOPAC Systems), and tests for statistical significance were made using Sigma Plot 5.0 (Jandel Scientific, San Rafael, CA). Statistical comparisons of data were performed using Student’s (paired or unpaired) t-tests or ANOVA, and a minimum level of significance was reached at \( P < 0.05 \). In RESULTS, \( n \) refers to the number of animals from which colons were taken. All data are presented as means \( \pm SE \).

Drugs and solutions. 5-HT, NANC-190 hydrobromide (1-(2-Methoxyphenyl)-4-(4-phthalimidobutyl)piperazine hydrobromide), (S)-WAY 100135 dihydrochloride (5-HT1A antagonists), SB 269970 hydrochloride, and SB 258719 hydrochloride (5-HT7 antagonists) were purchased from Tocris Bioscience (Ellisville, MO); apamin, atropine, 5-carboxamidotryptamine (5-CT), hexamethonium, granisetron, ondansetron, and 5-hydroxytryptophan (5-HTP) were purchased from Sigma-Aldrich (St. Louis, MO). The Krebs solution was (in mM) 120.35 NaCl, 5.9 KCl, 15.5 NaHCO3, 1.2 NaH2PO4, 1.2 MgSO4, 2.5 CaCl2, and 11.5 glucose, gassed continuously with a mixture of 3% CO2-97% O2 (vol/vol) to give a final pH of 7.3–7.4.

RESULTS

Effects of blocking 5-HT3 receptors in preparations without the mucosa. It has been shown previously that 5-HT3 receptor antagonists block spontaneous CMMCs in intact preparations containing the mucosa (18), as does removal of the mucosa (1, 8, 18). Similarly we found that, when the mucosa was removed from the colon, no spontaneous CMMCs were observed when electrical recordings were made from the CM (Fig. 1A; \( n = 18 \)). However, we did observe regular spontaneous IJPs in the CM that were similar in frequency to those in preparations with the mucosa intact (without mucosa 40.32 \( \pm 1.9/min \); with mucosa 38.8 \( \pm 3.1/min \); \( n = 18 \); \( P > 0.05 \); Fig. 1A). The spontaneous IJPs were blocked by apamin (0.1 \( \mu M; n = 3 \)), suggesting that they resulted from the opening of small-conductance calcium-activated potassium (SK) channels caused by the release of purines, as described previously (34). Furthermore, there was no difference in the resting membrane potential of the CM in preparations with or without the mucosa (without mucosa 56.4 \( \pm 1.1 \) mV; with mucosa 57.3 \( \pm 2.1 \) mV; \( n = 18 \); \( P > 0.05 \)) or in the number of spontaneous IJPs. This suggests that the removal of the mucosa has no effect on tonic inhibitory drive to the CM, suggesting that the nerve circuits mediating this activity do not require the mucosa (1, 8).

In the colon there are a number of different classes of ascending and descending interneurons; most interneurons contain acetylcholine (ACh) along with other potential neurotransmitters (25). We tested the hypothesis that tonic inhibition of the CM is driven by ongoing activity in descending serotonergic interneurons, which contain 5-HT and ACh that synapse with inhibitory motor neurons. Hexamethonium (100 \( \mu M \)), a nicotinic antagonist, significantly reduced the frequency of spontaneous IJPs (Fig. 1A). The further addition of the 5-HT3 antagonists ondansetron (3 \( \mu M; n = 5 \)) or granisetron (3 \( \mu M; n = 5 \), data not shown) completely blocked the remaining spontaneous IJPs (Fig. 1A and C). There was a depolarization associated with the addition of each drug (depolarization: hexamethonium 8.6 \( \pm 0.7 \) mV; ondanseretron 6.2 \( \pm 2.3\); \( n = 6 \)). The effect of these drugs on spontaneous IJPs and membrane potential was independent of the order in which they were applied (Fig. 1, B and C; \( n = 5 \)). This result strongly suggests that ongoing activity in descending serotonergic interneurons, which also contain ACh (25), drive inhibitory motor neurons to the CM.

We have previously shown that, when electrical recordings were made close to the site of nerve stimulation, electrical-field stimulation (EFS) evoked a CMMC in preparations devoid of the mucosa (8, 18). It is possible that long interneuronal nerve pathways may also use 5-HT (21). To determine whether this was the case, we evoked CMMCs some distance from the recording electrode (30 mm orally or anally) in preparations devoid of mucosa using EFS (Fig. 2). Ondansetron (3 \( \mu M \)) did
not affect the duration of the evoked CMMCs (duration: control 37.8 ± 2.8 mV; ondansetron 41.4 ± 3.6 mV; n = 5; P > 0.01) although it did significantly reduce the preceding inhibition (see Fig. 2). This experiment proves two crucial points: 1) the neural pathways are still intact and viable following the removal of the mucosa because a CMMC can be readily evoked at a long distance from the recording site and 2) descending serotonergic interneurons must be responsible for the preceding inhibitory component of the CMMC.

**Effect of 5-HT and EFS on intact colonic tubes devoid of mucosa.** We wanted to determine whether the absence of spontaneous CMMCs in preparations without the mucosa (1, 9, 18) was attributable to 1) dissection damage (21) or 2) descending serotonergic interneurons must be responsible for the preceding inhibitory component of the CMMC.

In sock preparations, a single spritz (~1 s) of 5-HT (10 or 100 μM) onto the preparation evoked a prolonged burst of CMMCs that lasted for ~20 min (Fig. 2C). These 5-HT-evoked CMMCs occurred at a frequency, amplitude, area, and duration of 16.8 ± 1.1 mN, 295.1 ± 24.3 mN, and 51.8 ± 2.7 s, respectively (n = 8). The CMMCs evoked by EFS or 5-HT (see below) were blocked by hexamethonium (100 μM; n = 3), suggesting that ascending excitatory nervous pathways that are responsible for activating excitatory motor neurons to the muscle (see Fig. 3D).

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duration that were not significantly different to spontaneous CMMCs in preparations with the mucosa intact (see Table 1). After washing out the 5-HT for 20 min, a second spritz of 5-HT gave a diminished response (Fig. 2C), suggesting that there was desensitization to 5-HT. Following the addition of ondansetron (3 μM), 5-HT still generated CMMCs with a frequency (0.26 ± 0.1 cycles/min), amplitude (13.0 ± 2.7 mV), area (294.8 ± 70.0 mV), and duration (47.8 ± 9.0 s) that was not significantly different from control (Fig. 3C; P > 0.05; n = 8).

Effect of blocking 5-HT1A receptors on the CMMC. Electrical stimulation of descending nerve pathways in the guinea pig ileum produces slow excitatory postsynaptic potentials (sEPSP) in AH neurons that are likely mediated by activation of 5-HT7 receptors (29). Because we have postulated that activation of myenteric Dogiel Type II/AH neurons are necessary for triggering the CMMC (1, 18), we wanted to determine whether activation of 5-HT7 receptors are involved in generating the CMMC.

SB 269970 (3–10 μM; n = 6) and SB 258719 (1–10 μM; n = 6), which are 5-HT7 receptor antagonists (12, 17, 24, 29), completely blocked spontaneous CMMCs in preparations with the mucosa (Fig. 4, A and B). SB 258791 (2 μM; 5-HT7 receptor antagonist; n = 6) also blocked both the 5-HT evoked CMMCs in preparations with and without the mucosa (Fig. 4B; ED50 for SB 269970 = 0.99 μM; ED50 for SB 258791 = 0.4 μM). In electrophysiological studies, both SB 269970 (5 μM; n = 6) and SB 258719 (0.4 μM; n = 6) blocked spontaneous CMMCs in the CM in preparations with the mucosa intact (Fig. 5, A and B).

Neither of these drugs affected the resting membrane potential (control 55.9 ± 3.1 mV; SB 269970 56.2 ± 3.7 mV; SB 258719 54.0 ± 4.2 mV; n = 7; P > 0.05) or the ongoing spontaneous IJPs (control 35.4 ± 2.3/min; SB 269970 35.2 ± 4.7/min; SB 258719 34.6 ± 3.8/min; n = 7; P > 0.05) in the CM (Fig. 5, A and B).

SB 269970 and SB 258719 also blocked CMMCs evoked by EFS in preparations without the mucosa and submucous plexus, without affecting the preceding hyperpolarization (Fig. 5C; n = 6); therefore, a primary site of action of these drugs is within the myenteric plexus.

Interestingly, SB 258719 blocked not only the spontaneously occurring CMMCs but also those evoked by spritzing 5-HT (10–100 μM) onto preparations both with (n = 4) and without (n = 4) the mucosa present (Fig. 6B).

Effect of blocking 5-HT1A receptors on the CMMC. Activation of 5-HT1A receptors hyperpolarizes myenteric AH neurons and causes presynaptic inhibition of fast and slow transmitter release from AH neurons to S neurons (11–14, 30, 31).

In tube preparations containing a fixed artificial pellet, where tension transducers monitored activity, application of either of the 5-HT1A receptor antagonists NAN-190 (5 μM; n = 10) or (S)-WAY 100135 (2 μM; n = 5) completely abolished spontaneous CMMCs (Fig. 6, A and B).

In other preparations where we used microelectrodes to record activity from the CM, we found that NAN-190 or (S)-WAY 100135 not only blocked CMMCs but also depolarized the CM and induced action potential firing in the CM [depolarization by NAN-190: 11.2 ± 1.0 mV; n = 10; depolarization by (S)-WAY 100135: 13.2 ± 1.3 mV; Fig. 7, A and C]. When NAN-190 (5 μM; n = 6) was added in the presence of atropine (1 μM), the depolarization was significantly reduced (depolarization by NAN-190: 11.2 ± 1.0 mV; depolarization by NAN-190 following atropine: 6.4 ± 0.7 mV; P < 0.05) and the spontaneous CMMCs continued, albeit at a reduced rate (Fig. 7B). Also, when NAN-190 was added in the presence of hexamethonium (100 μM; n = 3), which blocked CMMCs, it had no effect (data not shown).

In addition, in preparations without the mucosa, both NAN-190 (1 μM; n = 5) and (S)-WAY 100135 (1 μM; n = 3) blocked both the fast oscillations and slow-depolarization phases of the CMMC evoked by EFS but not the preceding hyperpolarization (Fig. 7D).

Caution is necessary because SB 269970 and NAN-190 may not be as specific as one thought since they have been shown to also block α2-receptors that are activated on submucosal neurons by norepinephrine (12). However, in our experiments, yohimbine (100 nM; n = 4) (37), which had no significant effect on CMMCs, did not appear to modify the effects of the 5-HT7 or the 5-HT1A antagonists (see Figs. 4B and 6B).
Effect of 5-HTP. The rate-limiting enzyme in the biosynthesis of 5-HT is tryptophan hydroxylase (TPH), which is bypassed by 5-HTP, the endogenous precursor of 5-HT (15). In the guinea pig colon, exogenous application of 5-HTP increases the luminal outflow of 5-HT (23). A possible source of this luminal 5-HT is likely to be overflow of 5-HT from EC cells because it occurs following neural blockade with TTX. Therefore, we tested the application of 5-HTP on spontaneous CMMCs in the murine colon and found that 5-HTP (10 μM) significantly increased the frequency of spontaneous CMMCs.

![Fig. 3. Tension recordings of activity in an intact, inverted colon devoid of the mucosa. A: spontaneous CMMCs in a colonic tube preparation with the mucosa intact. B: when the mucosa was removed from the whole colon, while maintaining its circumferential integrity (“sock preparation”), no spontaneous CMMCs were observed. However, EFS (duration 0.5 ms, 20 Hz for 1 s, 40 V) evoked a robust CMMC response at the 2 recording sites. C: a brief spritz of 5-HT (100 μM) onto the external surface of the circular muscle initiated CMMCs. 5-HT was found to initiate propagating CMMCs for a period of ~20 min, after a 20-min period of quiescence, and a second application of 5-HT once again generated CMMCs, albeit at a reduced frequency. D: 5-HT and EFS can both initiate CMMCs even in the presence of ondansetron (3 μM), whereas the addition of hexamethonium (100 μM) completely blocked all responses.](image-url)
in intact preparations (spontaneous: 0.37 ± 0.02 cycles/min; 5-HTP: 0.6 ± 0.01 cycles/min; \( P < 0.01; n = 5 \)). In three other preparations, 5-HTP (10 μM) also generated spontaneous CMMCs in intact preparations without spontaneous CMMCs. In these preparations with the mucosa, 5-HTP produced a maximal effect within 5.3 ± 1.0 min (\( n = 5 \)).

In preparations without the mucosa (sock preparation) where no spontaneous CMMCs were observed, the addition of 5-HTP initiated CMMCs after a period of \( \sim 20.2 ± 2.2 \) min (frequency 0.23 ± 0.03 cycles/min; \( n = 4 \); Fig. 8B). The addition of SB 258719 (2 μM) completely blocked all 5-HTP-induced CMMCs (Fig. 8B; \( n = 3 \)).

**Effect of 5-CT.** 5-CT (5 μM), which is a 5-HT1/5/7 receptor agonist (14, 29, 33), increased the frequency of spontaneous CMMCs (frequency: control 0.27 ± 0.05; 5-CT 0.49 ± 0.07 cycles/min; \( n = 6; P < 0.05 \)) but had no significant affect on the resting membrane potential (Fig. 9A). On occasion, 5-CT also increased the frequency of both IJPs (Fig. 9B; \( n = 2 \)) and excitatory junction potentials (EJPs) (Fig. 9A; \( n = 2 \)). Overall the effect of 5-CT on the frequency of spontaneous IJPs was not significant (IJPs: control 39.7 ± 2.2/min; 5-CT 44.6 ± 4.9 cycles/min).

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*All values are presented as means ± SE. 5-HT, 5-hydroxytryptamine; CMMC, colonic migrating motor complex.*

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**Table 1. Data for CMMCs with intact and removed mucosa**

**Fig. 4.** Effect of 5-HT7 receptor antagonists on spontaneous CMMCs. A: when tension recordings were made from tube preparations with the mucosa intact, the addition of SB 269970 in increasing doses abolished CMMCs. B: in another preparation with the mucosa intact, spontaneous CMMCs were abolished by increasing doses of SB 258719. C: a brief spritz of 5-HT (10 μM; see square) onto the preparation evoked a series of CMMCs. Application of SB 258719 (2 μM) not only abolished the spontaneous complexes but also the responses to 5-HT (10 μM). Yohimbine (100 nM), an α-2 antagonist, was present throughout the experiments.
cycles/min; \( n = 6; P > 0.05 \). However, 5-CT did increase spontaneous IJP amplitude (control 8.7 ± 0.6 mV; 5-CT 13.5 ± 0.8 mV; \( n = 6; P < 0.05 \)). 5-CT was also observed to induce CMMCs in two preparations with the mucosa intact that lacked spontaneous CMMCs (Fig. 9B).

In sock preparations devoid of mucosa, which exhibited no spontaneous CMMCs, 5-CT (5 \( \mu \)M) induced CMMCs that were similar to those evoked by 5-HT in similar preparations (\( n = 4; \) Fig. 9C).

**DISCUSSION**

All three 5-HT receptor subtypes (5-HT\(_{1A}\), 5-HT\(_{3}\), and 5-HT\(_{7}\)) we investigated appear to have important and distinct roles in initiating, generating, and propagating the spontaneous CMMC. Although antagonizing each one of these receptors alone can block the CMMC, their mechanisms of action are quite different. Despite the fact that a number of functional classes of enteric neurons appear to be contacted by serotonergic interneurons (30), the critical role of each of these receptors can be explained if we use Occam’s Razor and assume that the three receptors are mainly on the soma and processes of myenteric AH/Dogiel Type II sensory neurons in the murine colon (Fig. 10), as they appear to be in the guinea pig ileum (29–31). Blocking any one of these receptors inhibits the CMMC; therefore they appear to be in series on the AH neuron: 5-HT\(_{3}\) receptors on the processes in the mucosa, 5-HT\(_{7}\) receptors on the soma, and 5-HT\(_{1A}\) receptors on the output processes (1, 2, 14, 18, 29, 42). Physiological and immunohistochemical evidence demonstrates a structural and functional connection between 5-HT-containing interneurons and AH/Dogiel type II (calbindin positive) neurons in the guinea pig small intestine (30, 47). Our recent evidence suggests that myenteric Dogiel Type II neurons are not only likely to be the sensory neurons or first responders that lead to the generation of the CMMC but also cause neural activity to propagate slowly along the colon because AH neurons lying along the bowel are not only activated by local stimuli but also by interneurons (1). In addition, both nicotinic and 5-HT\(_{3}\) receptors appear to be on inhibitory motor neurons that are driven by activity in descending serotonergic interneurons that appear to contribute to tonic inhibition of the CM.
Role of 5-HT3 receptors in generating tonic inhibition and the initiation of the CMMC. In this study we show that spontaneous IJPs occur in preparations with or without the mucosa. We found that a combination of both hexamethonium (nicotinic antagonist) and ondansetron (5-HT3 antagonist) blocked the spontaneous IJPs and depolarized the CM, suggesting that inhibitory motor neurons are activated by descending interneurons that release 5-HT and ACh. Both 5-HT and ACh contribute to the fast excitatory postsynaptic potential (fEPSP) in some S neurons, which comprise interneurons and motor neurons, by activating 5-HT3 and nicotinic receptors, respectively (13, 14). Despite their low numbers, myenteric descending serotonergic interneurons, which must have intrinsic activity, appear necessary for exciting inhibitory motor neurons via both 5-HT3 and nicotinic receptors to produce ongoing tonic inhibition of the CM and the hyperpolarization that precedes the CMMC (see Fig. 10). However, parallel cholinergic and serotonergic descending interneurons cannot be ruled out. Presumably, it is the coordinated activation of these descending interneurons that initiates the preceding hyperpolarization associated with a CMMC (12, 18, 39; Fig. 10). That descending interneurons are involved in inhibitory nerve pathways is suggested by experiments where recordings were made at the extreme ends of the colon. Between CMMCs, ongoing EJPs and IJPs are recorded at the oral and anal ends of the colon, respectively, suggesting that long-descending inhibitory nerve pathways have been severed at the oral end and ascending excitatory nerve pathways have been severed at the anal end (39).

In contrast to Keating and Spencer (21), we found that blocking 5-HT3 receptors affected neither the neurally evoked CMMC nor CMMCs initiated by 5-HT in preparations without the mucosa; however, these responses were blocked by a 5-HT7 antagonist (see below).

Previously it has been shown that ondansetron and alosetron block spontaneous CMMCs generated in isolated flaccid tubes of murine colon (3, 10). We have recently shown that, in addition to ondansetron, spontaneous CMMCs are also abolished by removing the mucosa (1, 8, 18). This strongly suggests that spontaneous CMMCs are initiated by the release of 5-HT from EC cells in the mucosa that stimulate 5-HT3 receptors on the mucosal endings of Dogiel Type II neurons (1, 2), bringing them to threshold for the initiation of the CMMC (Fig. 7; 1). Recently, we have shown that myenteric Dogiel Type II neurons are the first responders underlying the CMMC when activated by brushing the mucosa over the recording site, and their prolonged responses to local stimulation are almost blocked by ondansetron (1).

Role of 5-HT7 receptors in generating the CMMC. In the guinea pig ileum, fast and slow depolarizing responses are
activated in some AH neurons by spraying 5-HT onto their cell bodies (27, 29, 31, 44). The fast response is mediated by activation of 5-HT\textsubscript{3} receptors, whereas the slow response is mediated by activation of 5-HT\textsubscript{1P} receptor (27) and 5-HT\textsubscript{7} receptors (29). 5-HT\textsubscript{7} receptors are expressed on the soma of AH neurons (42). Significantly, SB 269970, 5-HT\textsubscript{7} receptor antagonist, appears to reduce or abolish the slow depolarizing response to 5-HT and the sEPSP in AH neurons evoked by stimulating descending neural pathways (containing serotonergic interneurons) but has no effect on the sEPSPs evoked in AH neurons evoked by stimulation of circumferential nerve pathways that release tachykinins (29, 31). Furthermore, SB 269970 did not affect sEPSPs in S neurons evoked by stimulating descending nerve pathways, suggesting that they were mediated by other neurotransmitters released from other descending interneurons. These results strongly suggest that descending serotonergic interneurons release 5-HT to generate sEPSPs in AH neurons that are mediated by activation of 5-HT\textsubscript{7} receptors.

We found that two 5-HT\textsubscript{7} receptor antagonists (SB 269970 and SB 258719) completely blocked both the spontaneous CMMCs in preparations with the mucosa. Importantly, these antagonists also blocked the CMMCs evoked by nerve stimulation and exogenous 5-HT in preparations without the mucosa, suggesting that it was likely blocking sEPSPs on AH neurons rather than receptors on their mucosal endings. However, neither SB 269970 nor SB 258719 affected the resting membrane potential or the ongoing IJPs in the CM. Our results are consistent with the hypothesis that descending serotonergic interneurons are not only synapsing with inhibitory motor neurons but are also producing a 5-HT\textsubscript{7} receptor-mediated sEPSP in more distal AH neurons (Fig. 10). This sEPSP appears to be essential for CMMC generation. Unlike in the guinea pig ileum, we found no evidence for functional 5-HT\textsubscript{7} receptors.

Fig. 7. Effect of a 5-HT\textsubscript{1A} antagonists on CMMCs. A: intracellular recordings were made from the CM in the middle of an opened segment of colon. Spontaneous CMMCs were observed in this preparation, which had the mucosa intact. Ongoing IJPs were observed between and during spontaneous CMMCs. NAN-190 (5 \mu M; 5-HT\textsubscript{1A} antagonist) depolarized the CM and appeared to abolish the CMMCs although during the depolarization suprathreshold EJPs that gave rise to action potentials were observed. Following the washout of NAN-190 spontaneous CMMCs returned. B: spontaneous CMMCs were observed following the addition of atropine (1 \mu M). However, in atropine, NAN-190 (5 \mu M) produced a lesser depolarization of the CM that was associated with a reduction in the frequency of CMMCs. C: similarly, (S)-WAY 100135 (1 \mu M) abolished CMMCs and depolarized the CM. D: both these antagonists abolished CMMCs evoked by EFS (duration 0.5 ms, 20 Hz for 1 s, 40 V) in preparations without the mucosa, which had no spontaneous CMMCs. Both NAN-190 and (S)-WAY 100135 depolarized the CM by \sim\text{-}8 \text{mV}. Note that these drugs did not effect the preceding hyperpolarization.
receptors on the muscle or on inhibitory motor neurons (42) because the 5-HT$_7$ receptor antagonists did not affect the spontaneous IJPs, the resting membrane potential of the CM, or the hyperpolarization preceding the CMMC.

**Role of 5-HT$_{1A}$ receptors in generating the CMMC.** Both 5-HT$_{1A}$ receptor antagonists NAN-190 and (S)-WAY 100135 also blocked the CMMC. The role of 5-HT$_{1A}$ receptors is more difficult to explain because when these receptors are blocked rhythmic CMMC activity is impaired. Activation of these receptors on the cell body hyperpolarizes myenteric AH neurons, whereas activation of these receptors on the output processes of AH neurons presynaptically inhibits fast and slow neurotransmitter release from AH neurons onto S neurons (14, 29–31). The hyperpolarization in AH neurons that can be observed by 5-HT or 5-CT (5-HT$_{1,5,7}$ agonist) is blocked by NAN-190 (5-HT$_{1A}$ antagonist), as are synaptically evoked hyperpolarizations (12, 14, 30).

In electrophysiological experiments we found that NAN-190 and (S)-WAY 100135 depolarized the muscle by releasing excitatory neurotransmitters from excitatory motor neurons. The 5-HT$_{1A}$ receptors blocked by this drug to cause depolarization of the muscle are unlikely to be acting directly on excitatory motor neurons or ascending interneurons because NAN-190 had no affect after hexamethonium, which blocks the ascending excitatory nerve pathway that underlies the generation of the CMMC (Fig. 10; 1, 8, 18). Therefore, the sequence of events leading to this depolarization are likely to be 1) an increase in neurotransmitter release from the terminals of AH neurons, 2) a prolonged activation of ascending interneurons, and 3) activation of excitatory motor neurons. The release of ACh from excitatory motor neurons likely causes a large conductance change in the muscle that reduces the CMMC because after atropine the reduced depolarization, which was probably mediated by tachykinins (8), did not block the CMMC but reduced its frequency. Therefore, 5-HT$_{1A}$ receptors on the output terminals of Dogiel Type II/AH neurons in the myenteric plexus likely play an important role in limiting neurotransmitter output from AH neurons to ascending S type interneurons. Without this negative feedback, rhythmicity is unlikely to develop the CMMC motor pattern (31). 5-HT$_{1A}$ receptors on the soma of AH neurons may also be important contributors to the CMMC rhythm because activation of these receptors may reduce the size and duration of the sEPSP, also limiting the output of sensory AH neurons.

**Effect of 5-CT.** 5-CT, which is a 5-HT$_{1,5,7}$ receptor agonist, inhibits fEPSPs and sEPSPs evoked in S neurons, and this reduction in their amplitude is blocked by NAN-190 (13, 29). 5-CT also produces a slow-depolarizing response in AH neurons that is inhibited by SB 269970 (29). Therefore 5-CT is a mixed agonist, acting on both 5-HT$_{1A}$ and a 5-HT$_7$ receptors within the myenteric plexus.
We found that 5-CT increased the frequency of spontaneous CMMCs and induced CMMCs in preparations with the mucosa that were quiescent, without affecting the resting membrane potential. Importantly, 5-CT also produced robust CMMCs in sock preparations without the mucosa that were similar to those induced by 5-HT. This suggests that a dominant affect of 5-CT is its ability to depolarize AH neurons through activation of 5-HT7 receptors.

Are preparations without the mucosa damaged? To date, we have removed the mucosa from over 80 preparations and have never observed a spontaneous CMMC, suggesting to us that 5-HT released from EC cells must be important for generating the CMMC (1, 8, 18). In support of this conclusion, we found that 5-HTP increased the amplitude and frequency of CMMCs after a short exposure, suggesting that much of the effects of this drug were through increased release of 5-HT from EC cells. This conclusion is based on the fact that 5-HTP has been shown to increase luminal 5-HT in the guinea-pig colon by a TTX-insensitive mechanism that is independent of nerves (21). This suggests that in intact colons the effects of the increased release of mucosal 5-HT was likely stimulating the endings of AH neurons. It has been shown that the murine colonic mucosa releases large amounts of 5-HT and that increased 5-HT release from the mucosa occurs in phase with the CMMC (21).

Keating and Spencer (21) have recently proposed an alternative hypothesis to ours with regard to the generation of the CMMC. They came to the conclusion that 5-HT release from the mucosa was modulatory rather than essential for the initiation of CMMCs, as we proposed (1, 8, 18). They recorded CMMCs in preparations both with and without the mucosa although the frequencies of CMMCs were reduced in preparations without the mucosa. They also reported that ondansetron blocked CMMCs in both preparations, suggesting that descending serotonergic interneurons were essential for the propagation and generation of the CMMC. They explained the differences between the two studies as due to damage by our dissection technique. However, this sounds unlikely because in our present study we also found that when we removed the mucosa no spontaneous CMMCs occurred, either in colonic preparations used for electrophysiology or in whole sock-preparation colons from which the mucosa was removed. These preparations were viable and undamaged because CMMCs were readily evoked in these preparations by transmural nerve
induced CMMCs are blocked by a 5-HT7 antagonist (Fig. 10). CMMC, a conclusion based on the fact that these 5-HTP-to-trigger Dogiel type II neurons and initiate and propagate a subsequent release of 5-HT from these interneurons is enough synthesized in descending serotonergic interneurons, and the preparations devoid of the mucosa (albeit at a lower frequency). In support of this conclusion, we showed that 5-HTP can induce CMMCs in preparations where descending interneurons maybe excessive amounts of circumferential stretch in the generation of the CMMC in our preparations without the mucosa or for the spread of the CMMC along the enteric circuitry. However, these receptors are required for the preceding inhibition, which determines the direction of the apparent propagation of the CMMC (39). We have argued that Keating and Spencer (21) are unlikely to be studying the normal physiology or pharmacology of the CMMC because they are not recording normal “spontaneous” CMMCs, but CMMCs generated by excessive amounts of circumferential stretch in preparations where descending interneurons maybe excessive amounts of circumferential stretch in preparations where descending interneurons maybe excessive overloads with 5-HT (36). In support of this conclusion, we showed that 5-HTP can induce CMMCs in preparations devoid of the mucosa (albeit at a lower frequency). Presumably, 5-HTP causes an increase in the amount of 5-HT synthesized in descending serotonergic interneurons, and the subsequent release of 5-HT from these interneurons is enough to trigger Dogiel type II neurons and initiate and propagate a CMMC, a conclusion based on the fact that these 5-HTP-induced CMMCs are blocked by a 5-HT7 antagonist (Fig. 10). Previously, we have also shown that circumferential stretch alone can generate a CMMC that is independent of 5-HT release from the mucosa (18).

Limitations and future directions. Interpretations of our results rest on the specificity of the 5-HT7 and 5-HT1A receptor antagonists used in this study. Caution is necessary because both SB 269970 and NAN-190 may not be entirely specific because they have been shown to also block α2-receptors that are activated on submucosal neurons by norepinephrine (12); however, in our experiments, yohimbine had no significant affect on CMMCs or the effects of these antagonists on the CMMC.

In the future, to determine the importance of each of these receptors, it will be of interest to study the effects on colonic CMMCs in mice where each of the 5-HT3, 5-HT1A, and 5-HT7 receptors have been knocked out.

The rate-limiting enzyme in the biosynthesis of 5-HT is TPH (15, 48). There are two TPH isoforms, TPH1 and TPH2 (7, 15, 43). TPH1 is peripheral and critical for 5-HT biosynthesis in EC cells, whereas TPH2 is critical for 5-HT biosynthesis in neurons (7, 32, 43). When TPH1 is ablated in transgenic mice, brain 5-HT is unaffected, but peripheral 5-HT is eliminated, except for a residual store of 5-HT in the gut, which is compatible with enteric nervous system TPH2-dependent 5-HT biosynthesis (15). Furthermore, despite the apparent importance of serotonin released from EC cells in initiating the CMMC (1), it appears that knocking out TPH1, which is the rate-limiting enzyme necessary for synthesizing 5-HT in EC cells (7, 15, 43), or overexpressing Lrp5 (46), which inhibits TPH1 expression, may not have noticeable affects on gut motility. It is possible that under these circumstances genetic plasticity may occur, allowing other substances released from stimulation and by bath application of 5-CT or by spritzing 5-HT; the circuits mediating tonic inhibition of the CM are intact because rhythmically firing inhibitory motor neurons are observed (1), and Dogiel type II neurons are still active in preparations without the mucosa although they don’t appear to reach threshold for generating the CMMC (1). Unlike Keating and Spencer (21), we found that ondansetron had no detectable effect on the CMMC evoked at some distance from the recording site in preparations without the mucosa; however, ondansetron did block the preceding inhibition. This suggests that 5-HT3 receptors were not required for the generation of the CMMC in our preparations without the mucosa or for the spread of the CMMC along the enteric circuitry. However, these receptors are required for the preceding inhibition, which determines the direction of the apparent propagation of the CMMC (39). We have argued that Keating and Spencer (21) are unlikely to be studying the normal physiology or pharmacology of the CMMC because they are not recording normal “spontaneous” CMMCs, but CMMCs generated by excessive amounts of circumferential stretch in preparations where descending interneurons maybe excessive overloads with 5-HT (36). In support of this conclusion, we showed that 5-HTP can induce CMMCs in preparations devoid of the mucosa (albeit at a lower frequency). Presumably, 5-HTP causes an increase in the amount of 5-HT synthesized in descending serotonergic interneurons, and the subsequent release of 5-HT from these interneurons is enough to trigger Dogiel type II neurons and initiate and propagate a CMMC, a conclusion based on the fact that these 5-HTP-induced CMMCs are blocked by a 5-HT7 antagonist (Fig. 10). Previously, we have also shown that circumferential stretch alone can generate a CMMC that is independent of 5-HT release from the mucosa (18).

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EC cells to become dominant and compensate for a lack of mucosal 5-HT, or serotonergic interneurons, which express TPH2 (15), may become more active and drive AH neurons without input from the mucosa. Therefore, studying motility in TPH1 knockout mice could also give some important clues regarding the relative importance of 5-HT synthesized in EC cells by TPH1 compared with that synthesized in enteric neurons by TPH2.

Interestingly, when investigating the role of enteric neural of 5-HT in inbred mice it is important to select the appropriate strain. Recently it has been shown that inbred mice strains can exhibit functional polymorphisms in TPH2 that can lead to both anatomic and functional differences between different strains (32).

Circuitry underlying the CMMC. The initiation, generation, and propagation of the CMMC requires the coordinated activation of multiple 5-HT receptors. A better understanding of these receptors in generating such a primary motor event in the colon is important clinically because pharmacological manipulation of these receptors could alter colonic motility. Although these receptors may have more complex locations and functions in the nerve pathways than can be deduced from our studies, a simplified model is shown in Fig. 10.

Importantly, our results suggest that ongoing activity in descending serotonergic interneurons contributes to ongoing tonic inhibition of the muscle by activation of 5-HT3 and nicotinic receptors on inhibitory motor neurons. Tonic inhibition maintains the resting membrane potential in a hyperpolarized state, which is important for normal stool formation and propulsion (7).

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