5-Hydroxytryptophan activates colonic myenteric neurons and propulsive motor function through 5-HT₄ receptors in conscious mice

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Wang L, Martínez V, Kimura H, Taché Y. 5-Hydroxytryptophan activates colonic myenteric neurons and propulsive motor function through 5-HT₄ receptors in conscious mice. Am J Physiol Gastrointest Liver Physiol 292: G419–G428, 2007. First published September 21, 2006; doi:10.1152/ajpgi.00289.2006.—Serotonin [5-hydroxytryptamine (5-HT)] acts as a modulator of colonic motility and secretion. We characterized the action of the 5-HT precursor 5-hydroxytryptophan (5-HTP) on colonic myenteric neurons and propulsive motor activity in conscious mice. Fos immunoreactivity (IR), used as a marker of neuronal activation, was monitored in longitudinal muscle/myenteric plexus whole mount preparations of the distal colon 90 min after an intraperitoneal injection of 5-HTP. Double staining of Fos IR with peripheral choline acetyltransferase (pChAT) IR or NADPH-diaphorase activity was performed. The injection of 5-HTP (0.5, 1, 5, or 10 mg/kg ip) increased fecal pellet output and fluid content in a dose-related manner, with a peak response observed within the first 15 min postinjection. 5-HTP (0.5–10 mg/kg) dose dependently increased Fos expression in myenteric neurons, with a maximal response of 9.9 ± 1.0 cells/ganglion [P < 0.05 vs. vehicle-treated mice (2.3 ± 0.6 cells/ganglion)]. There was a positive correlation between Fos expression and fecal output. Of Fos-positive ganglionic cells, 40 ± 4% were also pChAT positive and 21 ± 5% were NADPH-diaphorase positive in response to 5-HTP, respectively. 5-HTP-induced defecation and Fos expression were completely prevented by pretreatment with the selective 5-HT₄ antagonist RS-39604. These results show that 5-HTP injected peripherally increases Fos expression in different populations of cholinergic and nitric myenteric neurons in the distal colon and stimulates propulsive colonic motor function through 5-HT₄ receptors in conscious mice. These findings suggest an important role of activation of colonic myenteric neurons in the 5-HT₄ receptor-mediated colonic propulsive motor response.

cholinergic neurons; NADPH-diaphorase; peripheral choline acetyltransferase; serotonin; serotonin receptors

IN MAMMALS, over 95% of the body’s serotonin [5-hydroxytryptamine (5-HT)] is produced and stored in the gastrointestinal tract (18, 19). 5-HT is localized mainly in enterochromaffin (EC) cells of the intestinal epithelium (4) but also, to a smaller extent, in neurons of the myenteric plexus (16, 52). EC cells respond to chemical and mechanical stimuli by releasing 5-HT, which acts on 5-HT receptors located in gut neural structures and smooth muscle cells (19). In the intestine, 5-HT has long been known to increase intestinal propulsive motility and the secretion of water and electrolytes, which lead to the induction of defecation and, in some cases, diarrhea in animal models and humans (2, 7, 18, 19, 54, 56).

5-HT has also been implicated in the pathophysiology of several intestinal disorders, including functional bowel disorders such as irritable bowel syndrome (IBS) (3, 19, 34). In postinfectious or diarrhea-predominant IBS patients, reports have indicated the existence of significant cellular and molecular alterations of gut serotonergic signaling mechanisms, including an increase in the number of rectal EC cells, enhanced postprandial release of 5-HT, and altered expression of the 5-HT transporter (3, 8, 10, 13, 14, 34). Similar changes in the gut serotonergic system, resulting in the increased availability of 5-HT, have also been described in a mouse model of postinfectious IBS (63).

The colonic motor and secretory changes associated with increased levels of gut 5-HT are largely mediated through 5-HT₃ (19, 59) as well as 5-HT₄ receptors (27, 47). In rodents, 5-HT₄ receptors mediate colonic propulsive motor activity and defecation in response to exogenous or endogenous 5-HT, as shown by the use of peripheral injection of selective 5-HT₄ agonists and antagonists under in vivo and in vitro conditions (2, 22, 29, 41, 54, 56, 58). Clinical observations have also demonstrated that selective 5-HT₄ receptor agonists accelerate colonic transit in healthy subjects (1, 6) and in patients with the constipation-predominant form of IBS (11, 19). On the other hand, 5-HT₃ antagonists have been reported to have antidiarrheic effects in diarrhea-predominant IBS patients (20, 25). However, 5-HT₃ antagonists failed to influence human in vivo colonic or in vitro jejunal peristalsis (5, 15). In rodents, 5-HT₃ antagonists blocked 5-hydroxytryptophan (5-HTP)- or stress-induced stimulation of defecation (40, 47), and, in guinea pigs, recruitment of both 5-HT₃ and 5-HT₄ receptors may be needed to fully elicit 5-HT-induced stimulation of colonic motor function (27, 28). The prokinetic effect of 5-HT₃ receptors is mediated by afferent signaling from the gut to the brain via extrinsic afferent nerves and submucosal sensory neurons (19, 39). Supporting evidence is that 5-HT₃ receptors are localized on intrinsic sensory neurons and extrinsic sensory nerve fibers that innervate the rat colon (35), whereas 5-HT₄ receptors exist in interneurons and motor and sensory neurons as well as in smooth muscle cells in the intestine of rodents, guinea pigs, and humans (26, 32, 48, 51). Therefore, 5-HT₄ receptors are likely to be primarily involved in local mechanisms mediating the motor and secretory effects of 5-HT at a peripheral level.
Convergent data have established that the direct activation of 5-HT4 receptors on colonic smooth muscle is inhibitory. Selective 5-HT4 agonists induce a TTX-independent relaxation of the isolated human circular smooth muscle that is blocked by selective 5-HT4 antagonists (49, 54, 59). By contrast, the neuronal action of 5-HT4 receptor agonists is excitatory and exerted mainly by facilitating fast excitatory synaptic transmission, as shown by electrophysiological monitoring of myenteric neuron activity (31, 32). However, these prior studies on 5-HT4-mediated myenteric actions have been all carried out in vitro using mainly guinea pig ileum longitudinal muscle/myenteric plexus (LMMP) preparations (31, 46), with the exception of one recent study (32) performed in cultured colonic myenteric neurons of young mice. We previously used Fos as a marker of neuronal activation (12) to establish that peripheral myenteric neurons of young mice. We previously used Fos as a marker of neuronal activation (12) to establish that peripheral injection of corticotropin-releasing factor (CRF) induces a CRF1 receptor-mediated activation of colonic myenteric neurons and propulsive motor function in conscious rats (36). This approach allowed us to monitor changes in the activity of myenteric neurons in conscious animals coupled with the simultaneous monitoring of functional changes of propulsive colonic activity leading to defection.

The aim of the present study was to examine the colonic myenteric and propulsive responses induced by peripheral injection of the 5-HT precursor 5-HTP in conscious mice. 5-HTP was chosen to mimic a state of endogenous 5-HT production and release (55) at intestinal sites that normally synthesize 5-HT (64). 5-HTP-induced neuronal myenteric activation was assessed using Fos immunohistochemistry. The neurochemical phenotype of 5-HTP-activated neurons was examined using double labeling with Fos and peripheral choline acetyltransferase (pChAT), a marker of peripheral cholinergic neurons (43), or with NADPH-diaphorase, a cofactor of nitric oxide (NO) synthase (24). 5-HT4 receptor-mediated actions of 5-HTP were assessed using the potent and selective 5-HT4 antagonist RS-39604 (23). Finally, the magnitude of 5-HTP-induced defection was compared with that of intra-peritoneal injection of CRF-induced myenteric-mediated stimulation of colonic motor function in rodents (33, 36).

MATERIALS AND METHODS

Animals

Adult male C57BL/6 mice (6–8 wk of age; Harlan, San Diego, CA) were maintained on a 12:12-h light-dark cycle with controlled temperature (21–23°C) and humidity (30–35%). Animals were housed in direct bedding cages with free access to food (Prolab RMH 2500, PMI Nutrition, Brentwood, MO) and tap water. Animals were moved to the experimental room 18–20 h before the experiments and were housed individually, with water and food ad libitum. All the experiments were performed between 8:00 and 11:00 AM. To reduce the number of animals, when functional experiments were performed on colonic motor function, the same animal was used three times with at least a 5-day interval between consecutive experiments with treatments randomly assigned to the animals. Immunohistochemical experiments were conducted in naïve mice. All protocols were approved by the Veterans Affairs Animal Component of the Research Protocol (Nos. 99-092-05, Veterans Affairs Greater Los Angeles Healthcare System).

Compounds

1. 2-Amino-3-(5-hydroxyindolyl)propionic acid (5-HTP; Sigma Chemical, St. Louis, MO) and rat/human CRF (r/hCRF; Clayton Foundation Laboratories for Peptide Biology, Salk Institute, La Jolla, CA) were dissolved in sterile saline. 1-[4-Amino-5-chloro-2-(3,5-dimethoxyphenyl)methoxy]-3-[1-(2-methylsulphonylamino)ethyl]piperidin-4-yl)propan-1-one hydrochloride (RS-39604, hydrochloride form; Tocris, Ellisville, MO) (23) was dissolved in 5% DMSO (Sigma)-5% Tween 80 (Sigma)-90% saline. Either sterile saline or 5% DMSO-5% Tween 80–90% saline, as appropriate, served as the vehicle control. All solutions were prepared immediately before use.

Defecation Score and Fecal Fluid Content

The numbers of fecal pellets excreted were determined at 15-min intervals for the 90-min period after treatments, and cumulative pellet output was calculated at the end of the experimental time. At every 15-min interval, all fecal pellets excreted were collected, weighed (net weight, in mg), desiccated in an oven (50°C, 6 h), and weighed again (dry weight, in mg). The fecal fluid content percentage was calculated according to the following equation: fluid content in (%) = 100 × (net weight − dry weight) ÷ net weight.

Tissue Preparation of the Distal Colonic LMMP

Mice were euthanized by cervical dislocation, and the abdominal cavity was opened. The distal colon was harvested, opened longitudinally along the mesenteric border, stretched, and pinned flat on a Sylgard-coated petri dish (Sylgard 184, Dow Corning, Midland, MI). Tissues were fixed by immersion with 4% paraformaldehyde and 14% saturated picric acid in 0.1 M phosphate buffer (pH 7.4) for 4–6 h at 4°C. Thereafter, tissues were washed several times with 0.1 M PBS (pH 7.4) and maintained in PBS at 4°C. The mucosa, submucosa, and circular muscle layers were carefully removed, yielding a whole mount preparation consisting of the serosa and longitudinal muscle layer with the myenteric plexus attached to its internal side.

Fos Immunohistochemistry

Free-floating LMMP preparations of the mouse distal colon were incubated overnight at 4°C with rabbit polyclonal anti-Fos antibody in 0.01 M PBS (pH 7.4) containing 0.3% Triton X-100 (1:10,000; Oncogene, Cambridge, MA) as a primary antibody. Sections were then incubated with biotinylated secondary goat anti-rabbit IgG (1:1,000; Jackson ImmunoResearch) followed by an incubation with the avidin-biotin-peroxidase complex (1:400; Vector, Burlingame, CA), followed by an incubation with the avidin-biotin-peroxidase complex (1:400; Vector, Burlingame, CA), as previously reported (43, 65). Other procedures were the same as those described above for Fos immunohistochemistry except that the chromogen was DAB with hydrogen peroxide (0.01%). After being washed, sections were mounted, air dried, dehydrated in ethanol, cleared in xylene, and coverslipped. The presence of Fos immunoreactivity (IR) was revealed as a dark brown precipitate located in neuronal nuclei.

Double Staining of Fos and pChAT

LMMP preparations of the distal colon were stained for Fos IR as described above except that the secondary antibody was a Fab fragment (1:1,000; Jackson ImmunoResearch) and DAB staining was enhanced with nickel ammonium sulfate. Free-floating LMMP preparations were incubated with specific pChAT antibody (Dr. H. Kimura) at 1:20,000 dilution in 0.1 M PBS containing 0.3% Triton X-100 for 48 h at 4°C, as previously reported (43, 65). Other procedures were the same as those described above for Fos immunohistochemistry except that the chromogen was DAB without color enhancement. With two cycles of immunostaining, Fos IR was detected as a dark blue nuclear reaction product, and pChAT-like IR (pChAT IR) appeared as brown staining in the cytoplasm. For specificity of the primary antibody, preabsorption with specific antigens was performed. Ten micrograms of Fos (Oncogene) or 5 µg of pChAT peptide were added to the corresponding antibody solutions diluted at the working titer (1:10,000 and 1:20,000, respectively) and incubated for 24 h at 4°C. The preabsorbed solutions were then

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applied to colonic LMMP preparations as the primary antibody incubation. The other immunohistochemical procedures were the same.

**Double Staining of Fos and NADPH-Diaphorase**

Distal colon LMMP preparations were incubated at 37°C for 40 min in a freshly prepared solution containing β-NADPH (0.5 mg/ml; Sigma) and nitroblue tetrazolium (0.1 mg/ml; Sigma) in 0.3% Triton X-100 in 0.1 M phosphate buffer (pH 7.4). After being thoroughly rinsed, tissue preparations were processed for Fos immunohistochemistry as described above. The presence of NADPH-diaphorase activity was detected as a diffused blue staining in the cytoplasm, whereas Fos was localized as a brown nuclear reaction product.

**Cell Counting**

Numbers of immunohistochemically-positive cells were counted under light microscopy in 20 ganglia randomly selected in 3–4 separate regions of each distal colon LMMP preparation. Cell counting was expressed as the mean number of Fos-positive cells per ganglia in each mouse. For the double staining of Fos IR plus pChAT IR and Fos IR plus NADPH diaphorase, data are expressed as percentages of Fos IR plus pChAT IR or Fos- plus NADPH-diaphorase-positive cells from total Fos-positive cells and from total pChAT- or NADPH-diaphorase-positive cells, respectively.

**Experimental Protocols**

**Effects of 5-HTP alone or with 5-HT4 antagonist on defecation and fecal fluid content.** 5-HTP (0.5, 1, 5, or 10 mg/kg), r/hCRF (20 μg/kg), or vehicle (0.1 ml/mouse) was administered intraperitoneally, and fecal pellet output and fecal fluid content were determined at 15-min intervals for the following 90 min. In a separate experiment, mice were injected intraperitoneally with the selective 5-HT4 antagonist RS-39604 (1 or 3 mg/kg) or vehicle (0.1 ml) and 10 min later with 5-HTP (0.5 or 10 mg/kg) or vehicle (0.1 ml). Fecal pellet output and fecal fluid content were determined for the following 90 min. In each daily experiment, vehicle controls and different doses of the test substances were included and repeated on multiple days in different animals in a randomized fashion. Doses of 5-HTP, RS-39604, and CRF were based on preliminary data and previous reports (2, 33, 47).

**Statistical Analysis**

Results are expressed as means ± SE. Comparisons within multiple groups were performed using one-way ANOVA followed by a Student-Newman-Keuls multiple-comparison test whenever appropriate. Comparisons between two groups were performed by Student’s t-test. The correlation between Fos expression and fecal pellet output was determined using linear regression. P values of <0.05 were considered statistically significant.

**RESULTS**

**Stimulatory Effects of 5-HTP and r/hCRF Injected Intraperitoneally on Fecal Pellet Output and Fecal Fluid Content**

In conscious mice, 5-HTP injected at 0.5, 1, and 5 mg/kg dose dependently increased fecal pellet output, with a plateau response at 5 and 10 mg/kg (Fig. 1 and Table 1). A time-course study showed a rapid onset with a peak increase of fecal pellet output and water content reached within 15 min postinjection. Thereafter, defecation scores returned to basal levels for the

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**Table 1. Effects of 5-HTP on fecal scores in mice**

<table>
<thead>
<tr>
<th>Number of mice</th>
<th>Number of fecal pellets</th>
<th>Fecal output, mg wet wt</th>
<th>Water content, %</th>
<th>Number of fecal pellets</th>
<th>Fecal output, mg wet wt</th>
<th>Water content, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HTP 0.5 mg/kg</td>
<td>6</td>
<td>0.8±0.7</td>
<td>19±13</td>
<td>49.9±4.3</td>
<td>3.3±0.7</td>
<td>106±28</td>
</tr>
<tr>
<td>1 mg/kg</td>
<td>6</td>
<td>6.0±0.6*</td>
<td>223±21*</td>
<td>77.5±1.0*</td>
<td>10.8±1.4*</td>
<td>339±37*</td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>6</td>
<td>9.3±1.7*</td>
<td>316±52*</td>
<td>70.0±3.0*</td>
<td>10.3±1.8*</td>
<td>337±53*</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>6</td>
<td>9.8±0.7*</td>
<td>412±29*</td>
<td>74.3±2.2*</td>
<td>12.8±1.0*</td>
<td>486±28*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.0±1.6*</td>
<td>261±76*</td>
<td>73.6±3.1*</td>
<td>10.8±1.1*</td>
<td>335±55*</td>
</tr>
</tbody>
</table>

Data represent means ± SE of fecal scores at 15 min and cumulative scores for the 90-min experimental time. Vehicle or 5-hydroxytryptophan (5-HTP) was administered intraperitoneally, and the fecal output was determined at 15-min intervals for the following 90 min. *P<0.05 vs. vehicle.
remaining 75-min period (Fig. 1 and Table 1). Although most of the fecal output was in the form of defined pellets, soft stools could be seen at all doses of 5-HTP. At 5 and 10 mg/kg, 5-HTP also induced diarrhea-like pellets (2–3 pellets of the total pellets from each mouse).

CRF (20 μg/kg ip) increased fecal pellet output to 7.9 ± 1.6 pellets/90 min (n = 7) with a time course similar to that described for 5-HTP. The peak defecatory response was observed within the first 15 min after administration [r/hCRF: 4.4 ± 1.0 pellets/15 min, P < 0.05 vs. vehicle (0.8 ± 0.7 pellets/15 min)]. The pellets produced by intraperitoneal r/hCRF showed no signs of diarrhea, although there was a trend of increase in fecal fluid content (62.1 ± 3.2% vs. 49.9 ± 4.3% in intraperitoneal vehicle, P = 0.071).

Based on the time course of 5-HTP-induced defecation, fecal pellet output and fluid content were determined only during the first 30-min period after 5-HTP in all subsequent experiments.

Fig. 2. Blockade of 5-HTP-induced defecation by the 5-HT₃ antagonist RS-39604 in conscious mice. RS-39604 (1 or 3 mg/kg ip) or vehicle (0.1 ml ip) was injected immediately before 5-HTP (0.5 or 10 mg/kg ip) or vehicle (0.1 ml ip), and fecal pellet output (A), fecal wet weight (B), and fecal water fluid content (C) were determined 30 min later. Data corresponding to the RS-39604 + vehicle group represent a pooled group for the two doses of RS-39604 tested. Each column represents the mean ± SE of n = 5–9 mice/group. *P < 0.05 vs. vehicle + vehicle; #P < 0.05 vs. the respective 5-HTP + vehicle group (by ANOVA).

RS-39604 Blocks Intraperitoneal 5-HTP-Induced Increases in Fecal Output and Fluid Content

In vehicle + vehicle-treated mice, pellet output during the 30-min experimental time was 2.4 ± 0.8 pellets/30 min (n = 7). RS-39604 injected intraperitoneally at either 1 or 3 mg/kg followed by intraperitoneal vehicle (n = 4 and 5, respectively) showed a similar tendency to reduce fecal pellet output, although statistical significance was not reached. Therefore, the two doses were pooled as a single group, with a 30-min cumulative pellet output of 0.7 ± 0.3 pellets/30 min (P > 0.05 vs. vehicle + vehicle; Fig. 2A). Pretreatment with RS-39604 at 1 mg/kg completely prevented the stimulatory effects of 5-HTP at 0.5 mg/kg (RS-39604 + 5-HTP: 1.4 ± 0.6 pellets/30 min, n = 5; vehicle + 5-HTP: 5.8 ± 0.9 pellets/30 min, n = 6; P < 0.05) while having only a partial antagonist action when 5-HTP was injected at 10 mg/kg (RS-39604 + 5-HTP: 5.3 ± 0.8 pellets/30 min, n = 9; vehicle + 5-HTP: 8.4 ± 1.2
pellets/30 min, n = 7; P < 0.05). RS-39604 at 3 mg/kg completely prevented the stimulatory effects of 5-HTP at 10 mg/kg (RS-39604 + 5-HTP: 1.6 ± 0.4 pellets/30 min, n = 5, P < 0.001 vs. vehicle + 5-HTP; Fig. 2A).

Similar inhibitions were observed for fecal wet weight and fluid content. RS-39604 per se, at either 1 or 3 mg/kg, had a tendency to reduce fecal wet weight and significantly reduced fluid content by 20% (Fig. 2, B and C). The 5-HT4 antagonist injected intraperitoneally at 1 mg/kg blocked the increase in fluid content and fecal wet weight induced by 5-HTP at 0.5 mg/kg; and, at 1 and 3 mg/kg, RS-39604 prevented, in a dose-related manner, the 5-HTP (10 mg/kg)-induced increase in fecal wet weight and fluid content (Fig. 2, B and C).

5-HTP Induces Fos Expression in Distal Colonic Myenteric Ganglia

In colonic LMMP preparations, myenteric ganglia were recognized as clearly delineated groups of neurons separated by well-defined internodal connections. The presence of Fos IR was restricted to cells located within the myenteric ganglia, and no Fos IR was observed in smooth muscle cells. LMMP preparations processed with the primary antibody preabsorbed with an excess of Fos protein did not show any Fos IR.

In mice injected intraperitoneally with vehicle, only a few scattered Fos-positive cells were observed in distal colonic myenteric ganglia (2.3 ± 0.6 cells/ganglion, n = 9). 5-HTP (0.5, 1, 5, or 10 mg/kg, n = 3–7 for each dose) dose dependently increased Fos expression by two-, two-, four-, and fivefold, respectively, over control values (Fig. 3). In the same animals, 5-HTP at 5 and 10 mg/kg also stimulated fecal pellet output to 13.7 ± 1.9 and 9.9 ± 1.0 pellets/30 min, respectively [both P < 0.05 vs. vehicle (3.0 ± 0.8 pellets/30 min), F(4,22) = 9.342, P < 0.001]. The peak defecatory response occurred during the first 15 min after 5-HTP administration. Regardless of the 5-HTP doses tested, there was a linear correlation (r = 0.62, P = 0.0028) between pellet output during the 30-min period and the mean numbers of Fos-positive cells observed in myenteric ganglia of the distal colon, as determined in the same animals (Fig. 4).

RS-39604 Prevents 5-HTP-Induced Fos Expression in Colonic Myenteric Ganglia

Fos expression in myenteric ganglia in mice treated with vehicle + vehicle was low (2.7 ± 0.5 cells/ganglion, n = 4; Fig. 5). In vehicle-pretreated animals, 5-HTP (10 mg/kg ip) increased the numbers of Fos-positive cells in the myenteric ganglia of the distal colon and fecal pellet output by 4.8- and 4.4-fold, respectively (Fig. 5). In accordance with previous experiments, the maximal pellet output response was observed during the 15-min period after 5-HTP administration. Pretreatment with RS-39604 prevented both the increase in Fos expression and stimulation of fecal pellet output induced by 5-HTP administration (Fig. 5, A and B). RS-39604, by itself, did not significantly modify Fos expression in distal colonic myenteric ganglia (Fig. 5A).

5-HTP Induces Fos-IR in a Population of pChAT-IR Myenteric Neurons in Mouse Distal Colonic LMMP Preparations

pChAT IR was found in numerous ganglion cells and was abundantly distributed in myenteric nerve fibers both within and between ganglia of the mouse distal colon (Fig. 6A). No pChAT-positive cell bodies were localized outside ganglionic
MOUSE COLONIC MYENTERIC NEURONS ACTIVATED BY 5-HTP

The present study demonstrates that 5-HTP injected intraperitoneally in conscious mice increases Fos expression in colonic myenteric ganglia, as detected by immunohistochemistry in LMMP preparations of the distal colon, indicative of enteric neuronal activation (36, 37). The few Fos-positive cells detected in myenteric neurons after intraperitoneal vehicle and the dose-related increase in Fos expression induced by intraperitoneal 5-HTP show the specificity of the 5-HT precursor action. Monitoring Fos expression has been used previously to gain insight into changes in colonic myenteric activity after various treatments. However, all previous studies were restricted to rat or guinea pig colonic LMMP preparations (36, 37, 60, 67). The present study shows that Fos expression can be monitored in distal colonic myenteric neurons of mice, allowing the combined characterization of colonic myenteric neuronal activity and functional changes.

Convergent neuroanatomical and pharmacological evidence supports that intraperitoneal 5-HTP-induced Fos expression is mediated through 5-HT action on colonic 5-HT₄ receptors. First, 5-HT can be synthesized from 5-HTP within the gut wall, including colonic myenteric cell bodies and fibers as well as within EC and mast cells (64). Second, transcripts encoding the 5-HT₄α and 5-HT₄β splice variants of the 5-HT₄ receptor are abundantly expressed in the mouse colon (9, 32). Immunohistochemistry also revealed dense 5-HT₄ receptor labeling in mouse colonic myenteric ganglia (32, 48). Finally, pretreatment with RS-39604, a potent and selective 5-HT₄ antagonist that displays a 1,000-fold selectivity on 5-HT₄ over 5-HT₁A, 5-HT₂C, and 5-HT₃ receptors (23), completely prevented the occurrence of Fos expression in distal colonic myenteric neurons in response to an intraperitoneal injection of 5-HTP. In previous studies, the 5-HT₄-mediated excitatory action of 5-HT on myenteric neurons was investigated mainly in vitro in guinea pig ileum LMMP preparations (31, 46). More recently, a report (32) using cultured colonic myenteric neurons of young mice indicated that 5-HT₄ receptor activation increases the amplitude of nicotinic-evoked postsynaptic currents, suggesting a presynaptic action that strengthens excitatory neurotransmission. In addition, 5-HT₄-containing neurons localized by immunohistochemistry in the mouse did not show either regional differences in the small intestine and colon (32) or species difference among rats, mice, and guinea pigs (48). This supports the evidence showing that mouse colonic LMMP preparation can be another model to study 5-HT₄ receptor-mediated modulation of colonic motor function.

The biochemical coding of 5-HT-activated neurons in the distal colonic LMMP preparation was investigated in relation to well-established cholinergic excitatory and NO inhibitory myenteric neurotransmitters involved in the regulation of the colonic peristaltic reflex in the mouse (21). The pChAT antisera used in the present study was previously characterized by Western blot to be selective for the peripheral variant of ChAT, which is primarily expressed in enteric neurons (43, 61, 65). In a whole mount preparation cultured with cholincine, ChAT-positive myenteric neurons were detected in the mouse colon, although under these conditions, neuronal terminals were weakly stained (53). In the present study, the use of pChAT not only displayed more abundant neuronal cell bodies and fibers in the mouse distal colon than ChAT labeling but also enabled us to visualize cholinergic neurons in noncholincine-treated LMMP preparations obtained after monitoring defeation in vivo and to correlate double labeling with Fos to functional changes and neuronal myenteric activity. We found that, after the 5-HTP injection, 40% of Fos-positive neurons were double labeled with pChAT and that Fos expression was prevented by a selective 5-HT₄ antagonist, showing 5-HT₄...
receptor-mediated activation of a population of cholinergic myenteric neurons. Most of the cholinergic cells in the myenteric plexus of the mouse distal colon displayed features of Dogiel type I neurons (53), which include motor neurons and interneurons (44). The colocalization of Fos IR and pChAT IR occurred mostly in this group of neurons. Although not further characterized, activated neurons are likely to encompass cholinergic myenteric motor neurons that are involved in the contractile response of the ascending component of the peristaltic reflex (21). Interestingly, 5-HT₄ receptors have recently been identified on Dogiel type II neurons of mouse colonic myenteric ganglia, which correspond to intrinsic primary afferent neurons whose activation triggers motility and secretory reflexes (48). Although we could identify, with relative frequency, pChAT-positive, Dogiel type II-like neurons in LMMP preparations, these neurons were mostly Fos negative, suggesting that most of them were not activated.

In the rat enteric nervous system, none of the pChAT-positive neurons were found double stained with NADPH-diaphorase (43), suggesting that the non-pChAT neurons activated by intraperitoneal 5-HTP in the colon may encompass, at least in part, a distinct population of NO-synthesizing neurons. Indeed, we demonstrated that ~21% of the 5-HTP-activated cells in myenteric ganglia of the distal colon were also NADPH-diaphorase positive. The ascending and descending components of the peristaltic reflex encompass excitatory (cholinergic/tachykinergic) and inhibitory (VIP/PACAP/NO) motor neurons that act orally and caudally, respectively, to coordinate propulsive motor responses in the mouse colon (21). We found that the 5-HT₄ antagonist completely prevented Fos expression in myenteric neurons, which include both pChAT- and NADPH-positive cells. In addition, there was a correlation between the Fos expression in distal colonic myenteric neurons and colonic propulsive motor function elicited by 5-HTP. Distention in isolated intestinal segments from the guinea pig has been reported to induce Fos expression in myenteric neurons (50), suggesting a possible role of mechanical events in the Fos response. However, it is unlikely that Fos expression in distal colonic myenteric neurons is secondary to increased colonic motility induced by intraperitoneal 5-HTP. First, 180 min of intestinal distention was required to induce the Fos response, whereas in our experiments, colonic propulsive motility was increased only for the first 15 min. In addition, in our previous studies, Fos expression occurring in response to...
central vagal activation in gastric or colonic myenteric neurons induced by intraperitoneal CRF was not modified by atropine at doses that blocked the increased motility (36, 38, 66). In addition to direct peripheral effects on the myenteric nervous system, whether 5-HTP, known to increase 5-HT in the central nervous system, could also influence Fos expression through central pathways needs to be investigated. However, kinetics of 5-HT occurrence in the brain after 5-HTP administration is more delayed compared with the viscera, and the doses at which central nervous system actions of 5-HTP have been reported are about 10-fold higher than those in the periphery (62). Taken together, these data provide strong support, in an in vivo model, of previous in vitro data pointing to the peripheral activation of 5-HT4 receptors being responsible for the initiation of the peristaltic reflex in the mouse distal colon (21).

In addition to enteric neurons, colonic smooth muscles, unlike epithelial cells, are also endowed with 5-HT4 receptors, as detected both at gene and protein levels in mice (32, 48). However, activation of 5-HT4 receptors located on smooth muscle cells results largely in an inhibitory response, as shown by the TTX-independent relaxation of isolated canine and human rectal circular smooth muscle and rat ileum longitudinal segments induced by selective 5-HT4 agonists and the selective blockade of these responses by 5-HT4 antagonists (30, 49, 54, 59). It cannot be ruled out that the recruitment of these inhibitory mechanisms occurring directly on smooth muscle cells may counteract the stimulatory response and plays a role in the short-lasting action of 5-HTP and the trend to have a maximum colonic motor effect (pellet output and weight) at 5 mg/kg and a lesser effect at 10 mg/kg.

In addition to its motor effects, 5-HTP injected intraperitoneally increased fecal fluid content, and, for the higher doses tested, diarrheic responses could be seen within 15–30 min. These data are consistent with previous observations in mice injected with 5-HTP subcutaneously or intraperitoneally (2, 42, 47). This probably reflects specific 5-HTP-mediated effects on colonic secretion of fluid and electrolytes rather than an increased passage of fecal content. 5-HT evokes fluid diarrhea through the stimulation of chloride and mucus secretion from the intestinal mucosa (7). Morphological studies have shown the presence of 5-HT4 receptors in colonic submucosal ganglia, although they were almost entirely absent from secretomotor...
neurons (48). Of note, the pattern of defecation and increased fecal water content induced by 5-HTP was similar to that observed by intraperitoneal CRF, reported to accelerate distal colonic transit in mice (33) and to act through the activation of myenteric neuronal mechanisms in the rat colon (36, 57). The actions of 5-HTP are 5-HT4 receptor mediated, as shown by the complete blockade of the 5-HTP-induced increase in fecal output and fluid content by RS-39604. Previous reports have shown that the 5-HT4 antagonist SB-207266, injected subcutaneously, prevented the subcutaneous 5-HTP-evoked increase in pellet output and fecal fluid content in conscious mice (2, 54, 56). Interestingly, the 5-HT4 antagonist by itself had a tendency to reduce pellet output and significantly reduced fecal fluid content compared with vehicle-treated mice. This suggests that 5-HT4 receptors are involved in the basal regulation of colonic motility and secretion, supporting recent functional observations in knockout mice lacking 5-HT4 receptors (19). These animals have an abnormally slow colorectal motility, with normal gastric emptying or small intestine motility, thus suggesting that 5-HT4 receptors are necessary to maintain a normal colonic motility (19). Similarly, in isolated longitudinal muscle strips of the rat distal colon, 5-HT4 receptors have been recently reported to participate in the regulation of spontaneous contractions (45). These data, however, contrast with previous observations in knockout mice lacking 5-HT4 receptors in myenteric neurons in the control of colonic transit (21). These observations support a role for 5-HT, acting through 5-HT4 receptors in myenteric neurons in the peristaltic reflex in LMMP preparations of the mouse distal colon to detect activation of myenteric cholinergic and nitrergic neurons in conscious mice. We demonstrated that the 5-HT precursor 5-HTP, injected intraperitoneally, induces a 5-HT4 receptor-mediated dose-related activation of distal colonic myenteric neurons, which correlated with the stimulation of colonic motor function. Fos expression in the distal colon encompasses separate populations of pChAT- and NADPH-diaphorase-positive neurons, known to be involved in the peristaltic reflex in mice (21). These observations support a role for 5-HT, acting through 5-HT4 receptors in myenteric neurons in the control of colonic motility, and suggest that these mechanisms might be implicated in the pathophysiology of IBS and/or other colonic disorders characterized by altered motility and defecation.

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