

## THE TERMINALS OF MYENTERIC INTRINSIC PRIMARY AFFERENT NEURONS OF THE GUINEA-PIG ILEUM ARE EXCITED BY 5-HYDROXYTRYPTAMINE ACTING AT 5-HYDROXYTRYPTAMINE-3 RECEPTORS

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**Abstract**—The aim of this study was to identify the receptor type(s) by which 5-hydroxytryptamine applied to the intestinal mucosa excites the terminals of myenteric AH neurons. The AH neurons have been identified as the intrinsic primary afferent (sensory) neurons in guinea-pig small intestine and 5-hydroxytryptamine has been identified as a possible intermediate in the sensory transduction process. Intracellular recordings were taken from AH neurons located within 1 mm of intact mucosa to which 5-hydroxytryptamine was applied. Trains of action potentials and/or slow depolarizing responses were recorded in AH neurons in response to mucosal application of 5-hydroxytryptamine (10 or 20  $\mu$ M) or the 5-hydroxytryptamine-3 receptor agonist, 2-methyl-5-hydroxytryptamine (1 or 3 mM), and to electrical stimulation of the mucosa. The 5-hydroxytryptamine-2 receptor agonist,  $\alpha$ -methyl-5-hydroxytryptamine (100  $\mu$ M), and the 5-hydroxytryptamine-1,2,4 receptor agonist, 5-methoxytryptamine (10  $\mu$ M), did not elicit such responses. The 5-hydroxytryptamine-3 receptor-selective antagonist, granisetron (typically 1  $\mu$ M), and the 5-hydroxytryptamine-3,4 receptor antagonist, tropisetron (typically 1  $\mu$ M), each reduced or abolished the responses to 5-hydroxytryptamine, while the selective 5-hydroxytryptamine-4 receptor antagonist, SB 204070 (1  $\mu$ M), did not.

It is concluded that application of 5-hydroxytryptamine to the mucosa activates a 5-hydroxytryptamine-3 receptor that triggers action potential generation in the mucosal nerve terminals of myenteric AH neurons. © 2000 IBRO. Published by Elsevier Science Ltd. All rights reserved.

**Key words:** serotonin, intestine, electrophysiology, sensory transduction, sensory neuron.

Serotonin (5-hydroxytryptamine, 5-HT) can exert a powerful influence upon the motility and secretory activity of the gastrointestinal tract. The exact nature of these changes depends upon the region of gut studied and the route by which 5-HT is administered.<sup>6–8,36</sup> The large number of 5-HT receptor subtypes present on gut tissues and their wide distribution across different functional parts of the enteric circuitry are likely to contribute to the observed diversity in responses elicited by 5-HT.<sup>12</sup> For example, the myenteric AH neuron, which functions as an intrinsic primary afferent neuron (IPAN; also referred to as a sensory neuron) in the enteric circuitry,<sup>11</sup> has 5-HT<sub>1A</sub>, 5-HT<sub>1P</sub> and 5-HT<sub>3</sub> receptors on its cell body,<sup>14,27</sup> and 5-HT<sub>1A</sub> and 5-HT<sub>4</sub> receptors on its terminals.<sup>12</sup> The submucosal AH neuron (submucosal IPAN<sup>21</sup>) has 5-HT<sub>1P</sub> and 5-HT<sub>3</sub> receptors on its cell body, but only 5-HT<sub>1P</sub> receptors on its terminals.<sup>30,35</sup> Extrinsic primary afferent neurons from the vagus have 5-HT<sub>1A</sub> and 5-HT<sub>3</sub> receptors on their terminals,<sup>15,16</sup> and the 5-HT-containing enterochromaffin (EC) cells have 5-HT<sub>3</sub> and 5-HT<sub>4</sub> receptors.<sup>13,32</sup>

5-HT, when applied to the mucosa, generates action potentials (APs) in the mucosal processes of myenteric AH neurons.<sup>1,4,34</sup> Electrical stimulation of the mucosa elicits a similar response, suggesting that, in addition to a direct effect on the nerve terminal, release of endogenous 5-HT contributes to this response.<sup>3,4</sup> The 5-HT-induced changes in motility

and secretion described above may be due to the actions of 5-HT on the terminals of myenteric AH neurons.

The purpose of this study is to characterize the receptor type(s) through which mucosal application of 5-HT activates the terminals of the IPANs with cell bodies in the myenteric plexus.

### EXPERIMENTAL PROCEDURES

#### Tissue preparation

All experiments were performed using guinea-pigs (either gender, 160–280 g, Hartley strain, from the University of Melbourne), which were fed a standard laboratory diet until the day of the experiment. Animals were stunned by a blow to the head and killed by severing the carotid arteries and spinal cord in accordance with the guidelines of the University of Melbourne Animal Ethics Committee. A 2- to 3-cm-long segment of ileum, approximately 10–20 cm from the ileocaecal junction, was removed and placed in oxygenated (95% O<sub>2</sub>/5% CO<sub>2</sub>) physiological saline of the following composition (in mM): NaCl, 117; NaH<sub>2</sub>PO<sub>4</sub>, 1.2; MgSO<sub>4</sub>, 1.2; CaCl<sub>2</sub>, 2.5; KCl, 4.7; NaHCO<sub>3</sub>, 25; glucose, 11. The physiological saline also contained nicardipine (3  $\mu$ M) and scopolamine (hyoscine, 1  $\mu$ M) to relax the smooth muscle and to minimize its movements. The segment was cut open along the line of the mesenteric attachment and pinned mucosal side up in a Petri dish lined with a silastic elastomer. The mucosa, submucosa and circular muscle were removed over half the circumference, leaving the myenteric plexus with attached longitudinal muscle exposed. The preparation was then transferred to the base of a small recording chamber (volume approximately 2 ml), stretched and pinned flat with 80- $\mu$ m pins. The preparation was superfused with warmed (35–36°C) physiological saline at a flow rate of 4–6 ml/min.

#### Electrophysiology

Myenteric ganglia were visualized at  $\times$ 200–300 magnification with differential interference contrast optics. Neurons were impaled with glass microelectrodes (120–200 M $\Omega$  tip resistance) containing 2% biocytin in 1 M KCl. Voltage recordings were made in bridge mode

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**Abbreviations:** AP, action potential; EC cells, enterochromaffin cells; EPSP, excitatory postsynaptic potential; HEPES, *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulphonic acid; 5-HT, 5-hydroxytryptamine (serotonin); IPAN, intrinsic primary afferent neuron; PPP, proximal process potential; RMP, resting membrane potential.

using an Axoclamp 2A amplifier, digitized at 2–20 kHz (Digidata 1200A), recorded on a personal computer using Axoscope 7.0 (all from Axon Instruments, USA) and then analysed with Origin 5.0 (MicroCal, Northampton, MA, USA). Measurements of AH and S neuron properties commenced 10 min or more after impalement (for classification criteria, see Refs 5, 17 and 33).

#### *Electrical stimulation of the mucosa*

Bipolar stimulating electrodes were made from two lengths of 114- $\mu$ m diameter stainless steel wire insulated with a layer of 15- $\mu$ m Teflon. Stimulus pulses from 0.5 to 2.0 mA (typically 1.0 mA) and 0.5-ms duration (Master-8 stimulator, ISO-Flex stimulus isolation unit, both from A.M.P.I., Israel) were used (all other protocols are detailed in Ref. 3).

#### *Agonist and antagonist solutions*

Stock solutions were made up in distilled water as follows: 5-HT (10 mM), 2-methyl-5-HT (10 mM),  $\alpha$ -methyl-5-HT (1 mM), 5-methoxytryptamine (1 mM), granisetron (10 mM), methiothepin (1 mM), SB 204070 (1 mM). Tropicisetron was first dissolved in a small volume of 1 N HCl and then diluted to 10 mM with distilled water.

Drugs were made up on the day of the experiment from stock solutions and diluted in distilled water or HEPES-buffered saline. Previous studies have found that application of small volumes of either carrier solution to the mucosa does not cause firing in AH neurons (see Refs 3, 4 and 25).

Agonists were applied by pressure ejection (10 p.s.i.) from one of two micropipettes (10–20  $\mu$ m tip diameter, 2 mm o.d., 1 mm i.d.) mounted on a micromanipulator and positioned approximately 0.5 mm above the intact mucosa and approximately 2 mm from the impaled neuron (Picospritzer II, General Valve Corp, USA). Pulse durations of 50–200 ms led to ejection volumes of 1–5  $\mu$ l (determined by measuring the accumulation of 1000 pulses) and produced non-equilibrium concentrations of agonist in small areas of mucosa. We have estimated previously that agonist concentrations at the site of action reach only one-tenth that value of the concentration in the micropipette.<sup>2</sup>

Application of agonists was at or near the position of the stimulating electrode and the micropipettes were withdrawn from the superfusing solution between trials to guard against leakage of agonist into the bath. Desensitization of responses was observed when agonists were applied more frequently than once every 2 min (see Results).

The spread of agonist, either across the mucosa or toward the cell body of the impaled neuron, was assessed in two ways. First, it was noted that regions from which trains of APs could be elicited were usually small and circumscribed (see Ref. 3), suggesting that agonist was not spreading over large areas of mucosa. Second, when APs were recorded, they were always found to be resistant to hyperpolarization of the cell body, suggesting that agonist was not reaching and directly depolarizing the cell body.

Antagonists were added to the superfusing physiological saline and were allowed to equilibrate with the tissue for 10–15 min before further measurements were taken. SB 204070 has been reported to bind to certain types of plastic;<sup>37</sup> thus, effluent containing 1  $\mu$ M SB 204070, that had been used during an electrophysiological experiment, was collected. This effluent was successfully used at concentrations of 1  $\mu$ M and at a dilution of 0.1  $\mu$ M to block 5-HT (10  $\mu$ M) induced contractions of longitudinal muscle, myenteric plexus preparations from guinea-pig distal colon in the presence of methiothepin and granisetron ( $n = 6$  from two animals; for details, see Ref. 38).

#### *Materials*

Drugs were obtained as follows: tetrodotoxin was purchased from Alomone (Israel); SB 204070 and granisetron were the kind gifts of SmithKline Beecham (Middlessex, UK); tropisetron was a kind gift from Sandoz (Basel, Switzerland). All other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA).

#### *Quantification of the responses*

Trains of APs with, or without, slow depolarizing potentials [i.e. slow excitatory postsynaptic potential (EPSP)-like events] were recorded in AH neurons in response to mucosal application of agonist.

Slow EPSP-like events began 1–2 s after application of agonist and were quantified by measuring the peak amplitude relative to the pre-stimulus resting membrane potential (RMP). The extents to which the excitability and the input resistance of the cell were altered during the slow depolarization were assessed by passing 500 ms positive and negative current pulses through the recording electrode.<sup>24</sup> The amplitude of slow EPSP-like events in some neurons may have been reduced by the presence of scopolamine in the physiological saline.<sup>28</sup> Slow EPSP-like events are the likely result of the stimulation of a population of mucosal sensory fibres that then feed on to the impaled neuron; the fibres could originate from submucosal, myenteric or extrinsic sensory neurons.<sup>4,11</sup>

APs and proximal process potentials (PPPs), elicited by a single agonist application to the mucosa, were counted and the combined total was expressed as APs within a response. Return maps were generated by plotting the interval from the preceding AP against the interval to the next AP (interval + 1; see Fig. 1). For example, for the second AP in a train, the points on a return map are the time between the second AP and the first, which is plotted as the  $x$ -value, and the time between the second AP and the third, which is plotted as the  $y$ -value. Return maps were used to assess the pattern of discharge (e.g. bursting, non-bursting, frequency of discharge; see the legend of Fig. 1 and Results). The latency to the first AP, the number of APs and the duration over which AP generation took place were also measured, and used to determine the extent to which antagonists were effective in blocking the actions of the agonists.

Some AH neurons spontaneously fired bursts of APs; the majority, however, did so only infrequently and it was possible to avoid counting spontaneous activity as part of agonist-induced responses.

#### *Morphological analysis*

After electrophysiological testing, most neurons were injected with biocytin, then fixed and visualized as described before.<sup>4</sup> All AH neurons were found to have Dogiel type II morphology and had one or more projections running toward or entering the circumferentially located mucosa; the morphology of these cells was not analysed further.

#### *Statistics*

All numbers are given as mean  $\pm$  S.E.M. Changes in the counts of APs were assessed using a Wilcoxon signed rank test with pairing and an alpha of 0.05 taken as the cut-off for significance. All other comparisons were performed using Student's  $t$ -tests which, unless otherwise noted, were one-tailed and paired; Bonferroni's correction for multiple comparisons was used where appropriate.

## RESULTS

### *Electrophysiology*

Intracellular recordings were made from 64 myenteric AH neurons (see Experimental Procedures) and six S neurons. All cells studied were within 1 mm of the intact mucosa in the circumferential direction. Receptive fields of AH neurons were tested at the beginning of an impalement by stimulating different locations on the mucosa with single electrical pulses (0.5 ms duration); neurons responding did so with one or more short-latency ( $>10$  ms) APs.<sup>3,4</sup> These AH neurons, and the regions of mucosa from which APs were evoked, were then preferentially used for further pharmacological studies.

### *Responses to electrical stimulation of the mucosa*

In 38 of 44 AH neurons, a single electrical stimulus applied to the mucosa evoked one or more APs (1 AP,  $n = 16$ ;  $>1$  AP,  $n = 22$ ). The bursts of APs consisted of an average of  $5 \pm 1$  APs and included both a directly evoked AP and a later train of APs.<sup>3,4</sup> This late train of APs was investigated together with the trains of APs elicited by the 5-HT receptor agonists (for example, see Fig. 6).

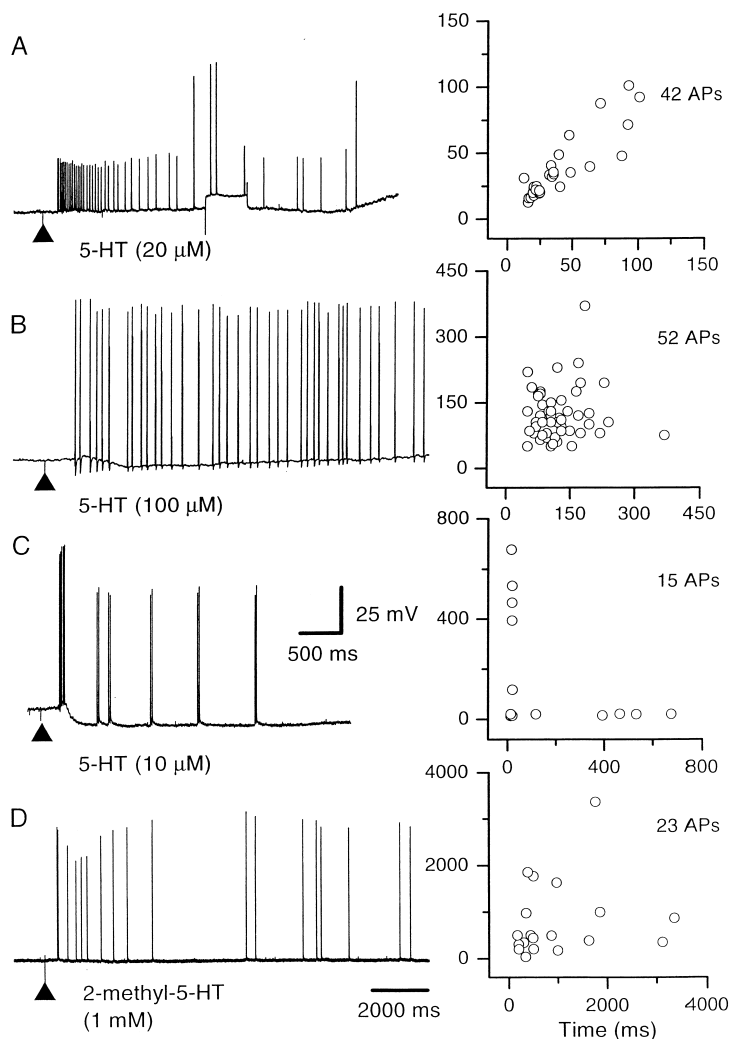


Fig. 1. Patterns of AP discharge in AH neurons elicited by 5-HT or 2-methyl-5-HT, applied to the mucosa. Left: voltage traces from four AH neurons showing responses to 5-HT (three representative patterns, A–C) and 2-methyl-5-HT (D). Agonists were applied in 100-ms pulses starting at the filled triangle. The vertical scale for A–D is the same, the time base for D is longer. Right: a return map showing the interval between APs versus the next AP interval (interval + 1) of APs in the traces on the left; the *x*- and *y*-axes are each time (ms); see Experimental Procedures. The total number of APs (including PPPs) is shown to the right. (A) Application of 5-HT (20  $\mu$ M in the micropipette) elicited a high-frequency train of PPPs that showed a marked slowing in frequency over time. A 500-ms depolarizing current pulse was passed through the recording electrode in the middle of the recording (RMP =  $-55$  mV). (B) 5-HT (100  $\mu$ M) elicited a long train of APs that fired at a slow, relatively constant frequency (RMP =  $-63$  mV). (C) 5-HT (10  $\mu$ M) elicited a train of APs that appeared to be made up of individual bursts of APs; the intra-burst frequency did not change (RMP =  $-76$  mV). (D) In contrast, 2-methyl-5-HT (1 mM) usually elicited a single pattern of discharge, a long train of APs with some irregular variation in frequency (RMP =  $-75$  mV).

Trains of electrical stimuli (10 pulses, 10 Hz), applied to the mucosa, were used evoke slow EPSPs in AH neurons; 10 of 11 neurons tested responded to such stimuli with a 5–15 mV slow EPSP.

#### *Responses to 5-hydroxytryptamine and the 5-hydroxytryptamine-3 receptor agonist, 2-methyl-5-hydroxytryptamine*

5-HT, applied to the mucosa, elicited a train of APs with, or without, a later slow depolarizing potential in 21 of 45 (46%) AH neurons, while only a slow depolarizing potential was elicited in a further 13 of 45 (29%). Previous observations indicate that these responses were due to an action of 5-HT on the mucosa rather than at the cell body; 5-HT applied to the cell body does not cause a train of APs and 5-HT applied to the mucosa elicits slow depolarizing responses that can be blocked by tetrodotoxin.<sup>4</sup> In general, the trains of APs elicited by 5-HT had initial latencies of under 500 ms and contained up to 50 APs over several seconds (see Fig. 1); for a single

neuron, the pattern of response and the number of APs were stable over time and for repeated application of agonist.

5-HT (3  $\mu$ M in the micropipette) had no effect on three AH neurons that had electrically evoked APs from the mucosa; accordingly, higher concentrations were used for the remainder of the study. 5-HT, at 5  $\mu$ M ( $n=2$ ), 10  $\mu$ M ( $n=10$ ), 20  $\mu$ M ( $n=8$ ) or 100  $\mu$ M ( $n=1$ ; but see Ref. 4) in the micropipette, elicited trains of APs that varied little in number with agonist concentration (data not shown). Overall, responses appeared as a long, high-frequency train of APs that either decreased in frequency (Fig. 1A), was relatively fixed in frequency (Fig. 1B) or had multiple, high-frequency bursts of APs (Fig. 1C); during responses with bursts of APs, the inter-burst frequency decreased over time, while the intra-burst frequency appeared to stay constant (Fig. 1C). This information can also be displayed by plotting the AP interval versus the next AP interval (interval + 1) to yield a return map. For example, in Fig. 1C (right panel), the absence of points in the middle of the plot (where a long interval would

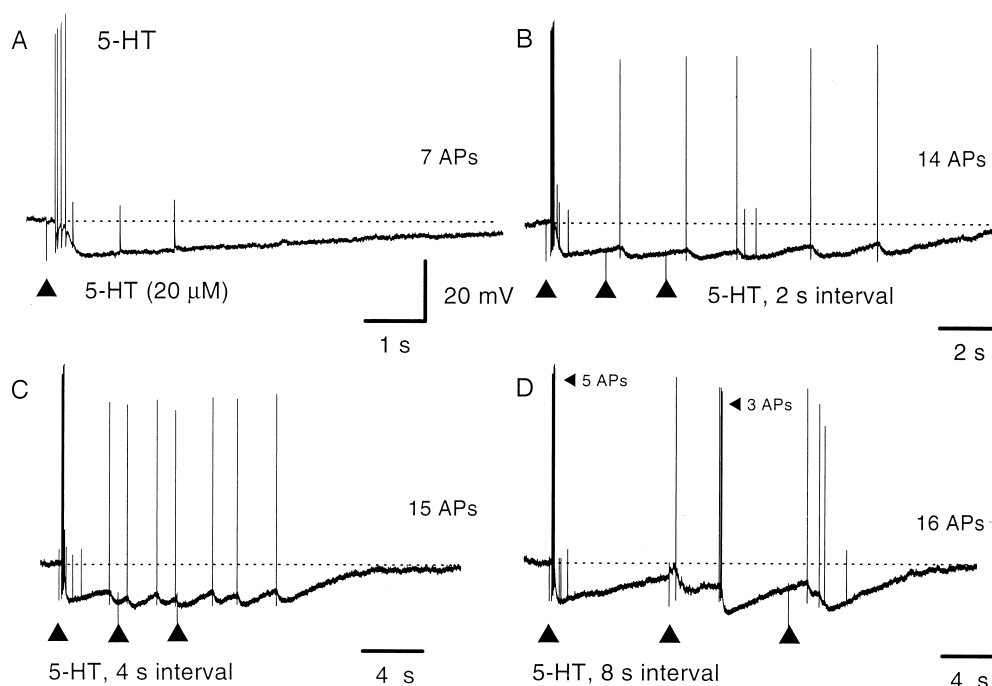


Fig. 2. Effect of repeated 5-HT application on the number and pattern of APs. Voltage traces from a single AH neuron showing responses to 5-HT application to the mucosa (starting at the filled triangle); the dotted line indicates the RMP. The vertical scale is the same throughout, while the time base increases from A to B and from B to C. The total number of APs for each trace is shown to the right. Note that the long after hyperpolarization following AP generation does not prevent incoming APs from the distal processes from evoking PPPs. (A) In control, 5-HT ( $20 \mu\text{M}$ ) elicited a train of seven APs at an average instantaneous frequency of 11.0 Hz for a duration of 2.1 s ( $\text{RMP} = -66 \text{ mV}$ ). Over seven trials on this same neuron, 5-HT elicited a train of  $7 \pm 1$  APs. (B–D) 5-HT was applied three times, in rapid succession with 2-, 4- or 8-s intervals between applications ( $\text{RMP} = -65$  to  $-67 \text{ mV}$ ). In D, the number of APs within bursts has been noted separately.

be measured on either side of an AP) indicates that all APs had a short interval before the AP, after the AP or both; this corresponds to an AP which was at the beginning, the end or in the middle of a burst.

The 5-HT<sub>3</sub> receptor agonist, 2-methyl-5-HT (1 or 3 mM in the micropipette), elicited trains of APs with or without slow depolarizing potentials in 10 of 21 AH neurons. In general, responses were longer in latency than 5-HT responses and consisted mainly of low-frequency trains of APs with little evidence of bursting (Fig. 1D). 2-Methyl-5-HT (0.1 mM) was not effective in any of three neurons tested.

Partial desensitization of both 5-HT and 2-methyl-5-HT responses was observed when either were applied at an interval of less than 2 min; complete desensitization of responses was not seen even when one response was run directly into another (i.e. an interval of less than about 30 s). This is illustrated in Fig. 2, where 5-HT caused a 2-s train of APs. A response to a single application of 5-HT is shown in Fig. 2A; over seven similar applications separated by 5-min intervals, the number of APs varied little ( $7 \pm 1$  APs) and the burst of APs always occurred at a fixed time after the stimulus (i.e. stimulus locked). In Fig. 2B–D, 5-HT was applied three times, with 2-, 4- or 8-s intervals. The first application of 5-HT elicited a short-latency train of APs that was identical to the control response; subsequent applications of 5-HT, however, elicited fewer APs that were poorly stimulus locked.

#### Responses to $\alpha$ -methyl-5-hydroxytryptamine and 5-methoxytryptamine

Neither  $\alpha$ -methyl-5-HT (100  $\mu\text{M}$  in the micropipette), a 5-HT<sub>2</sub> receptor agonist ( $n = 6$ ), nor 5-methoxytryptamine

(10  $\mu\text{M}$  in the micropipette), a 5-HT<sub>1,2,4</sub> receptor agonist ( $n = 6$ ), elicited a train of APs in the AH neurons tested, although 5-HT (10 or 20  $\mu\text{M}$ ), tested in parallel, was able to elicit responses in most of these neurons.

#### Effect of 5-hydroxytryptamine-3 receptor antagonists on agonist-induced trains of action potentials

The 5-HT<sub>3</sub> receptor-selective antagonist, granisetron (0.3–10  $\mu\text{M}$ ), and the mixed 5-HT<sub>3,4</sub> receptor antagonist, tropisetron (1–10  $\mu\text{M}$ ), were each superfused into the organ bath. Neither antagonist changed the resting membrane properties of the neurons nor the shape of the APs evoked by somal current injection. Each antagonist reduced, or in most cases abolished, the trains of APs and the slow EPSP-like responses evoked by 5-HT (10–20  $\mu\text{M}$ ) or 2-methyl-5-HT (1 mM) applied to the mucosa.

In nine AH neurons, 5-HT elicited bursts of  $19 \pm 3$  APs (see Table 1). In the presence of granisetron (1  $\mu\text{M}$ ), eight of nine AH neurons no longer responded (i.e. 0 APs), while the remaining neuron responded with only two APs ( $0.2 \pm 0.2$  APs,  $n = 9$ ). Partial recovery was observed in five of nine neurons after an average washout of 50 min; these bursts consisted of  $8 \pm 1$  APs ( $n = 5$ ; Fig. 3). In three of the same AH neurons, 2-methyl-5-HT elicited a similar response to 5-HT that consisted of  $27 \pm 11$  APs. In the presence of granisetron (1  $\mu\text{M}$ ), two of three AH neurons did not respond to 2-methyl-5-HT, while the remaining neuron responded with only two APs ( $0.7 \pm 0.8$  APs,  $n = 3$ ; see Table 1). Partial recovery was observed in all three neurons after an average of 100 min; these bursts consisted of  $12 \pm 2$  APs ( $n = 3$ ).

The effects of tropisetron were tested in four AH neurons

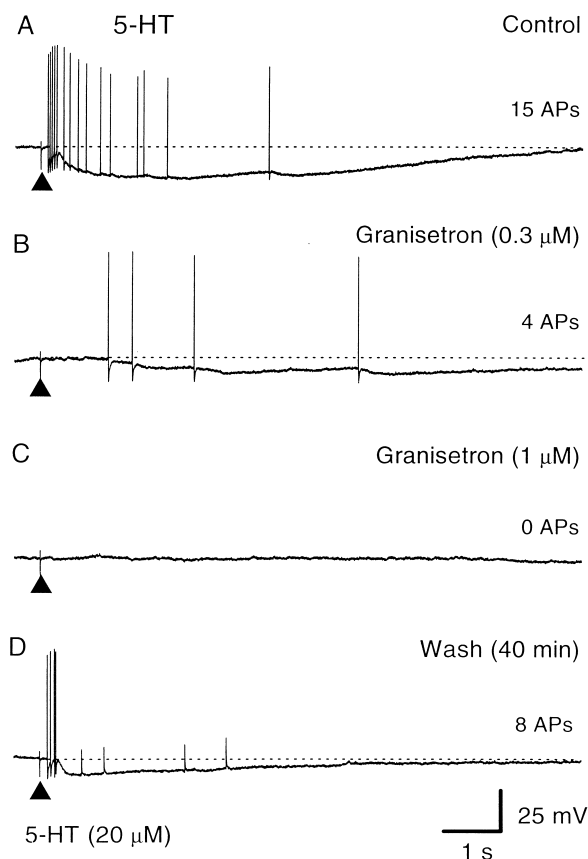


Fig. 3. The effects of granisetron on 5-HT responses are concentration dependent. Voltage traces from a single AH neuron showing responses to 5-HT application to the mucosa (starting at the filled triangle); the dotted line indicates the RMP. The scale is the same for all traces. The total number of APs for each trace is shown to the right. (A) In control, 5-HT ( $20 \mu\text{M}$ ) elicited a train of 15 APs at an average instantaneous frequency of 9.9 Hz for a duration of 3.9 s. The train consisted of an initial high-frequency burst followed by a slowing of the train over the duration of the response (RMP =  $-61 \text{ mV}$ ). (B) Granisetron ( $0.3 \mu\text{M}$ ) reduced the number of APs in the train. There was, in addition, a characteristic increase in the latency to the first AP of the train; it was increased from 0.12 s in control to 1.2 s in the presence of granisetron (RMP =  $-64 \text{ mV}$ ). (C) A higher concentration of granisetron ( $1 \mu\text{M}$ ), added cumulatively, abolished the response to 5-HT (RMP =  $-66 \text{ mV}$ ). Responses were typically abolished by all concentrations of granisetron or tropisetron of  $1 \mu\text{M}$  or greater. (D) Forty minutes after antagonist had been washed from the bath, 5-HT again produced short-latency trains of APs, though of a reduced number (RMP =  $-67 \text{ mV}$ ).

( $1 \mu\text{M}$  in two neurons and  $10 \mu\text{M}$  in two neurons), all of which were tested with 5-HT and two of which were also tested with 2-methyl-5-HT. 5-HT elicited bursts of  $13 \pm 2$  APs (Table 1), while in the presence of tropisetron, three of four AH neurons failed to respond, with the remaining neuron responding with only two APs (tropisetron,  $10 \mu\text{M}$ ). Partial recovery was observed in two of four neurons after 25 and 45 min; these bursts consisted of 10 and six APs (tropisetron, 1 and  $10 \mu\text{M}$ ). Similarly, 2-methyl-5-HT elicited bursts of APs in two AH neurons. The number of APs in control was 10 and 10; in the presence of tropisetron ( $1 \mu\text{M}$ ) there were two and no APs. No recovery was observed in these two neurons.

#### Effect of 5-hydroxytryptamine-3 receptor antagonists on agonist-induced slow excitatory postsynaptic potential-like responses

Slow depolarizing responses elicited by application of

5-HT to the mucosa are believed to be due to the simultaneous activation of sensory neurons in the area, that then feed on to the impaled myenteric sensory neuron (see Ref. 4). The effects of granisetron ( $1$  or  $10 \mu\text{M}$ ) on the slow, depolarizing responses elicited by either 5-HT or 2-methyl-5-HT were tested in a total of 10 AH neurons. 5-HT, applied to the mucosa, elicited slow depolarizations in eight of the 10 AH neurons. Control responses were  $9 \pm 1 \text{ mV}$ , while in the presence of granisetron ( $1 \mu\text{M}$ ) they were reduced to  $2 \pm 1 \text{ mV}$  ( $n = 8$ ,  $P < 0.05$ ). Full recovery was observed in two cells after 10 and 45 min (Fig. 4). These two neurons plus a further two neurons were tested with granisetron ( $10 \mu\text{M}$ ), which reduced 5-HT-elicited depolarizations (from  $9 \pm 1$  to  $2 \pm 1 \text{ mV}$ ,  $n = 4$ ,  $P < 0.05$ ). Partial recovery was observed in one cell after 25 min. Furthermore, 2-methyl-5-HT elicited slow depolarizations in four of the 10 AH neurons tested; control responses of  $12 \pm 2 \text{ mV}$  were reduced in the presence of granisetron ( $1 \mu\text{M}$ ) to  $3 \pm 1 \text{ mV}$  ( $n = 4$ ,  $P < 0.05$ ). Full recovery of the slow depolarizing response was observed in one cell, after 74 min. This neuron plus two further neurons were tested with granisetron ( $10 \mu\text{M}$ ), which abolished the 2-methyl-5-HT-elicited depolarizations (from  $11 \pm 2$  to  $0 \pm 0 \text{ mV}$ ,  $n = 3$ ,  $P < 0.05$ ); no recovery was observed.

#### Effect of 5-hydroxytryptamine-3 receptor antagonists on responses to electrical stimulation of the mucosa

In seven AH neurons, electrical stimulation of the mucosa elicited bursts of  $6 \pm 8$  APs; these bursts were not significantly modified in the presence of granisetron ( $1$ – $10 \mu\text{M}$ ;  $5 \pm 4$  APs,  $n = 7$ ,  $P = 0.3$ ; see Fig. 5C). In a further five AH neurons, electrical stimulation of the mucosa elicited a burst of  $5 \pm 5$  APs which were unaffected by tropisetron ( $1$ – $10 \mu\text{M}$ ;  $5 \pm 8$  APs,  $n = 5$ ,  $P = 0.9$ ; Fig. 6). Slow EPSPs, evoked by electrical stimulation of the mucosa ( $10 \text{ Hz}$ ,  $1 \text{ s}$ ), were also unchanged by either  $10 \mu\text{M}$  granisetron or  $10 \mu\text{M}$  tropisetron ( $n = 3$ ; data not shown).

#### Effect of the 5-hydroxytryptamine-4 receptor antagonist, SB 204070

The effects of SB 204070 ( $1 \mu\text{M}$ ) on responses elicited by either 5-HT or 2-methyl-5-HT were tested in a total of five AH neurons, four of which were also tested with electrical stimulation of the mucosa (see Fig. 7). Control bursts of APs in response to 5-HT had a latency to the first AP of  $1.6 \pm 1.3 \text{ s}$ , consisted of  $13 \pm 4$  APs and had a duration of  $8.0 \pm 3.9 \text{ s}$  ( $n = 4$ ). SB 204070 ( $1 \mu\text{M}$ ) produced no significant changes in the latency ( $0.7 \pm 0.4 \text{ s}$ ), number ( $17 \pm 6$  APs) or duration ( $25.5 \pm 14.5 \text{ s}$ ) of the response ( $P > 0.05$ ,  $n = 4$ ). Similarly, control bursts of APs in response to 2-methyl-5-HT had a latency to the first AP of  $0.3 \pm 0.1 \text{ s}$ , consisted of  $21 \pm 6$  APs and had a duration of  $6.9 \pm 3.6 \text{ s}$  ( $n = 3$ ). In the presence of SB 204070 ( $1 \mu\text{M}$ ), the latency to the first AP was  $0.7 \pm 0.2 \text{ s}$ , the burst consisted of  $27 \pm 11$  APs and had a duration of  $22.3 \pm 7.5 \text{ s}$  ( $n = 3$ ).

In four AH neurons, electrical stimulation of the mucosa elicited bursts of  $9 \pm 10$  APs that were unchanged in the presence of SB 204070 ( $1 \mu\text{M}$ ;  $9 \pm 10$  APs,  $n = 4$ , paired  $t$ -test, two tailed,  $P = 0.8$ , data not shown).

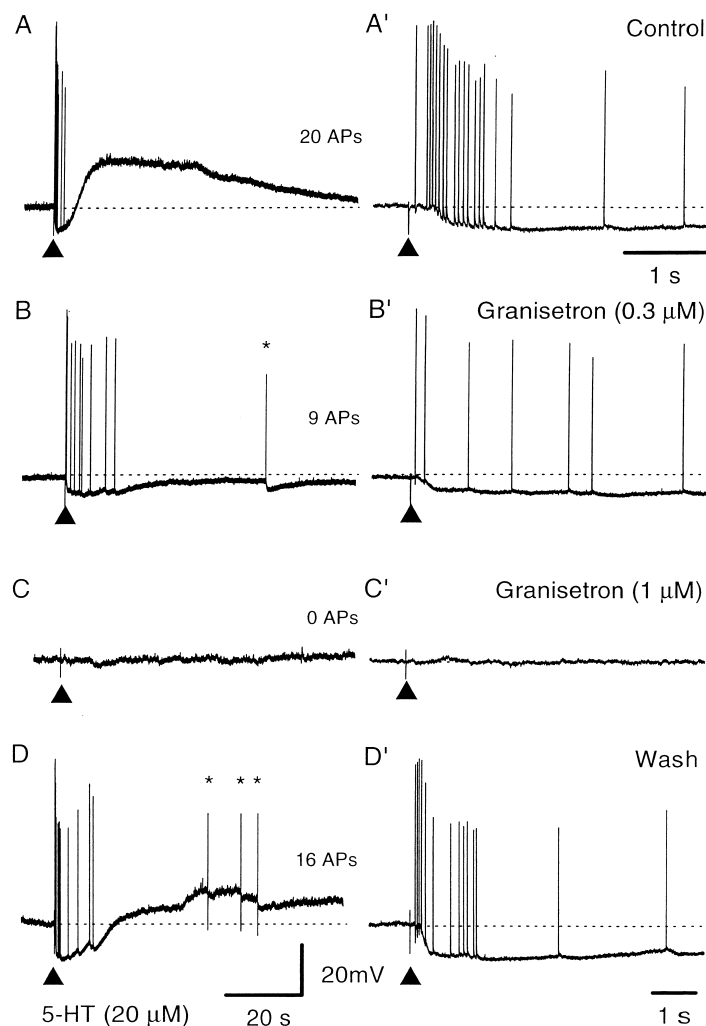


Fig. 4. Effect of granisetron on the train of action potentials and the slow depolarizing response elicited by 5-HT. Voltage traces from a single AH neuron; the dotted line indicates the RMP. Full traces are on the left, while expanded traces on the right show the burst of APs in more detail; all vertical scales are the same. The asterisks represent APs that were not counted as part of the response to agonist, either because they were evoked by a depolarizing current pulse (passed through the recording electrode) or because they were evoked by the slow depolarizing potential. (A, A') 5-HT ( $20 \mu\text{M}$ , 100 ms, at the filled triangle) elicited a train of 20 APs at an average instantaneous frequency of 11.8 Hz in control for a duration of 3 s and slow EPSP-like depolarization of 18 mV at  $-75 \text{ mV}$ . (B, B') Superfusion with  $0.3 \mu\text{M}$  granisetron reduced the number of APs to nine, increased the duration of the response to 13 s and reduced the average instantaneous frequency of the response to 1.3 Hz, while the slow depolarization was abolished at a membrane potential of  $-77 \text{ mV}$ . (C, C') Granisetron ( $1 \mu\text{M}$ ) fully blocked the train of APs. (D, D') Twenty-five minutes after washout of granisetron, the number of APs had increased to 16, but the duration was still 10 s and the average instantaneous frequency had risen to 8.6 Hz; the slow depolarization has also recovered and is 12 mV at  $-75 \text{ mV}$ .

#### Effects of agonists and antagonists on second-order neurons

Six S neurons were tested with 5-HT applied to the mucosa and two of these cells were also tested with 2-methyl-5-HT; each cell responded to either agonist with an extended burst of fast EPSPs (Fig. 8). Four of the six cells were tested with granisetron ( $0.3$  or  $1 \mu\text{M}$ ) or tropisetron ( $1 \mu\text{M}$ ); these results were pooled. In control, each burst of fast EPSPs had a latency to the first distinct EPSP of  $280 \pm 160 \text{ ms}$  and consisted of  $37 \pm 8$  distinct EPSPs which occurred within a window of  $8.7 \pm 2.1 \text{ s}$  from the start of the burst; the average amplitude of fast EPSPs counted was  $5.3 \pm 1.3 \text{ mV}$  ( $n = 4$ ). In the presence of 5-HT<sub>3</sub> receptor antagonists, the number of distinct EPSPs was reduced ( $7 \pm 2$ ,  $P < 0.05$ ), but not the average amplitude ( $3.5 \pm 0.5 \text{ mV}$ ,  $P > 0.05$ ,  $n = 4$ ; see Fig. 8). Partial recovery was observed following an average of 35 min after washout of the antagonist in three of four cases. In these same cells, 5-HT<sub>3</sub> receptor antagonists did not change the size or

number of fast EPSPs elicited by electrical stimulation of the mucosa ( $n = 4$ ; data not shown).

#### DISCUSSION

This study shows that 5-HT excites the mucosal nerve terminals of myenteric intrinsic primary afferent (sensory) neurons via 5-HT<sub>3</sub> receptors. APs generated in the mucosal processes of the sensory neurons propagate back to their cell bodies in the myenteric plexus.<sup>4</sup> These APs arise non-synaptically; they persist in a high  $\text{Mg}^{2+}$ , low  $\text{Ca}^{2+}$  saline solution and during somatic hyperpolarization.<sup>4</sup> They are, moreover, initiated with a short latency and with no indication of an underlying synaptic potential. The generation of APs by 5-HT is mimicked by a 5-HT<sub>3</sub> receptor agonist and blocked by 5-HT<sub>3</sub> receptor antagonists. By contrast, responses to electrical stimulation of the mucosa are not reduced by 5-HT<sub>3</sub> receptor antagonists.

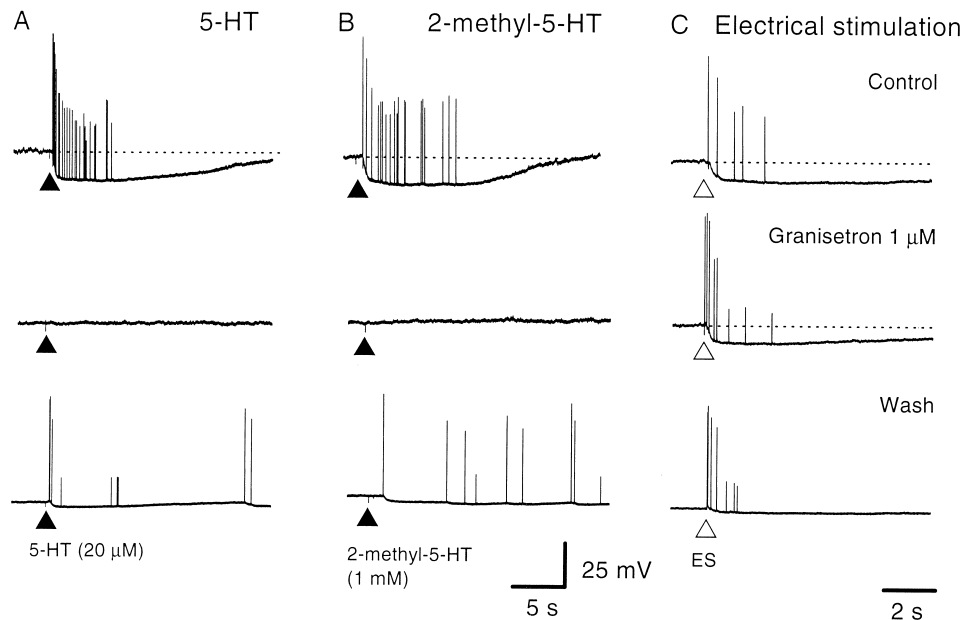


Fig. 5. Effect of granisetron on responses to 5-HT, 2-methyl-5-HT and electrical stimulation. Voltage traces from a single AH neuron; the dotted line indicates the RMP. All vertical scales are the same; time base in C is shorter. (A) 5-HT (20  $\mu$ M, 100 ms, at the filled triangle) elicited a train of 24 APs at an average instantaneous frequency of 6.0 Hz for a duration of 6 s (top) that was blocked by 1  $\mu$ M granisetron (middle). Fifty minutes later (bottom), there was a partial recovery of the 5-HT response (RMP: control = -67 mV; granisetron = -71 mV; recovery = -81 mV). (B) 2-Methyl-5-HT (1 mM, 100 ms, at the filled triangle) elicited a train of 19 APs in control (top) that was abolished during superfusion with granisetron (middle) and recovered with a time-course similar to that of 5-HT (RMP: control = -69 mV; granisetron = -69 mV; wash = -81 mV). (C) A single electrical pulse (0.5 ms, at the open triangle) was applied to the mucosa in the same location as were the agonists and elicited a train of five APs lasting more than 2 s. Granisetron did not reduce the number of APs elicited by electrical stimulation. Note that, during the long period of antagonist washout, the cell membrane became hyperpolarized due to a transient instability in the impalement.

Table 1. Effect of 5-hydroxytryptamine-3 receptor blockade on 5-hydroxytryptamine- and 2-methyl-5-hydroxytryptamine-elicited trains of action potentials

	Count of neurons responding	Average number of APs	Average latency (s) to first AP	Average duration (s) of burst
<b>5-HT</b>				
Control	9/9	15	0.4	11.4
Granisetron (1 $\mu$ M)	1/9	0.2 (2)	3.5	< 0.1
Wash	5/9	8	1.6	10.9
<b>Control</b>				
Tropisetron (1 $\mu$ M)	4/4	13	3.2	7.1
Wash	1/4	0.5 (2)	0.5	< 0.1
Wash	2/4	8	7.8	4.7
<b>2-Methyl-5-HT</b>				
Control	3/3	27	0.7	22.3
Granisetron (1 $\mu$ M)	1/3	0.7 (2)	7.4	< 0.1
Wash	3/3	12	2.3	24.3

5-HT or 2-methyl-5-HT were applied to the mucosa in control conditions, during superfusion with 5-HT<sub>3</sub> receptor antagonists and after washout of these antagonists. The number of neurons responding to agonist versus those tested has been expressed as a count (x/x). The number of APs is the average number of APs for all neurons tested, while the numbers in parentheses refer only to the average number of APs for neurons that responded. The latency and the duration of the trains of APs are also only averages for neurons that responded.

#### 5-Hydroxytryptamine acts at 5-hydroxytryptamine-3 receptors

Pharmacological evidence presented in this study indicates that 5-HT, applied to the mucosa, activates the mucosal processes of myenteric AH neurons through a 5-HT<sub>3</sub> receptor. First, agonists active at 5-HT<sub>3</sub> receptors, 5-HT and 2-methyl-5-HT, reliably elicited responses. Second, agonists that are not active at 5-HT<sub>3</sub> receptors, such as  $\alpha$ -methyl-5-HT (5-HT<sub>2</sub> receptor selective) and 5-methoxytryptamine (5-HT<sub>1,2,4</sub> receptor selective) did not elicit any responses. Third, both

granisetron, which is a selective 5-HT<sub>3</sub> receptor antagonist, and tropisetron, which is a 5-HT<sub>3,4</sub> receptor antagonist, blocked 5-HT- and 2-methyl-5-HT-elicited responses. Both antagonists did so quickly and potently; furthermore, they did not wash out readily. SB 204070, which is a selective 5-HT<sub>4</sub> receptor antagonist, did not affect any responses, even though it was applied at concentrations well above those needed to block 5-HT<sub>4</sub> receptors.<sup>37</sup> Finally, only relatively high concentrations of 5-HT were effective. These data suggest a low-affinity receptor with properties similar to those reported for

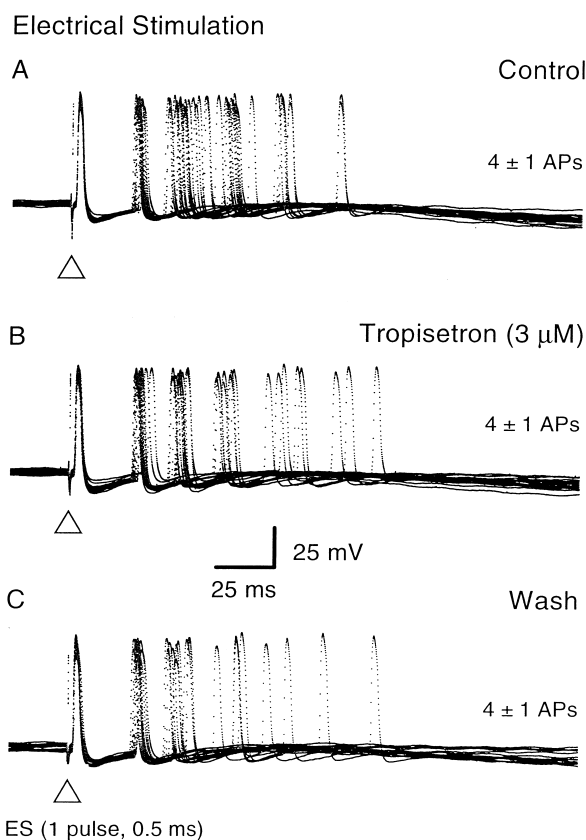


Fig. 6. Effect of tropisetron on responses to electrical stimulation of the mucosa. Fifteen to 20 overlapping voltage traces per panel from a single AH neuron showing responses to electrical stimulation of the mucosa (at the open triangle). The scale is the same for all traces. Data are displayed as individual points. The average number of APs for each set of traces is shown to the right. (A) In control, electrical stimulation (0.5 ms duration) evoked a single antidromic AP followed by one to five extra APs. (B) Tropisetron (3 μM) did not reduce the number of APs in the train. (C) Fifteen minutes after wash of antagonist.

5-HT<sub>3</sub> receptors on enteric neuron cell bodies or on EC cells.<sup>12,13,27,32</sup>

Recently, Tuladhar *et al.*<sup>36</sup> re-examined an effect first described by Bülbiring and Crema,<sup>6</sup> whereby lumenally applied 5-HT decreases the threshold for the initiation of peristalsis in the guinea-pig ileum. Tuladhar *et al.* found that this was due to activation of 5-HT<sub>3</sub> receptors and was dependent upon the integrity of the myenteric plexus. Our results are consistent with Tuladhar *et al.*'s study; thus, a site of action for luminal 5-HT-induced sensitization may be 5-HT<sub>3</sub> receptors on the mucosal terminals of the myenteric IPANs.

Other studies that have investigated the actions of 5-HT on the initiation of peristalsis have implicated the 5-HT<sub>3</sub> receptor, as well as the 5-HT<sub>4</sub> and 5-HT<sub>1P</sub> receptors. Whether these receptors are on the myenteric IPANs or on a similar population of neurons with cell bodies in the submucosal plexus<sup>21</sup> is not clear. Studies in the guinea-pig colon have suggested that 5-HT, released from EC cells, participates in the initiation of peristalsis via 5-HT<sub>3</sub> and 5-HT<sub>4</sub> receptors on unspecified IPANs.<sup>10,18</sup> This is supported by Kadowaki *et al.*,<sup>19</sup> who, in the same tissue, found that a combination of 5-HT<sub>3</sub> and 5-HT<sub>4</sub> receptor antagonists blocked peristalsis. In the guinea-pig ileum, however, Yuan *et al.*<sup>39</sup> found no reduction in the initiation of reflexes due to 5-HT<sub>3</sub> or 5-HT<sub>4</sub> receptor antagonists.

Our results support the idea that activation of 5-HT<sub>3</sub> receptors within the lumen could initiate or modulate peristalsis and suggest that 5-HT<sub>4</sub> receptors are not on the IPAN terminals. The involvement of a 5-HT<sub>4</sub> receptor may, however, lie upstream from the 5-HT<sub>3</sub> receptor identified here (Bornstein J. C., unpublished data).

The 5-HT<sub>1P</sub> receptor has been implicated in the initiation of motor reflexes via IPANs of the guinea-pig colon,<sup>10</sup> secretory reflexes via submucosal IPANs of the guinea-pig ileum<sup>9</sup> and the activation of submucosal IPANs of the guinea-pig ileum.<sup>20,21</sup> More recently, Pan and Gershon<sup>30</sup> have attempted to make electrical recordings from the submucosal IPANs. Although they were unable to record directly from the IPANs being stimulated, as was done in the present study, they were able to demonstrate convincing responses in second-order neurons to mucosal application of 5-HT. These responses were resistant to the 5-HT<sub>3</sub> receptor antagonist ondansetron, but were reduced by higher concentrations of tropisetron. These, and other data, suggest that the 5-HT<sub>1P</sub> receptor, rather than the 5-HT<sub>3</sub> receptor, is the primary 5-HT receptor on the submucosal IPANs. Our study suggests that the 5-HT<sub>1P</sub> receptor, like the 5-HT<sub>4</sub> receptor, does not participate in the activation of the nerve terminals of myenteric IPANs.

These findings suggest that the submucosal and the myenteric IPANs may respond to increasing concentrations of 5-HT differently. While the submucosal IPANs may respond to lower concentrations of 5-HT via low-affinity 5-HT<sub>1P</sub> or 5-HT<sub>4</sub> receptors, these receptors may become desensitized by the higher concentrations of 5-HT needed to activate the 5-HT<sub>3</sub> receptors on the myenteric IPANs.

#### Other substances that may be involved

Electrical stimulation of the intestine has been shown to release 5-HT<sup>31</sup> and may release other intermediates in the sensory transduction process. We have shown that electrical stimulation of the mucosa elicits a train of APs in myenteric sensory neurons, a finding supported in the colon.<sup>29</sup> The first AP is likely to be due to a direct stimulation of the nerve terminal, while we hypothesize that the later APs are due to release of an intermediary substance.<sup>4</sup> We report here that the number of APs evoked by electrical stimulation was not reduced in the presence of 5-HT<sub>3</sub> receptor antagonists (Figs 5, 6). If, as we suggest, the later APs are due to an intermediary substance, then these data argue that 5-HT is not primarily responsible for activation of the nerve terminal; furthermore, electrical stimulation must release something other than, or in addition to, 5-HT that is responsible for activation of the sensory nerve terminals.

Substances that may activate the IPAN nerve terminals include those that activate the extrinsic sensory nerve terminals. For instance, in the rat jejunum, extrinsic primary afferent neurons respond to 5-HT via a 5-HT<sub>3</sub> receptor and to agonists at cholecystokinin, adenosine, bradykinin and histamine receptors.<sup>16,22,23,26</sup>

#### The schema

When exogenous 5-HT is applied to the mucosal epithelium, as shown in Fig. 9, it may gain access through the epithelium (this is more likely *in vitro*, where the epithelium may be damaged) and directly activate 5-HT<sub>3</sub> receptors

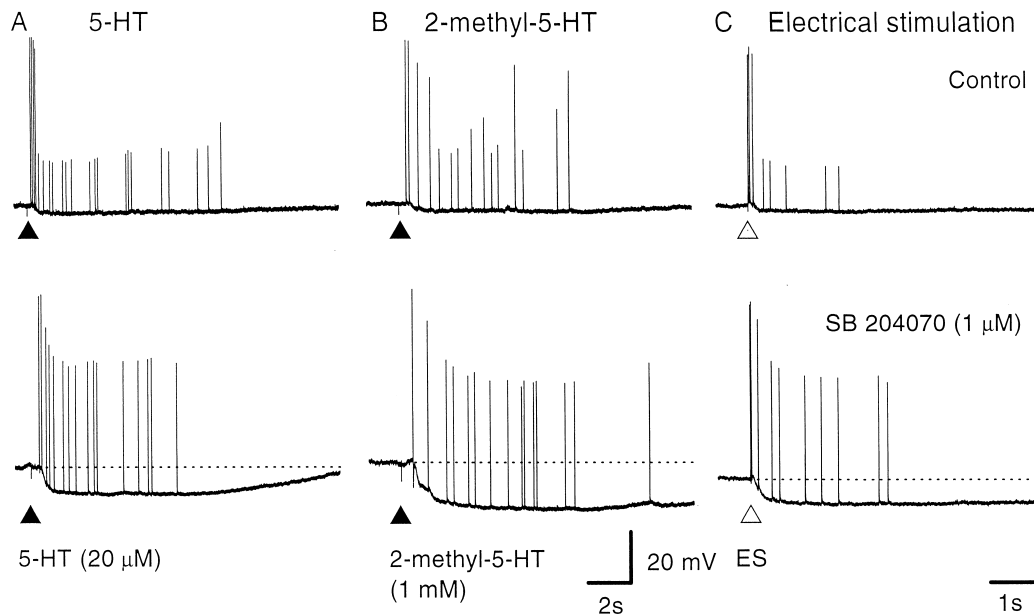


Fig. 7. Effect of SB 204070 on responses to 5-HT, 2-methyl-5-HT and electrical stimulation. Voltage recordings from a single AH neuron; the dotted line indicates the RMP. All vertical scales are the same, while the time base in C is shorter. (A) 5-HT ( $20 \mu\text{M}$ , 100 ms, applied at the filled triangle) elicited a train of four APs followed by 18 PPPs at an average instantaneous frequency of 6.1 Hz in control over a duration of 9 s (top). The membrane potential was  $-79 \text{ mV}$ , the afterhyperpolarization was small and there was no sign of a slow depolarization. Superfusion with  $1 \mu\text{M}$  SB 204070 (bottom) did not significantly change the number of APs (16), the average instantaneous frequency (3.9 Hz) or the duration of the response (6 s). Note that the membrane potential has now depolarized to  $-62 \text{ mV}$  and the afterhyperpolarization is prominent. (B) 2-Methyl-5-HT ( $1 \text{ mM}$ , 100 ms, applied at the filled triangle) elicited a train of 15 mixed APs and PPPs at an average instantaneous frequency of 2.4 Hz in control over a duration of 7.7 s (top) that were not significantly affected by superfusion of SB 204070 (bottom). (C) A single electrical stimulus to mucosa in the same location as the agonists were applied elicited a train of eight APs at an average instantaneous frequency of 8.3 Hz in control over a duration of 2.1 s (top). Superfusion with SB 204070 did not affect the number, duration or instantaneous frequency of the electrical response.

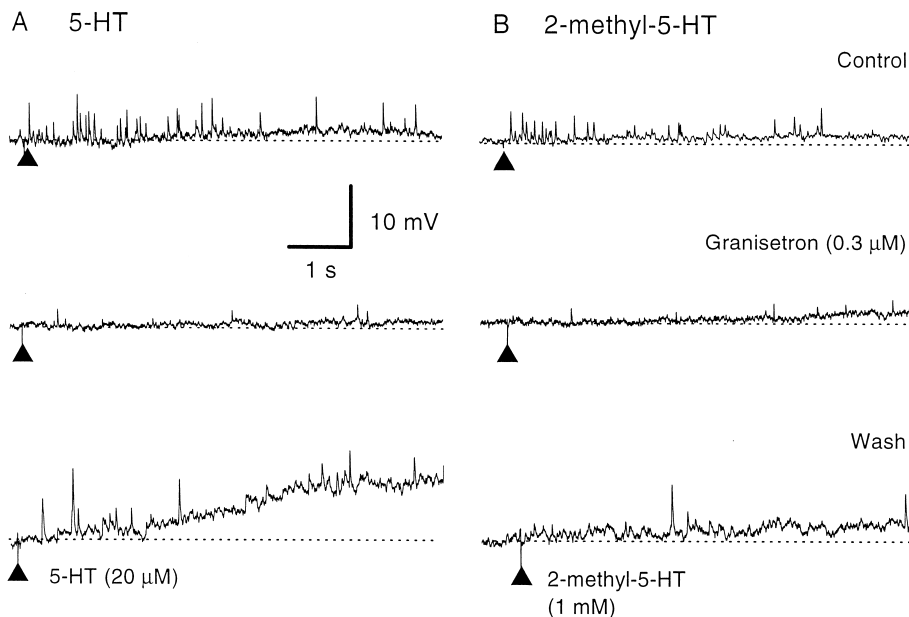


Fig. 8. Responses in second-order neurons to 5-HT. Voltage traces from a single S neuron; the dotted line indicates the RMP; all scales are the same. (A) 5-HT ( $20 \mu\text{M}$ , 100 ms, applied at the filled triangle) elicited a burst of fast EPSPs that lasted for more than the 8-s window of time illustrated. Within the first 20 s, there were 49 peaks at an average instantaneous frequency of 2.7 Hz. There was also a small sustained depolarization that lasted for 16 s (top). Granisetron ( $0.3 \mu\text{M}$ ) clearly reduced the number of fast EPSPs and their frequency (middle). Within the first 20 s, there were only 13 EPSPs counted. Twenty minutes after washout, there is partial recovery with 29 EPSPs counted. The large depolarization appeared to be a slow EPSP-like event. (B) 2-Methyl-5-HT also elicited a burst of fast EPSPs; in the first 20 s after application there were 58 peaks at an average instantaneous frequency of 2.6 Hz (top). Granisetron reduced the 2-methyl-5-HT response to 11 EPSPs, and it was washed out with a similar time-course to 5-HT, with 30 EPSPs counted (middle and bottom).

located on the nerve terminals of the myenteric IPANs (Fig. 9, Path A).

5-HT may activate 5-HT<sub>3</sub> receptors on EC cells (or other

closely related structures), which results in the release of endogenous 5-HT and/or another mediator that then acts upon the IPAN nerve terminals (Fig. 9, Path B); this could

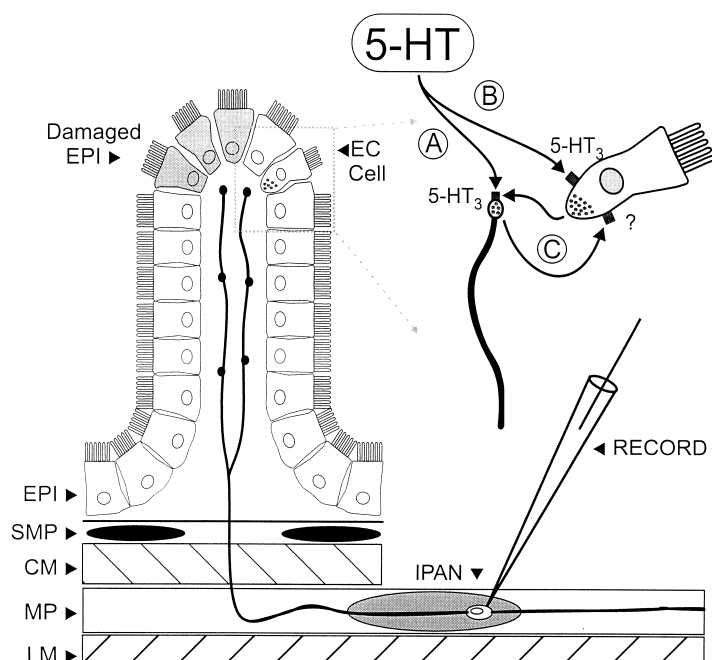


Fig. 9. An illustration of the possible locations of the 5-HT<sub>3</sub> receptors in relation to the mucosal epithelium and the sensory nerve terminals. CM, circular muscle; EPI, epithelium; LM, longitudinal muscle; MP, myenteric plexus; SMP, submucosal plexus. The circular muscle, submucosal plexus and epithelium have been dissected away from the right half of the preparation to allow an intracellular electrode (RECORD) to record from a myenteric AH neuron (IPAN) that has one or more projections to intact mucosa. These fibres are in the lamina propria adjacent to EC cells. Inset: the 5-HT<sub>3</sub> receptor (5-HT<sub>3</sub>) identified in this study may be on the IPAN nerve terminal or on the EC cell. The involvement of other mediators cannot be ruled out. Exogenous 5-HT (at the top), transiently applied to the mucosa, could interact with 5-HT<sub>3</sub> receptors on the nerve terminal via a break in the epithelium (Path A) or on the EC cells directly (Path B). Further release of transmitter from the EC cell may interact with the IPAN nerve terminal or, alternatively, transmitter from the nerve terminal may interact with the EC cell (Path C).

also excite nearby EC cells. Whether the IPAN nerve terminals or the adjacent EC cells then release transmitter is not known (Fig. 9, Path C).

#### CONCLUSIONS

The mucosal processes of myenteric IPANs in the guinea-pig small intestine are excited via activation of 5-HT<sub>3</sub> receptors. 5-HT or the 5-HT<sub>3</sub> receptor agonist, 2-methyl-5-HT, cause generation of APs in the mucosal processes which

then propagate back to the cell body and into other processes located in the myenteric and possibly submucosal ganglia. The initiation or enhancement of reflexes by luminal 5-HT in the guinea-pig small intestine may be dependent on these 5-HT<sub>3</sub> receptors.

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