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# Inhibitory effect of YM060 on 5-$HT_3$ receptor-mediated depolarization in colonic myenteric neurons of the guinea pig

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**Abstract**

We used conventional intracellular recording methods to examine the effects of YM060 {(−)-(*R*)-5-[(1-methyl-1*H*-indol-3-yl)carbonyl]-4,5,6,7-tetrahydro-1*H*-benzimidazole monohydrochloride}, a novel 5-$HT_3$ receptor antagonist, on 5-hydroxytryptamine (5-HT, serotonin)-evoked fast membrane depolarization in myenteric neurons of the guinea pig distal colon, and compared its potency to that of other 5-$HT_3$ receptor antagonists. Microapplication of 5-HT from fine-tipped pipettes evoked both fast and slowly activating depolarizing responses in 78% and 40% of colonic myenteric neurons, respectively. The selective 5-$HT_3$ receptor agonist 2-methyl-5-HT applied with short pressure pulses (100–300 ms) mimicked the fast but not the slow response. The 5-$HT_3$ receptor antagonists YM060, granisetron and ondansetron suppressed the 5-HT-evoked fast response in 98% of colonic myenteric neurons in a concentration-dependent manner with $pIC_{50}$ values of 8.62, 7.77 and 6.90, respectively. Methysergide and GR113808 did not affect the fast responses at concentrations sufficient to block 5-$HT_1$, 5-$HT_2$ and 5-$HT_4$ receptors, respectively. YM060 did not affect the slowly activating response to 5-HT or any other electrophysiological parameter of the neurons including resting membrane potential, input resistance and the amplitude of action potentials evoked by injection of depolarizing current. Stimulus-evoked fast excitatory postsynaptic potentials were unchanged by YM060 at concentrations up to $10^{-8}$ M, excluding any possible local anesthetic or anticholinergic effects of YM060. The results confirm that the fast component of the two depolarizing responses to 5-HT in colonic myenteric neurons is mediated by 5-$HT_3$ receptors. They also demonstrate that YM060 is a potent and selective 5-$HT_3$ receptor antagonist.

*Keywords:* 5-$HT_3$ receptor; Enteric nervous system; Myenteric neuron; Distal colon; YM060 {(−)-(*R*)-5-[(1-methyl-1*H*-indol-3-yl)carbonyl]-4,5,6,7-tetrahydro-1*H*-benzimidazole monohydrochloride}; (Guinea pig)

## 1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is a putative neurotransmitter in the enteric nervous system (Lee, 1960; Talley et al., 1990; Wood, 1987). 5-HT is synthesized and stored in myenteric neurons and released in association with peristaltic propulsion (Gershon et al., 1965; Lee, 1960), which suggests that it plays an important role in the neural mechanisms of gastrointestinal motility.

As many as four distinct 5-HT receptor subtypes (5-$HT_{1A}$, 5-$HT_{1P}$, 5-$HT_3$ and 5-$HT_4$) have been identified on enteric neurons in the guinea pig small intestine (Mawe et al., 1986; Galligan and North, 1991; Pan and Galligan, 1994). Intracellular recording studies have shown that 5-HT evokes fast and slow depolarizing responses that are mediated by 5-$HT_3$ and 5-$HT_{1P}$ receptors, respectively, in small intestinal myenteric neurons (Mawe et al., 1986). The 5-$HT_3$ receptor-mediated fast response involves receptor gating of non-selective cation channels, whereas slow signal transduction for the slow response involves receptor-activation of adenylate cyclase and the 2nd messenger function of cAMP (Derkach et al., 1989; Palmer et al., 1986; Xia et al., 1994). 5-$HT_{1A}$ and 5-$HT_4$ receptors located on presynaptic terminals at nicotinic cholinergic synapses suppress or facilitate synaptic transmission, respectively, in the myenteric plexus of the guinea pig small intestine (Pan and Galligan, 1994). 5-$HT_{1A}$ receptors

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are also expressed on the neuronal cell soma and evoke hyperpolarizing responses when activated (Galligan and North, 1991).

5-$HT_3$ receptors have been suggested to play a role in physiological and pathophysiological conditions in the distal colon. 5-$HT_3$ receptor antagonists suppress nerve-mediated contraction of isolated longitudinal muscle-myenteric plexus preparations (Miyata et al., 1991; Woollard et al., 1994). 5-$HT_3$ receptor antagonists suppress colonic motility in healthy humans (Talley et al., 1990) and decrease sensitivity to rectal distention in patients with the irritable bowel syndrome (Prior and Read, 1993). It has also been reported that selective 5-$HT_3$ receptor antagonists suppress stress-induced defecation in rats (Miyata et al., 1992). The effects of microapplication of 5-HT on the electrophysiological properties of myenteric neurons in the guinea pig distal colon and rectum have been reported (Wade and Wood, 1988a; Tamura and Wood, 1989). Nevertheless, no precise characterization of the 5-HT receptor subtypes that mediate 5-HT actions in this region has been made. Therefore, the aim of this study was to identify the 5-HT receptor subtype that mediates rapidly activating depolarizing responses evoked by 5-HT in myenteric neurons of the guinea pig distal colon. We also determined the selectivity and potency of YM060 {(−)-(*R*)-5-[(1-methyl-1*H*-indol-3-yl) carbonyl]-4,5,6,7-tetrahydro-1*H*-benzimidazole monohydrochloride}, a newly developed 5-$HT_3$ receptor antagonist.

## 2. Materials and methods

### 2.1. Preparations

Thirty-three male guinea pigs (300–500 g) were used. They were stunned by a blow to the head and killed by bleeding from the cervical vessels as approved by the Animal Experimentation Committee of the School of Medicine, Tokai University. A segment of the distal colon (2–5 cm) was removed and flat-sheet preparations of longitudinal muscle with attached myenteric plexus were prepared by dissection of the mucosa and circular muscle layers (Wade and Wood, 1988b). Preparations were then mounted in a recording chamber, superfused with Krebs solution warmed to 37°C and gassed with 95% $O_2$:5% $CO_2$ (pH 7.3–7.4) at a rate of 10 ml/min. Myenteric ganglia were viewed with an inverted microscope (Nikon TMD; Tokyo, Japan) equipped with Hoffman Modulation contrast optics (Tamura, 1992). The composition of the Krebs solution was (in mM) 120.9 NaCl, 5.9 KCl, 1.2 $MgCl_2$, 14.4 $NaHCO_3$, 1.33 $NaH_2PO_4$, 2.5 $CaCl_2$ and 11.5 glucose. Nifedipine (1 μM) and atropine (1 μM) were added to the solution to suppress muscle movement.

### 2.2. Electrophysiological recording

Intracellular recordings were made with glass microelectrodes containing 2 M KCl with tip resistances ranging from 100 to 200 MΩ. The amplifier (Nihon Kohden CEZ-3100; Tokyo, Japan) contained bridge circuitry for intrasomal injection of electrical current. Interganglionic fiber tracts were focally stimulated with 300-ms square pulses by means of electrodes made of Teflon-coated platinum wire (diameter 20 μm) to evoke synaptic potentials. The records were reproduced either on a digital storage oscilloscope (Hitachi VC-6075; Tokyo, Japan) or on a thermal array recorder (Nihon Kohden RTA-1100M) from original records on videotape.

5-HT and 2-methyl-5-HT were applied from fine-tipped pipettes (tip diameter 5–10 μm) by pressure microejection with controlled pulses of nitrogen gas. The micropipettes contained fast green dye in order to assess the distribution of the ejected substances in the chamber during pressure application. The concentration of agonists in the pipettes was 1 mM. Antagonists were applied by addition to the superfusion solution for 10 min.

### 2.3. Statistical analysis

Effects of the antagonists were normalized as percentage inhibition of depolarizing responses to 5-HT. $IC_{50}$ values were calculated by Probit analysis and converted to $pIC_{50}$ values (negative logarithm of $IC_{50}$ values). Data are shown as the mean ± S.E.M. or mean with 95% confidence limits. Statistical significance was estimated using Student's *t*-test. Probabilities less than 5% ($P < 0.05$) were considered significant.

### 2.4. Drugs

YM060, granisetron HCl, ondansetron HCl, GR113808 and 2-methyl-5-HT were prepared by Yamanouchi Pharmaceutical Co. (Tsukuba, Japan). Methysergide was a gift from Sandoz (Basel, Switzerland). 5-HT creatinine sulfate was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All drugs were dissolved in Krebs solution.

## 3. Results

Results were obtained from 103 colonic neurons. Of these, 27 were characterized as having action potentials accompanied by a long-lasting (> 2 s) after-hyperpolarization and were classified as AH/Type 2 neurons (Wood, 1994). The remaining 76 neurons that showed no long-lasting after-hyperpolarizations were further subdivided into S/Type 1, Type 3, and Type 4 neurons

(Wood, 1994). S/Type 1 ($n = 52$) neurons discharged multiple action potentials in response to a depolarizing current pulse, while Type 4 ($n = 21$) neurons fired only one action potential at the onset of a 950 ms current pulse and no hyperpolarizing after-potentials characteristic of AH/Type 2 neurons were associated with the spikes. Type 3 ($n = 3$) neurons could not be induced to discharge action potentials by injection of a depolarizing current pulse. Robust nicotinic excitatory postsynaptic potentials (EPSPs) identified them as neurons. The electrophysiological properties of the various types of neurons, including resting membrane potential, input resistance and amplitude and duration of action potentials, were compatible with values reported in previous studies of myenteric neurons in this region (Wade and Wood, 1988a) and in the rectum (Tamura and Wood, 1989).

### 3.1. Actions of 5-HT and 2-methyl-5-HT

Eighty-nine (86%) of 103 colonic neurons responded to microapplication (30–500 ms) of 5-HT with fast or slowly activating membrane depolarization, or with both responses (Fig. 1A). These responses were reminiscent

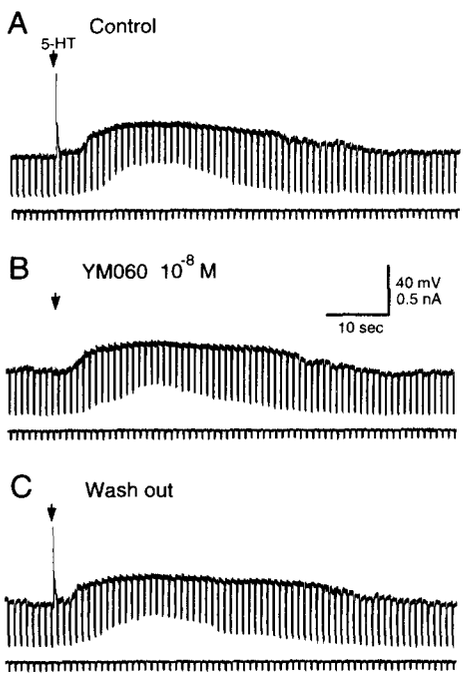


Fig. 1. Fast and slowly activating membrane depolarization evoked in an AH/Type 2 colonic myenteric neuron by 5-HT and selectivity of YM060 for inhibition of the fast response. A: 5-HT (1 mM, 200 ms 'puff') evoked fast and slow membrane depolarization. Constant current hyperpolarizing pulses were (downward deflections) injected through the microelectrode to assess changes in input resistance. B: The fast but not the slow response was abolished by application of YM060 ($10^{-8}$ M). C: Recovery of fast response 10 min after washout of YM060. Upper traces are transmembrane voltage; lower traces are injected current. Arrow indicates the time of 5-HT microejection.

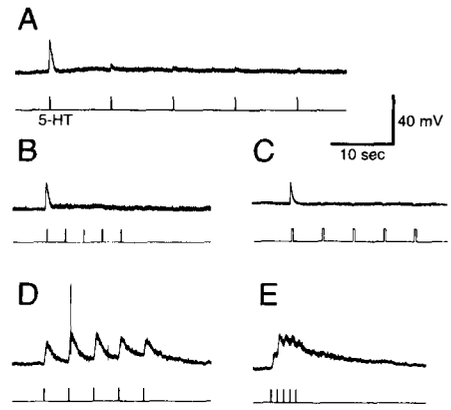


Fig. 2. Desensitization of 5-HT-evoked fast depolarizing responses. A and B: Reduction of fast membrane depolarization occurred in an AH/Type 2 neuron when 5-HT was applied repeatedly at intervals of 10 s (A) and 3 s (B). C: Complete suppression of fast response occurred in a Type 3 colonic neuron (interval: 5 s). D and E: No desensitization (D) but summation (E) of fast depolarization occurred in an S/Type 1 neuron with intervals of 4 and 1 s, respectively. Upper traces are transmembrane voltage; lower traces indicate timing of micropressure pulses of 5-HT.

of 5-HT-induced fast and slow membrane depolarizations in myenteric neurons of the small intestine mediated by $5\text{-HT}_3$ and $5\text{-HT}_{1P}$ receptors, respectively (Mawe et al., 1986). Fast responses always occurred within 1 s of the onset of the 'puff' of 5-HT. They were always accompanied by a decrease in input resistance and occasionally by the discharge of action potentials. Slowly activating membrane depolarization started more than 2 s after 5-HT application and lasted for 2–10 s. Slow responses were accompanied by both increases and decreases in input resistance.

5-HT evoked fast membrane depolarizations in 40 (77%) S/Type 1, 26 (96%) AH/Type 2, 2 (67%) Type 3 and 12 (57%) Type 4 neurons, whereas slow responses were observed in 22 (42%) S/Type 1, 12 (44%) AH/Type 2, and 7 (33%) Type 4 neurons. Both fast and slow responses occurred in 18 (35%) S/Type 1, 11 (41%) AH/Type 2, and 3 (14%) Type 4 neurons (Fig. 1). None of the AH/Type 2 neurons failed to respond to 5-HT.

As shown in Fig. 2 (A, B and C), the 5-HT-evoked fast responses desensitized rapidly when 5-HT was applied at intervals of less than 10 s. This was characteristic of $5\text{-HT}_3$ receptor-mediated responses and was probably due to desensitization of the receptor (Derkach et al., 1989). However, some S/Type 1 neurons had longer fast responses than the other types and did not show the rapid desensitization characteristically found in other regions of the digestive tract (Fig. 2D, E). The mean duration of the fast responses in S/Type 1 neurons was significantly longer than that in the other subtypes (8.23 ± 1.52 s for 11 neurons vs. 1.63 ±

0.25 s for 12 neurons when 5-HT was applied with a pulse durations of 100 ms).

Microapplication of 2-methyl-5-HT with short pulse durations (100–300 ms) mimicked fast responses to 5-HT, but not slow responses. The properties of the desensitization of fast responses to 2-methyl-5-HT were similar to those of the responses to 5-HT.

### 3.2. Effect of YM060 on 5-HT-induced depolarizations

Application of YM060 in the superfusion solution at concentrations from $3 \times 10^{-10}$ to $3 \times 10^{-8}$ M suppressed 5-HT-evoked fast responses in a reversible and concentration-dependent manner in neurons of all electrophysiological subtypes (Figs. 1 and 3). In contrast, YM060 had no significant effect on 5-HT-evoked slow response at concentrations up to $10^{-8}$ M (Fig. 1). The fast responses evoked by 2-methyl-5-HT were also suppressed by YM060 reversibly and concentration-dependently. There were no significant differences among the 4 electrophysiological subtypes in the inhibitory effects of YM060 on 5-HT-evoked fast responses.

The blocking action of YM060 was unaffected by the presence of tetrodotoxin at $2 \times 10^{-7}$ M in the superfusion solution ($n = 3$), indicating that suppression of 5-HT-evoked fast responses by YM060 was by direct competitive blockade of 5-$HT_3$ receptors and not through any indirect action, such as the release of other neurotransmitters from neurons projecting onto the impaled neurons. YM060 did not affect electrophysiological parameters of the impaled neurons, including resting membrane potential, input resistance, and amplitude and duration of action potentials. Fast EPSPs evoked by stimulation of fiber tracts were unchanged by YM060 during the inhibition of 5-HT-induced fast responses. This confirms the absence of any local anesthetic effect on action potential initiation and propagation in the axons.

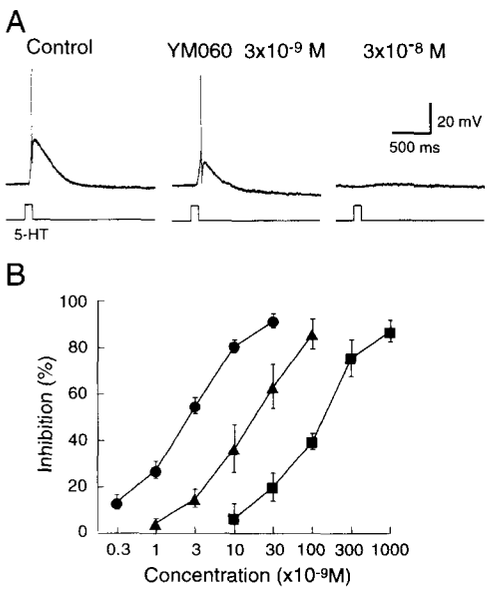


Fig. 3. Concentration-dependent suppression of 5-HT-evoked fast membrane depolarization by 5-$HT_3$ receptor antagonists. A: Inhibition of fast response to 5-HT (1 mM, 100 ms) by YM060 at concentrations of $3 \times 10^{-9}$ and $3 \times 10^{-8}$ M in an AH/Type 2 colonic neuron. Upper traces are transmembrane voltage; lower traces indicate timing of micropressure pulses of 5-HT. B: Concentration-response curves for 5-$HT_3$ receptor antagonists, YM060 (●), granisetron (▲) and ondansetron (■) suppression of the amplitude of 5-HT-evoked responses in myenteric neurons of the guinea pig distal colon. The results are the mean ± S.E.M. in 4–21 neurons.

Table 1
Potencies of 5-$HT_3$ receptor antagonists in myenteric neuron of the guinea pig distal colon

| Compound | $pIC_{50}$ (95% confidence limits) | Number of experiments |
|---|---|---|
| YM060 | 8.62 (8.52–8.92) | 7–21 |
| Granisetron | 7.77 (7.70–7.84) | 4 |
| Ondansetron | 6.90 (6.73–7.06) | 4–7 |

$pIC_{50}$: negative logarithmic value of the concentration required to inhibit 5-HT response by 50%.

### 3.3. Effects of 5-$HT_3$ receptor antagonists other than YM060

Granisetron and ondansetron, like YM060, suppressed 5-HT-evoked fast responses in a concentration-dependent and reversible manner (Fig. 3). The potency of these antagonists as compared by $pIC_{50}$ values (Table 1) was strongest for YM060, at approximately 7 and 53 times greater potency than granisetron and ondansetron, respectively.

Neither methysergide at a concentration of $10^{-5}$ M nor GR113808 at $10^{-7}$ M had any significant effect on the fast 5-HT-evoked responses, in contrast to YM060 which abolished the responses in the same neurons (data not shown). These concentrations of methysergide and GR113808 effectively antagonize responses mediated by 5-$HT_1$ and 5-$HT_2$ receptors, and 5-$HT_4$ receptors, respectively.

## 4. Discussion

In most mammalian species, approximately 90% of endogenous 5-HT is estimated to occur within the gastrointestinal tract (Erspamer, 1966; Furness and Costa, 1982). Although most is present in enterochromaffin cells in the epithelial layer, it has been estab-

lished that 5-HT meets the basic functional criteria for classification as a neurotransmitter within the enteric nervous system (Wood, 1987).

Microapplication of 5-HT from fine-tipped microelectrodes has been used in several published studies to characterize the effects of 5-HT on the electrical and synaptic properties of myenteric neurons and the 5-HT receptor subtypes in the enteric nervous system. By adjusting the pulse duration for pressure microejection or by iontophoresis it is possible to effectively control the concentration of 5-HT reaching the impaled neurons. Initial studies in myenteric neurons of the small intestine (Wood and Mayer, 1979; Johnson et al., 1980) failed to show fast membrane depolarization mediated by 5-$HT_3$ receptors, whereas subsequent studies confirmed that most AH/Type 2 neurons in the myenteric plexus (Mawe et al., 1986) and neurons in the submucous plexus (Surprenant and Crist, 1988; Frieling et al., 1991) respond to microapplication of 5-HT with both fast and slowly activating depolarization. In the present study, we confirmed the characteristic effects of 5-HT on colonic myenteric neurons (Wade and Wood, 1988a). Fast membrane depolarization was observed in most AH/Type 2 neurons and in the majority of non-AH type neurons.

The fast response evoked by 5-HT was mimicked by the selective 5-$HT_3$ receptor agonist 2-methyl-5-HT when applied in short (100–300 ms) 'puffs'. The potency of ondansetron, a specific 5-$HT_3$ receptor antagonist, against 5-HT-evoked fast responses was similar to that found in submucous neurons of the small intestine (Vanner and Surprenant, 1990). YM060, granisetron and ondansetron suppressed the fast responses evoked by 5-HT, with antagonistic potencies in good agreement with those obtained for inhibition of 5-HT-induced contractions in the same preparations (Miyata et al., 1991). These results provide further evidence that 5-HT-evoked fast depolarizing responses are mediated by the 5-$HT_3$ receptor subtype on colonic myenteric neurons.

The slowly activating response to 5-HT mimicked slow synaptic excitation (slow EPSP) in AH/Type 2 neurons (Wood, 1987). Neither YM060, granisetron nor ondansetron suppressed the slowly activating responses in the present study. The receptor subtype which mediates 5-HT-evoked slow responses as well as some slow EPSPs in myenteric neurons has been identified as the 5-$HT_{1P}$ receptor (Mawe et al., 1986). The receptor subtype responsible for slow EPSP-like responses to 5-HT in colonic myenteric neurons is not the 5-$HT_3$ receptor subtype and remains unidentified.

The fast responses to 5-HT in the colonic S/Type 1 neurons differed from those of the other types: these had a longer durations and were resistant to desensitization. On the other hand, the potency of each 5-$HT_3$ receptor antagonist was identical for all types of neurons. This suggests the possibility that different types of 5-$HT_3$ receptors exist on colonic myenteric neurons. Apparent splice variants of the murine 5-$HT_3$ receptor subunit termed the 5-$HT_3$ R-A and 5-$HT_3$ R-As were recently reported (Hope et al., 1993; Downie et al., 1994). Compared with 5-$HT_3$ R-A, 5-$HT_3$ R-As had deletions of six consecutive amino acids in the putative large intracellular loop between the predicted third and fourth transmembrane regions. This resulted in the production of a lower maximum current during activation by 2-methyl-5-HT when expressed in *Xenopus* oocytes. This suggests that the different properties of the fast responses found in the present study might be based on differences in the amino acid sequence of the receptors.

Recently, Miyata et al. (1992) suggested that stress-induced gastrointestinal dysfunction is, at least in part, attributable to 5-HT via 5-$HT_3$ receptors. In the study, the selective 5-$HT_3$ receptor antagonists YM060, granisetron and ondansetron inhibited stress-induced defecation in restrained rats. In humans, stress is commonly associated with gastrointestinal disorders such as the irritable bowel syndrome (Thompson, 1984). Previous studies have shown that post-prandial plasma concentration of 5-HT was increased in patients with this condition (Bearcroft et al., 1994). Moreover, rectal sensitivity and post-prandial motility were improved by a 5-$HT_3$ receptor antagonist (Prior and Read, 1993) These observations suggest that 5-HT might be involved in this disorder. In view of the findings of our study, we are inclined to believe that the mechanism underlying the inhibitory effect of 5-$HT_3$ receptor antagonists on stress-induced defecation may be the antagonism of the 5-HT-evoked fast response in colonic myenteric neurons.

In the present study, YM060 up to $10^{-8}$ M had no significant effect on fast EPSP. It has been shown that other 5-$HT_3$ receptor antagonists including granisetron, ondansetron and tropisetron have nicotinic receptor blocking actions at concentrations approximately 100–300 times higher than those required to block 5-$HT_3$ receptors in myenteric and submucous neurons of the small intestine (Sanger and Nelson, 1989; Vanner and Surprenant, 1990). YM060 $10^{-5}$ M, however, did not affect nicotinic depolarization in rabbit nodose ganglion and rat vagus nerve (Ito et al., 1992,1995), and our preliminary data showed that YM060 up to $10^{-5}$ M did not suppress the contraction of isolated guinea pig distal colon evoked by dimethylphenyl piperazinium, a nicotinic agonist. Therefore, the selectivity of YM060 for 5-$HT_3$ receptors over nicotinic receptors is considered to be much greater than that of other 5-$HT_3$ receptor antagonists.

In conclusion, functional 5-$HT_3$ receptors that mediate 5-HT-evoked fast membrane depolarization exist on myenteric neurons of the guinea pig distal colon, as

well as on neurons of the small intestine and stomach. Moreover, YM060 is a potent and selective 5-$HT_3$ receptor antagonist of these receptors. Unlike other neuronal types in the colon and all of the kinds of neurons elsewhere in the digestive tract, 5-$HT_3$ receptor-mediated responses in S/Type 1 neurons do not desensitize rapidly when exposed to 5-HT.

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