Expression and function of 5-HT$_4$ receptors in the mouse enteric nervous system

Mintsai Liu, Matthew S. Geddis, Ying Wen, Wanda Setlik, and Michael D. Gershon

Department of Anatomy and Cell Biology, College of Physicians and Surgeons, Columbia University, New York, New York

Submitted 26 May 2005; accepted in final form 14 July 2005

Liu, Mintsai, Matthew S. Geddis, Ying Wen, Wanda Setlik, and Michael D. Gershon. Expression and function of 5-HT$_4$ receptors in the mouse enteric nervous system. Am J Physiol Gastrointest Liver Physiol 289: G1148–G1163, 2005. First published July 21, 2005; doi:10.1152/ajpgi.00245.2005.—The aim of the current study was to identify enteric 5-HT$_4$ splice variants, locate enteric 5-HT$_4$ receptors, determine the relationship, if any, of the 5-HT$_4$ receptor to 5-HT$_1P$ activity, and to ascertain the function of 5-HT$_4$ receptors in enteric neurophysiology. 5-HT$_{4a}$, 5-HT$_{4b}$, 5-HT$_{4c}$, and 5-HT$_{4f}$ isoforms were found in mouse brain and gut. The ratio of 5-HT$_3$ expression to that of the neutral marker, synaptophysin, was higher in gut than in brain but was similar in small and large intestines. Submucosal 5-HT$_4$ expression was higher than myenteric. Although transcripts encoding 5-HT$_{4a}$ and 5-HT$_{4b}$ isoforms were more abundant, those encoding 5-HT$_{4c}$ and 5-HT$_{4f}$ were myenteric plexus specific. In situ hybridization revealed the presence of transcripts encoding 5-HT$_4$ receptors in subsets of enteric neurons, interstitial cells of Cajal, and smooth muscle cells. IgY antibodies to mouse 5-HT$_4$ receptors were raised, affinity purified, and characterized. Nerve fibers in the circular muscle and the neuropil in ganglia of both plexuses were highly 5-HT$_4$ immunoreactive, although only a small subset of neurons contained 5-HT$_4$ immunoreactivity. No 5-HT$_4$-immunoreactive nerves were detected in the mucosa. 5-HT and 5-HT$_1P$ agonists evoked a G protein-mediated long-lasting inward current that was neither mimicked by 5-HT$_4$ agonists nor blocked by 5-HT$_4$ antagonists. In contrast, the 5-HT$_4$ agonists renzapride and tegaserod increased the amplitudes of nicotinic evoked excitatory postsynaptic currents. Enteric neuronal 5-HT$_4$ receptors thus are presynaptic and probably exert their prokinetic effects by strengthening excitatory neurotransmission.

5-HT plays many critical roles in the physiology of the bowel (43–45). To understand these functions, it is thus necessary to identify the subtypes of 5-HT receptor that are expressed in the gut and to determine their locations and actions. The 5-HT receptors that are known to be expressed by enteric neurons include 5-HT$_{1A}$ (40, 60, 61), 5-HT$_{2A}$ (13, 35), 5-HT$_{2B}$ (14, 36), 5-HT$_3$ (29, 67, 69, 102), and 5-HT$_4$ (26, 27, 41, 59, 65, 79). In addition to these, another 5-HT$_4$ receptor activity, called 5-HT$_1P$, has been identified in the enteric nervous system (ENS) (15, 69). This receptor activity has thus far only been characterized operationally and with respect to its intracellular transduction coupling to G$_o$ (43, 44, 70, 80). The 5-HT$_1P$ receptor has been proposed by some authors to be identical to the 5-HT$_4$ receptor (49, 62, 63) but has been thought to be unrelated to 5-HT$_4$ by others (39, 41, 44). The 5-HT$_3$ (47–49, 55, 87, 92, 93) and 5-HT$_1P$ (44, 81) receptors are important because each has been implicated in the initiation of peristaltic reflexes. The hypothesis that 5-HT$_3$ receptors activate enteric neurons has not received any electrophysiological support but could be tested by determining the location and actions of 5-HT$_3$ receptors in the ENS. The 5-HT$_4$ receptor is also of considerable clinical significance because a 5-HT$_4$ agonist, tegaserod, has proven to be a useful prokinetic agent with which to treat the constipation-predominant form of the irritable bowel syndrome (IBS-C) (20, 21, 50, 75, 77, 85, 90) and chronic constipation (56). Unfortunately, enteric 5-HT$_4$ receptors have yet to be definitively localized, their functions in normal ENS physiology are not entirely clear, and the relationship of 5-HT$_4$ receptors to the 5-HT$_1P$ receptor activity remains unclear.

Several splice variants of the 5-HT$_4$ receptor have been described since the sequences of “short” (5-HT$_{4s}$) and “long” (5-HT$_{4l}$) forms were first determined in the rat (42). These newer splice variants include the a, b, and e isoforms in rats (24), a, b, e, and f isoforms in mice (23–25), and a, b, c, d, g, n, h, and i isoforms in humans (3, 6, 8, 9, 16, 24, 73, 97, 98). The 5-HT$_{4s}$ splice variants differ from one another in the sequence of their COOH-terminal domain. Pharmacological properties of the 5-HT$_4$ receptor in a given tissue may reflect the expression levels of the various 5-HT$_4$ isoforms (6, 16, 24, 72, 98). The 5-HT$_4$ receptor is thought to be coupled by G$_o$ to the stimulation of adenylyl cyclase (AC), increase in cAMP, and activation of PKA (6, 10, 12, 68). This coupling suggests that 5-HT$_4$ receptors may not be identical to the G$_o$-coupled 5-HT$_1P$ activity of the ENS. Because most studies of 5-HT$_4$ receptors have concerned the central nervous system (CNS) where they are widely distributed (11, 31, 64), the splice variants that characterize 5-HT$_4$ expression in the ENS and other peripheral sites (51) must still be identified.

The current study was designed to identify the 5-HT$_4$ splice variants expressed in the gut, to locate enteric sites of 5-HT$_4$ expression, to clarify the relationship of the 5-HT$_4$ receptor to 5-HT$_1P$ activity, and to determine the function of 5-HT$_4$ receptors in enteric neuronal signaling. Observations made in the current study suggest that 5-HT$_1P$ activity and 5-HT$_4$ receptors are not related to one another. The enteric 5-HT$_4$ receptor is presynaptic and acts to enhance the secretion of excitatory neurotransmitters, whereas 5-HT$_1P$ activity evokes a postsynaptic G protein-mediated slow sustained inward current. The data are compatible with the suggestion that 5-HT$_4$ agonists are prokinetic because they strengthen neurotransmission in excitatory pathways.
MATERIALS AND METHODS

Animals. Experiments were carried out with CD-1 mice of either sex (1–2 mo of age). Procedures and care followed guidelines of the National Institutes of Health and were approved by the Animal Care and Use Committee of Columbia University. The small and large intestines were removed from the mice, and dissected laminar preparations of longitudinal muscle with adherent myenteric plexus (L MMP) and submucosa (containing the submucosal plexus) were prepared. Control material was also obtained from dissected preparations of hippocampus, frontal cortex, atrium, and liver.

RNA isolation and RT-PCR. Total RNA (2 μg) was prepared from tissues by using a commercial reagent (RNA STAT-60; Tel-Test, Friendswood, TX) according to the directions of the manufacturer. cDNA was prepared from RNA by reverse transcription at 50°C (30 min) in the presence of random primers and Moloney Murine Leukemia Virus reverse transcriptase (SuperScript III; Invitrogen, Carlsbad, CA). Reverse transcriptase was omitted from control samples, which were employed for the detection of contamination of samples with genomic DNA. All experiments were carried out with 0.1% diethyl pyrocarbonate (DEPC; Sigma, St. Louis, MO)-treated distilled water. The cDNA was amplified by using PCR with Tag DNA polymerase (Promega, Madison, WI) in a Gene-Amp PCR System 9700 (Applied Biosystems, Foster City, CA). Initial denaturation was carried out at 94°C for 1 min. The touchdown method (30) was employed using a number of annealing temperatures in a single PCR (63–58°C for 10 cycles at 45 s/cycle). Final amplification was carried out with 20 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 45 s, and elongation at 72°C for 1 min. After the final PCR cycle, the reaction was extended for an additional 7 min at 72°C after which the reaction products were cooled to 4°C. Primer pairs are listed in Table 1. PCR products were resolved by electrophoresis through 1% agarose gels containing ethidium bromide (0.3 μg/ml) in Tris/acetic acid/EDTA (TAE) electrophoresis buffer. The identity of the PCR products was verified by sequencing. For this purpose, the PCR products were cloned using a commercial kit (TOPO TA; Invitrogen). PCR products were cloned using a commercial kit (TOPO TA; Invitrogen). The cloning products was verified by sequencing. For this purpose, the PCR products were cloned using a commercial kit (TOPO TA; Invitrogen). Sequencing of the plasmid DNA was accomplished by dye termination (ABI Automated Sequencer, Applied Biosystems) in the core facility of Columbia University. The final cDNA sequences were compared with those in the GenBank (BLAST search at National Center for Biotechnology Information, Bethesda, MD). The fluorescence of ethidium bromide in agarose gels was photographed, scanned, and digitized. The optical density of the digitized images was quantified by computer-assisted densitometry (Digital Science ID Image Analysis 1.51; Eastman Kodak, Rochester, NY). To assess relative quantities of transcripts encoding 5-HT4 receptor isoforms were normalized to those encoding synaptophysin, which was used as a neuron-specific product. Data were derived from three separate RNA preparations. Relative abundance of 5-HT transcripts was compared by one-way repeated-measures ANOVA. Data are reported as means ± SE (unless otherwise noted).

In situ hybridization. Transcripts encoding 5-HT4 receptors were located in tissues by in situ hybridization as described previously (67). Small segments of ileum were fixed for 3 h with 4% (wt/vol) formaldehyde (from paraformaldehyde) in PBS (pH 7.4). The tissue was cryoprotected, embedded in Neg-50 Frozen Section Medium (Richard-Allen Scientific), frozen in liquid N2, and sectioned (8 μm) at −20°C using a cryostat-microtome. All solutions were made with 0.1% DEPC-treated distilled water.

Digoxigenin-labeled cRNA probes were prepared from mouse cDNA encoding a region of the mouse 5-HT4 receptor between the third and the seventh transmembrane domains. PCR was used to obtain the cDNA (sense primer: 5′-TTCCTTGACAGGTATTAGCGC-3′, antisense primer: 5′-ATAGCAAACAGGAGAAAG-3′). Antisense and sense probes were linearized from plasmid DNA using HindIII (antisense) or XhoI (sense) and purified. The T7 and SP6 RNA polymerases were then used to transcribe the riboprobes, which were quantified by dot blotting (DIG-RNA labeling kit, Roche, Indianapolis, IN). Frozen sections were cut with a cryostat-microtome and incubated with sense or antisense probes (0.5 ng/ml) in a moist chamber at 60°C for 16 h. Bound digoxigenin was detected with alkaline phosphatase-coupled sheep antibodies to digoxigenin (diluted 1:1.500; Roche). Alkaline phosphatase activity was demonstrated and visualized with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (Roche) in a buffer containing NaCl (100 mM), MgCl2 (50 mM), Tween-20 (1%), and Tris·HCl (100 mM), pH 9.5. Levamisole (0.25 mg/ml; Sigma) was added to inhibit endogenous neuronal intestinal alkaline phosphatase. The blue reaction product representing the sites in the tissue sections containing mRNA encoding the 5-HT4 receptors were visualized by bright-field microscopy (Leica, Allendale, NJ).

Antibody production. A peptide sequence found within the second extracellular loop of the mouse 5-HT4 receptor was selected for use as an immunogen. This peptide, with an added NH2-terminal cysteine and an aminocaproic acid spacer “Z” (CZDVIEKRKFHSN) was synthesized and conjugated to maleimide-activated keyhole limpet hemocyanin. Two hens (at Aves Labs, Tigard, OR) were immunized intramuscularly with the conjugated peptide suspension. The IgY fractions were then isolated from the yolks of eggs collected from the immunized hens. IgY fractions were further purified by affinity chromatography using Sepharose beads coupled to the immunizing peptide. The depleted IgY fraction served as a negative control.

DNA constructs and transfection of COS-7 cells. Antibody specificity was tested with transfected cells that expressed 5-HT4 receptors. The 5-HT4b isoform, which is the longest of the 5-HT4 splice variants

Table 1. Primers used for PCR

<table>
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<tr>
<th>Product</th>
<th>Accession Number</th>
<th>S or AS</th>
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<td>S</td>
<td>5′- CCAAGGCGAAGGAGCTTTA-3′</td>
<td>327</td>
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<td></td>
<td></td>
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<td>386</td>
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<tr>
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<td>S</td>
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<td>340</td>
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<tr>
<td>5-HT4c</td>
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<tr>
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<td>AJ011369</td>
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<td>330</td>
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<tr>
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<td>5′- ACATGGGAGAAGAACTGAGG-3′</td>
<td>464</td>
</tr>
<tr>
<td>β-Actin</td>
<td>X03765</td>
<td>S</td>
<td>5′- GGATCACTCACTGAGGAG-3′</td>
<td>540</td>
</tr>
</tbody>
</table>

S, sense; AS, antisense.
5-HT_{4} RECEPTORS IN MOUSE ENS

and contains sequences found in all other 5-HT_{4} isoforms, was used for this purpose. The full-length 5-HT_{4}a sequence, with added PstI and SalI sites, was amplified by PCR. The resulting PCR product was digested with PstI and SalI for ligation into a gWIZ-green fluorescent protein (GFP) vector (Gene Therapy Systems, San Diego, CA) using T4 DNA ligase (Roche). Plasmids were sequenced before use to verify that they contained the correct inserts. COS-7 cells (ATCC, Manassas, VA) were transfected with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions and cultured on acid-washed glass coverslips for an additional 24–48 h. In most experiments, the cells were then fixed with methanol for 10 min at −20°C or 4% formaldehyde (from paraformaldehyde) with PBS for 15 min at room temperature. Alternatively, to determine whether cell surface 5-HT_{4}a expression could be detected without permeabilization, living cells were exposed to antibodies to the 5-HT_{4}a receptor or to the control depleted IgY fraction. The fixed cells were washed three times with PBS, permeabilized with 0.05% (vol/vol) Triton X-100 (Sigma), and blocked with a fish serum BlokHen II (diluted 1:10; Aves Labs) and 10% normal horse serum in PBS for 30 min. For immunostaining, cells were incubated with the affinity-purified anti-5-HT_{4} receptor IgY antibodies (0.35 μg/ml) in a solution containing 0.05% Triton X-100 (Sigma) and 10% normal horse serum in PBS at room temperature for 3 h. When live cells were examined, the primary antibody concentration was 1 μg/ml (4°C, 30 min, without agitation) and Triton X-100 was omitted. After being washed with PBS, the sites of bound primary antibody were detected by incubation with goat anti-chicken secondary antibody conjugated to Alexa 488 (1:250, 4°C for live cells; Molecular Probes, Eugene, OR) for 30 min. Live cells were fixed with methanol at −20°C for 10 min after exposure to secondary antibodies. Detection of GFP expression was enhanced in some experiments by immunostaining preparations with Alexa 488-labeled rabbit antibodies to GFP (diluted 1:1,000; Molecular Probes). Control preparations were treated similarly but were incubated with the depleted IgY from which the immunogen had been adsorbed. All preparations were washed extensively in PBS after immunostaining and mounted in Vectashield (Vector, Burlingame, CA). Immunofluorescence was observed with a vertical fluorescence microscope (DMR RXA2, Leica) equipped with a cooled charge-coupled device camera (Retiga EXi; Qimaging, Burnaby BC, Canada) and a computer-assisted video-imaging system software (Openlab 3.1; Improvision, Lexington, MA). Photomicrographs of preparations were also obtained with a laser-scanning confocal microscope (LSM 510, Axiovert IM100; Carl Zeiss, Thornwood, NY). Confocal images were obtained at 512 × 512 pixels. Contrast of images was adjusted using Adobe Photoshop 7 software (Adobe Systems, San Jose, CA), which was also used to arrange images in figures. No other image manipulations were used.

Western blotting. Membrane proteins were extracted from brain (hippocampus and frontal cortex) and small intestinal LMMP with 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; Pierce, Rockford, IL) (32). Tissues from six mice were pooled and homogenized (Dounce glass homogenizer) in 10 volumes (vol/vt) of ice-cold 50 mM Tris·HCl with 0.1 mM PMSF (Sigma) buffer, pH 7.4, containing a cocktail of protease inhibitors (Roche). The lysate was centrifuged at 40,000 g for 20 min at 4°C. The pellet was then washed twice by resuspension and centrifugation in 10 volumes of the same buffer. The final washed pellet was resuspended in 2 volumes of homogenizing solution. Nine volumes of the final suspension were mixed with one volume of 0.1 M CHAPS (in 50 mM Tris·HCl, pH 7.4), sonicated for 10 s (20 W), and left to stand at 4°C for 60 min. The suspension was again sonicated and centrifuged at 100,000 g for 60 min at 4°C. The clear supernatant was dialyzed overnight at 4°C in PBS with 0.1 mM PMSF buffer (pH 7.4) to remove residual CHAPS. The protein concentration of the final mixture was measured (Bio-Rad protein assay; Bio-Rad, Richmond, CA), and the preparation was stored at −80°C until used as the source of solubilized membrane protein.

Immunoblot assays were carried out as previously described (67). Briefly, membrane proteins (30 μg) were mixed with SDS sample buffer (Bio-Rad) containing dithiothreitol (350 mM), incubated at 70°C for 15 min, and then resolved on a 10% polyacrylamide gel (SDS-PAGE). Proteins were electrophoretically transferred from the gel to a nitrocellulose membrane (Bio-Rad). The blots were blocked by incubation for 1 h in fish serum (diluted 1:10; BlokHen II, Aves Labs) containing Tris-buffered saline (TBS), and then incubated in buffer containing 25 mM Tris, 150 mM NaCl, and 0.05% Tween-20 (TBST), with 5% (wt/vol) nonfat milk powder (Bio-Rad) at room temperature for another hour. The blots were probed with purified chicken antibodies to the 5-HT_{4} receptor (70 ng/ml) or with the same concentration of the depleted IgY fraction overnight at 4°C. Immunoreactivity was identified with goat anti-chicken secondary antibodies conjugated to horseradish peroxidase (HRP; diluted 1:5,000; Aves Labs). HRP activity was detected using enhanced chemiluminescence (Pierce). All immunoblot assays were repeated in three separate preparations.

Immunocytochemistry. Acetone fixation (at −20°C for 15 min) or fixation by perfusion with 2% acrolein in PBS was used for immunocytochemistry because formaldehyde and glutaraldehyde were both found to eliminate immunoreactivity. Frozen sections of acetone-fixed duodenum, ileum, proximal, and distal colon were obtained with a cryostat-microtome (8-μm sections) and incubated with fish serum (diluted 1:10 in PBS; BlokHen II, Aves Labs) for 30 min (at room temperature). Tissues were then permeabilized with 0.05% Triton X-100 and blocked with 10% normal horse serum in PBS for another hour. The preparations were exposed overnight to chicken anti-5-HT_{4} receptor antibodies (0.35 μg/ml; room temperature) and/or rat anti-c-Kit (ACK2) antibodies (5 μg/ml; at room temperature; ebIobioscience, San Diego, CA) in a solution that contained Triton X-100 and normal horse serum. After being washed with PBS, the sites of bound primary antibody were detected by incubation with goat anti-chicken secondary antibodies coupled to Alexa 488 (diluted 1:600; Molecular Probes) and/or goat anti-rat secondary antibodies coupled to Alexa 594 (diluted 1:600; Molecular Probes) for 3 h at room temperature. The preparations were washed again with PBS and mounted in Vectashield (Vector). Acrolein-fixed distal colon was frozen and sectioned as described above. Residual acrolein in the fixed tissues was destroyed by quenching sections with 1% (wt/vol) sodium borohydride in PBS at room temperature for 1 h. The sections were blocked and immunostained as described above, except that 0.1% saponin, rather than Triton X-100, was used for permeabilization. Sites of primary antibody binding were detected by incubation with donkey secondary antibodies to chicken IgY coupled to HRP (diluted 1:100; 3 h; room temperature; Jackson ImmunoResearch, West Grove, PA). Immunoreactivity was visualized with 3,3'-diaminobenzidine (DAB; Vector Laboratories DAB kit used according the manufacturer’s instructions). Control preparations were identically immunostained except that they were exposed to the depleted IgY fraction instead of antibodies to the 5-HT_{4} receptors. Alternatively, for ACK2, control sections were incubated only with secondary antibodies.

Electron microscopy. Small pieces (1 cm³) of LMMP from the small intestine were fixed for electron microscopy (EM) in a mixture of 4% formaldehyde (from paraformaldehyde) and 0.01% glutaraldehyde (Sigma) for 3 h at room temperature. After being rinsed in PBS buffer and dehydration in cold ethanol, the tissues were embedded in LR Gold resin (Electron Microscopy Sciences, Fort Washington, PA), and polymerized under ultraviolet light (365 nm) at −20°C. Ultrathin sections were collected on Formvar-coated nickel grids and treated with 10% fish serum (BlokHen II; Aves Labs) and 10% donkey serum with TBST for 30 min at room temperature to block nonspecific binding. The sections were incubated with chicken anti-5-HT_{4} receptor antibodies (0.35 μg/ml) in 4% donkey serum in TBST for 60 min at room temperature. Incubation with the antibody-depleted IgY fraction was used as a control. After being rinsed with TBS, the grids were incubated for 60 min at room temperature with donkey anti-
chicken IgY labeled with 12-nm gold particles (1:40; Jackson ImmunoResearch) in TBST. The grids were washed with TBS and postfixed with 2% glutaraldehyde and then washed with distilled water, stained with osmium tetroxide, uranyl acetate, and lead citrate (Electron Microscopy Sciences) and observed and photographed using a JEOL 1200EX microscope (JEOL, Peabody, MA).

Electrophysiological acquisition and data analysis. Myenteric neurons from mice approximately 1 mo of age were cultured as described previously (67). Neurons were investigated 2–5 days after plating. Whole cell patch-clamp recordings were obtained as previously described (67). Briefly, patch pipettes (~5 MΩ) were filled with an internal pipette solution containing (in mM): 140 potassium gluconate, 0.1 CaCl₂, 2 MgCl₂, 1 EGTA, 2 Na₂ATP, 0.2 Na₃GTP, and 10 HEPES, pH 7.25 with KOH. The bath solution contained (in mM): 145 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 11 glucose, and 10 HEPES, pH 7.35, with NaOH. A HEKA EPC-9B amplifier (HEKA Instruments, Southboro, MA) was used to voltage clamp neurons at ~60 mV. Recordings were carried out at room temperature (20–22°C) on the stage of an inverted microscope (Axiovert IM35, Zeiss). Electrodes were filled with submaximal concentrations of agonists. When antagonists were added, the currents were allowed to stabilize in amplitude on successive stimuli was determined and used in all regions of the bowel and more pronounced in the submucosal than in the known isoforms of mouse 5-HT₄ receptor (a, b, e, and f) identities of the PCR products. Transcripts encoding each of the 5-HT₄f isoforms, which have previously been considered to be agonist. The amplitudes of four consecutive evoked EPSCs were averaged and recorded at 60-s intervals. Experiments were completed <1 h after recordings were begun to minimize time-dependent changes in response properties or deterioration of cells. Off line data analysis was carried out with software programs A xoGraph 4.8 (Axon Instruments) or MiniAnalysis 5.8 (Synaptosoft, Decatur, GA).

Compounds used. All drugs were prepared as stock solutions in distilled water, ethanol, 1-methyl-2-pyrrolidinone, or DMSO at greater than 10⁷ times the highest experimental concentration according to the instruction of manufacturer and stored at ~20°C until use. The stock solution was subsequently diluted in the external solution for the experimental use. 5-HT creatinine sulfate, 5-methoxytryptamine (5-MeOТ), 1-(m-chlorophenyl)-biguanide, guanosine-5’-O-(3-thiorthophosphate; GTPS), guanosine-5’-O-(2-thiodiphosphate; GDPS), hexamethonium, tetrodotoxin, 1-methyl-2-pyrrolidinone, and DMSO were purchased from Sigma/RBI (Natick, MA). WAY 100325 was obtained from Wyeth (Madison, NJ). 5-HTP-DP, N-acetyl-5-hydroxytryptophan-5-hydroxytryptophan amide, was obtained from Dr. H. Tamir (New York State Psychiatric Institute, New York, NY). Endo-N-(8-methyl-8-azabicyclo[3,2,1]oct-3-yl)-2,3-di hydro-3-ethyl-2-oxo-1H-benzimidazole-1-carboxamide (BIMU 1) was obtained from Boehringer-Ingelheim (Milan, Italy). Rennazpride (BRL 24924; (±)-endo-4-amo nin-5-chloro-2-methoxy-N-[1-(azabicyclo[3,3,1]non-4-yl)benzamide monohydrochloride], [1-[2-(methylsulphonylamino)ethyl]-4-piperidinyl]methyl 1-methyl-1H-indole-3-carboxylate maleate (GR 113808), and 8-amino-7-chloro-(N-buty1-4-piperide) methylbenzo-1,4-dioxan-5-carboxylate hydrochloride (SB 204070) were obtained from GlaxoSmithKline (Harlow, UK). 5-Hydroxydindalpine (5-OHIP) was obtained from Rho-paneul Sante (Gennevilliers, France). Tegaserod [3-(5-methoxy-1H-indol-3-ylmethylene)-N-pentylcarbazimidamide hydrogen maleate] was obtained from Novartis (Basel, Switzerland).

RESULTS

Transcripts encoding four 5-HT₄ receptor isoforms are present in the mouse gut. RT-PCR (touchdown method of amplification) was used with primers corresponding to sequences found in the mouse 5-HT₄ receptor to identify the subtypes of 5-HT₄ receptor that are transcribed in the gut. Each primer pair was selected so as to amplify selectively a single cDNA corresponding to one of the 5-HT₄ isoforms. cDNA, extracted from the brain and atrium, which are known to express 5-HT₄ receptors, were investigated as positive controls, whereas the liver, which is thought not to express 5-HT₄ receptors, served as a negative control. As an additional negative control, to rule out potential contamination from genomic DNA, reverse transcriptase was omitted before amplification. Loading of gels was standardized so that an approximately equal amount of β-actin PCR product was present in each lane. Subcloning and sequencing were carried out to confirm the identities of the PCR products. Transcripts encoding each of the known isoforms of mouse 5-HT₄ receptor (a, b, e, and f) were found in the gut, brain, and atrium but not in the liver (Fig. 1). Transcripts encoding synaptophysin were also amplified and used to semiquantitatively normalize the data to the relative amount of mRNA encoding a synapase-specific gene product (Fig. 1). Notably, transcripts encoding the 5-HT₄a and 5-HT₄d isoforms, which have previously been considered to be brain-specific (24), were detected in the mouse gut. Transcripts encoding 5-HT₄ receptors, normalized to those encoding synaptophysin, were highly abundant in the gut, even compared with the brain and the atrium (Fig. 2). Expression of the 5-HT₄a and 5-HT₄b receptors was dominant in all regions of the bowel and more pronounced in the submucosal than in
the LMMP preparation. The apparent expression of mRNA encoding 5-HT4 receptors were myenteric plexus specific (Fig. 2). No significant difference was observed in the expression pattern of 5-HT4 transcripts in the small and large intestines.

Enteric neurons contain transcripts encoding the 5-HT4 receptor. In situ hybridization was used to identify the cells that contained transcripts encoding 5-HT4 receptors in the mouse gut. mRNA encoding 5-HT4 receptors was detected in enteric neurons and smooth muscle. A cRNA probe specifically labeled subsets of neurons in both the myenteric and submucosal plexuses (Fig. 3, A and B). The same cRNA probe also labeled a subset of smooth muscle cells (Fig. 3C). No labeling of any epithelial cells was detected (Fig. 3C). As a control, sections were hybridized with a riboprobe in the sense configuration; in contrast to the experimental antisense cRNA, no labeling with the sense probe was obtained (Fig. 3D).

Preparation and characterization of antibodies to the 5-HT4 receptor. Polyclonal antibodies to the mouse 5-HT4 receptor were generated in chickens to ascertain that not just mRNA, but also 5-HT4 receptor protein is expressed in the bowel and to make it possible to identify the cells in which this protein is expressed. A synthetic peptide corresponding in sequence to amino acids (168–179; mouse 5-HT4 receptor; GenBank accession no. NP 032339) was used as the immunogen. This sequence is found in the second extracellular domain of the 5-HT4 receptor. The IgY fraction of the resulting antiserum

Fig. 1. Transcripts encoding splice variants (a, b, e, and f) of 5-HT4 receptors are present in the mouse gut. 5-HT4 receptors were detected in the indicated tissues by means of RT-PCR. The expected size of the PCR products is shown to the right of the gels. Transcripts encoding 5-HT4a and 5-HT4b receptors were detected in the brain and atrium (positive controls) and in the longitudinal muscle with adherent myenteric plexus (LMMP) and submucosa [containing the submucosal plexus (SMP)] of the small intestine (SI), proximal colon (PC), and distal colon (DC), but not in the liver (negative control). Transcripts encoding 5-HT4e and 5-HT4f receptors were largely restricted to the brain and the LMMP of the SI and PC; very little was found in the SMP. The amount of cDNA added to each lane was normalized to that encoding β-actin amplified from each tissue. Reverse transcriptase (RTase) was omitted in controls to rule out contamination by genomic DNA. Transcripts encoding the synaptic protein synaptophysin (SYP) were simultaneously amplified to provide an indicator of the cDNA from each tissue that is derived from neurons.
was obtained and affinity purified. The utility of the antibodies (anti-5-HT₄) for the immunocytochemical detection of 5-HT₄ receptors was evaluated with transfected COS-7 cells that expressed the 5-HT₄b receptor tagged with GFP (Fig. 4). The chicken anti-5-HT₄ reacted with the 5-HT₄b GFP-expressing COS-7 cells (Fig. 4, A and B). In contrast, no immunofluorescence was seen when the 5-HT₄b GFP-expressing cells were exposed to a depleted IgY control fraction (anti-5-HT₄ adsorbed with the immunogen; Fig. 4, C and D) or when cells that expressed only GFP were exposed to anti-5-HT₄ IgY (Fig. 4, E and F). When the COS-7 cells were permeabilized, 5-HT₄ immunoreactivity was predominantly intracellular (Fig. 4A); however, when nonpermeabilized 5-HT₄b GFP-expressing cells were exposed to anti-5-HT₄ IgY (Fig. 4G), the immunofluorescence was prominent on the cell surface. No surface immunofluorescence was detected when nonpermeabilized 5-HT₄b GFP-expressing cells were exposed to the depleted IgY fraction (Fig. 4H). These observations suggest that the anti-5-HT₄ reagent can be employed to locate 5-HT₄ receptors by immunocytochemistry, that the receptors reach the cell surface even when they are expressed as an exogenous protein in a cell line, and that the antibodies react with the ectodomain of the 5-HT₄ receptor and thus can detect 5-HT₄-expressing cells without prior permeabilization.

An immunoblot was carried out with membrane proteins extracted from the small intestinal LMMP and from the brain to verify the specificity of the anti-5-HT₄ reagent. The membrane proteins were separated by SDS-PAGE, and the resulting blots were probed with anti-5-HT₄ and the depleted IgY control fraction. A single major immunoreactive band (46 kDa), corresponding in size to that of the mouse 5-HT₄ receptor, was found in membrane extracts of the small intestine LMMP and brain. In contrast, no labeling was observed when the blots were probed with the depleted IgY fraction (Fig. 5).

5-HT₄ receptor protein is specifically expressed in a subset of enteric neurons. Anti-5-HT₄ was used to investigate the distribution of 5-HT₄ immunoreactivity in the bowel wall. Tissue was fixed either with acetone (Fig. 6, A-D) or acrolein (Fig. 6, E-H). Subsets of neurons, and especially the ganglionic neuropil, were found to be 5-HT₄-immunoreactive in both the myenteric (Fig. 6, A-F) and submucosal plexuses. 5-HT₄ immunoreactivity was observed in all regions of the small intestine from the duodenum (Fig. 6A) through the terminal ileum (Fig. 6B) and was also seen in both the proximal (Fig. 6C) and distal regions (Fig. 6D).
Smooth muscle cells were 5-HT4 immunoreactive, and patches of immunoreactivity outlined the cells, suggesting that the receptors are concentrated in the plasma membranes. No structures were immunostained in the mucosa (Fig. 6F). Neither mucosal nerves nor epithelial cells were 5-HT4-immunoreactive. No immunostaining was obtained when tissue sections were incubated with the depleted IgY fraction instead of anti-5-HT4 (Fig. 6H). Sections of small intestine were coimmunostained with antibodies to Kit (ACK2) and 5-HT4 receptors to determine whether interstitial cells of Cajal (ICC) express 5-HT4 receptors. Kit is an ICC marker in the bowel (19, 99), and ICCs were most prominent bordering myenteric ganglia (IC-MY) and in the deep muscle plexus (IC-DMP) at the junction of the circular muscle layer and the submucosa (Fig. 6J). IC-DMP had tapering long processes that paralleled the long axis of smooth muscle fibers, and IC-MY often formed branches around neurons in the myenteric plexus. Coincident localization of 5-HT4 and Kit immunoreactivity was found in IC-MY (Fig. 6, I–K) and IC-DMP, although 5-HT4 immunoreactivity was very sparse in IC-DMP. No immunostaining was obtained when control tissue sections were incubated without ACK2 and with the depleted IgY fraction of anti-5-HT4 (Fig. 6L).

At the electron microscopic level, 5-HT4 immunoreactivity was found in axons of the myenteric plexus and in smooth muscle cells (Fig. 7). Axonal immunoreactivity was concentrated in subsets of varicosities, which contained both large dense cored and small electron lucent synaptic vesicles (Fig. 7A). Within these axons, large, dense, cored granules were often labeled (arrows). In contrast to the immunoreactivity found when sections were exposed to anti-5-HT4 IgY, no labeling was obtained when sections were incubated with the depleted IgY fraction (Fig. 7B). In smooth muscle cells, 5-HT4 immunoreactivity was found in scattered elements of rough endoplasmic reticulum and in caveolae in or near the plasma membrane (Fig. 7A, inset). Only a subset of the axons in the ganglionic neuropil contained 5-HT4 immunoreactivity (Fig. 8). Within these axons, 5-HT4 immunoreactivity was most concentrated inside regions of varicose expansion (Fig. 8, A and B, insets).
5-HT4 receptors facilitate EPSCs evoked in myenteric neurons. Although both fast and slow depolarizing responses to 5-HT have been observed when recordings have been made from enteric neurons impaled with sharp microelectrodes (39), only a fast inward current has previously been observed with the patch-clamp technique to be evoked by 5-HT (67, 101, 102). The fast responses, however recorded, are 5-HT3 mediated (67, 101, 102). The 5-HT3 receptor is a ligand-gated ion channel (29); all of the other subtypes of 5-HT receptor are G protein coupled (39, 53). It is possible that the dialysis of intracellular contents that occurs when neurons are patched obscures or interferes with the intracellular events of signal transduction from G protein-coupled receptors. If so, then the changes in transmembrane current resulting from G protein-mediated signal transduction might become apparent if G protein coupling could be potentiated or prolonged. To do so, the solution inside of the pipettes used to patch enteric neurons was layered so that an internal solution containing the nonhydroyzable GTPγS was layered over a non-GTPγS-containing solution at the pipette tips. These pipettes allowed an initial recording of the response to 5-HT to be made without internal GTPγS (Fig. 9A) while subsequent recordings from the same neurons of the response to 5-HT were obtained in its presence (Fig. 9B). As noted previously, 5-HT evoked only a fast inward current in the absence of GTPγS (Fig. 9A); however, after 10–15 min, when GTPγS had diffused into the cells, the 5-HT-elicited fast inward current was now followed by a prolonged inward current that was sustained for >15 s. The fast inward current evoked by 5-HT before the diffusion of GTPγS into the cells was blocked by 1.0 μM granisetron, a 5-HT3 antagonist (data not illustrated). The 5-HT-induced slow sustained inward current obtained in the presence of GTPγS (Fig. 9B) was granisetron resistant, averaged −137.6 ± 16.0 pA (n = 8), and was detected in ~20% of myenteric neurons (n = 70). To verify that the GTPγS-dependent manifestation of the 5-HT-evoked slow sustained inward current is related to the effect of GTPγS on G protein coupling, responses to 5-HT in neurons that were patched with pipettes containing GTPγS were compared with those in neurons patched with pipettes containing GDPβS. When GTPγS was included in the patch pipette, 5-HT evoked a sustained inward current with a rapid onset (Fig. 9C). In contrast, when GDPβS was included in the patch pipette, the inward current elicited by 5-HT decayed rapidly and was not sustained (Fig. 9D). The application of TTX (1 μM) affected neither the rapid onset nor the slow sustained component of the inward current associated with responses to 5-HT in the presence of internal GTPγS (Fig. 9, E and F). The failure of TTX to affect either component of the inward current evoked by 5-HT suggests that the cells from which recordings were obtained respond directly to 5-HT rather than indirectly to a transmitter released from other cells in the cultures; however, agonists may release a neurotransmitter from terminals via a TTX-insensitive mechanism (89). To determine which G protein is involved in mediating the slow sustained component of the 5-HT-evoked inward current, antibodies to Gαo or Gαs were included in the patch pipettes. Antibodies to Gαo and Gαs were investigated because slow depolarizing responses of enteric neurons have been reported to be Gα coupled (81), whereas 5-HT4-mediated responses are coupled to Gs (41). The human 5-HT4b receptor, however, couples to Gαo, in addition to Gαs, when transiently expressed as an exogenous receptor in HEK 293 cells (83). When antibodies to Gαo were included with GTPγS in the patch pipette, the fast inward current elicited by 5-HT decayed rapidly, as with GDPβS, and was followed by virtually no sustained current (Fig. 9G). The sustained current was still noticed when the heat-inactivated antibody to Gαo was included with GTPγS in the patch pipette (data not shown). In contrast, when a dialyzed rabbit antibody to Gαs was included with GTPγS in the patch pipette, the fast inward current did not decay as rapidly as with the antibody to Gαo, and a small current was sustained despite the absence of the antibody (Fig. 9H). The inclusion of the antibody to Gαs, however, did reduce the amplitude of the sustained current from that seen with GTPγS alone. The specificity of antibodies to Gαo (40 kDa) (66) and

Fig. 7. A 5-HT4-immunoreactive axonal terminal varicosity of the myenteric plexus is illustrated (A) and the plasma membrane of a smooth muscle cell is shown in the inset. Sites of bound IgY were detected with secondary antibodies coupled to colloidal gold (12 nm diameter). The varicosity contains both large, dense, cored, and small electron lucent synaptic vesicles. Note that immunogold particles are concentrated over large, dense, cored granules (arrows) and other vesicular structures. A caveolus of the smooth muscle cell (arrow; inset contains 5-HT4 immunoreactivity; scale bar = 100 nm). Note the close association of the immunogold particles with the membrane of the caveolus. Almost no labeling with gold (B) was seen when sections were exposed to deleted IgY from which antibodies were adsorbed with the immunogen instead of anti-5-HT4. Scale bars = 200 nm.
Gαs (42 and 45 kDa; 2 isoforms of Gαs) (88) was confirmed by immunoblotting with LMMP of small intestines (Fig. 9I). These observations suggest that one or more G protein-coupled receptors contribute to the sustained phase of the inward current evoked by 5-HT.

In the presence of GTPγS, WAY 100325 (50 μM), a specific 5-HT1P agonist (81), induced a slow sustained inward current (Fig. 10A), which was blocked by 5-HTP-DP (5 μM), a 5-HT1P antagonist (Fig. 10B). In the presence of GTPγS, the 5-HT4 antagonists 5-MeOT (50 μM; Fig. 10C) and BIMU 1 (50 μM; Fig. 10D) evoked only low-amplitude fast transient inward currents when first applied (respectively, −30.5 ± 4.5 or −10.6 ± 2.2 pA; n = 4). Neither of these compounds, nor another 5-HT4 agonist, renzapride (5-HT4 agonist/5-HT3 antagonist; 1.0 μM; n = 4; not illustrated), evoked a sustained inward current; moreover, no significant change in the current-voltage relationship was detected in the presence or absence of 5-MeOT, BIMU 1, or renzapride (data not shown). The 5-HT4 antagonists SB 204070 (1 μM; Fig. 10F) and GR 113808 (1 μM; data not shown) failed to affect the GTPγS-dependent slow sustained inward current evoked by 5-HT (50 μM; n = 3). In a subset of cells, many spontaneous low-amplitude transient fast currents (Fig. 11A), which resembled fast EPSCs, were superimposed on the slow sustained inward current evoked by the local application of 5-HT. Although the 5-HT4 antagonist GR 113808 (1 μM) did not block the slow sustained inward current, GR 113808 irreversibly abolished the superimposed low-amplitude transient fast currents (Fig. 11, B and C). The low-amplitude transient fast currents, therefore, are likely to have been EPSCs evoked presynaptically by 5-HT (see below; Fig. 12). The 5-HT1P agonist 5-OHIP (50 μM), similar to 5-HT, evoked a GTPγS-dependent slow sustained inward current (−55.5 ± 14.7 pA; n = 3; Fig. 11D). This effect was not shared by the 5-HT1P-agonist tegaserod (1.0 μM) applied to the same cells (Fig. 11E). The slow sustained inward current evoked by 5-OHIP was blocked by 5-HTP-DP (5 μM; Fig. 11F). These observations confirm that the GTPγS-dependent slow sustained inward current evoked by 5-HT is a 5-HT1P-mediated event. It is not related to 5-HT4 receptors.

Experiments were carried out to test the hypothesis, suggested by the low-amplitude transient fast currents seen in the presence of 5-HT4 agonists (Figs. 10, C and D, and 11A), that 5-HT4 receptors act presynaptically to evoke or potentiate fast EPSCs. Most fast excitatory neurotransmission in the myenteric plexus is mediated by ACh and is nicotinic. We therefore investigated the ability of hexamethonium to block putative fast EPSCs. Stimulating fibers that innervated patched neurons evoked fast EPSCs. The resulting evoked EPSCs were reversibly blocked by the nicotinic antagonist hexamethonium (300...
The 5-HT₄ agonists renzapride (100 nM; Fig. 12B) and tegaserod (100 nM; Fig. 12C) each significantly increased the amplitude of evoked EPSCs (154.4 ± 12.1 and 155.0 ± 10.6% of control, respectively; n = 6; P < 0.05). Responses to both agonists desensitized rapidly, making it difficult to obtain responses to higher concentrations of either compound. The 5-HT₄ antagonist GR 113808 (10 nM) did not affect EPSCs; however, GR 113808 blocked the facilitation of fast EPSCs elicited either by renzapride or tegaserod to 14.1 ± 1.4 and 12.2 ± 0.3% of the control, respectively (n = 3; Fig. 12D). These data confirm that 5-HT₄ receptors act presynaptically to increase the amplitude of cholinergic (nicotinic) EPSCs in mouse myenteric neurons. These observations are consistent with those made on other species in which both facilitation of fast EPSPs (41, 65, 79) and release of ACh have been reported (47).

**DISCUSSION**

The current study was designed to identify the 5-HT₄ splice variants expressed in the gut, to locate enteric sites of 5-HT₄ expression, to clarify the relationship of the 5-HT₄ receptor to 5-HT₁P activity, and to determine the function of 5-HT₄ receptors in enteric neuronal signaling. With regard to the expression of splice variants of the 5-HT₄ receptor, our data indicate that each of the four isoforms (a, b, e, and f) of the mouse 5-HT₄ receptor that have been found in the brain (24) are also expressed in the gut. Expression of the 5-HT₄a and 5-HT₄b isoforms predominated in all regions of the bowel and in both plexuses; however, the level of expression of each of these isoforms, normalized to the expression of synaptophysin to correct for the proportion of transcripts derived from neurons, was greater in the submucosal than the myenteric plexus. The
imidazole-1-carboxamide (BIMU 1; 50 \( \mu \)M) evokes a sustained inward current (Fig. 10A). A specific 5-HT1P agonist, WAY 100325, evokes a sustained inward current (Fig. 10A). A specific 5-HT1P agonist, WAY 100325 (50 \( \mu \)M), evokes a slow sustained inward current (A), which is blocked by the selective 5-HT1P receptor antagonist 5-HTP-DP (5 \( \mu \)M; B) or BIMU 1 (5 \( \mu \)M; C), both of which are 5-HT4 agonists, evoke a sustained inward current. D: neuron patched with pipettes containing GTP\( \gamma \)S. Neither 5–5-methoxytryptamine (5-MeOT; 50 \( \mu \)M) nor endo-N-(8-methyl-8-azabicyclo[3,2,1]oct-3-yl)-2,3-dihydro-3-ethyl-2-oxo-1H-benzimidazol-1-carboxamide (BIMU 1; 5 \( \mu \)M; D), both of which are 5-HT4 agonists, evokes a sustained inward current. E: neuron patched with a pipette containing GTP\( \gamma \)S and exposed to 5-HT for 10 s (dashed line). E: 5-HT evokes a sustained inward current that is maintained in the presence of 5-HT. F: the 5-HT-evoked slow inward current is not affected by the presence of the 5-HT4 antagonist SB 204070 (1 \( \mu \)M).

Relatively high concentration of the predominant 5-HT4 isoforms in the submucosal plexus is consistent with the possibility that 5-HT4 functions are particularly critical for events that occur in this region of the ENS (see below). Although expression of the 5-HT4a and 5-HT4f isoforms was far less than that of 5-HT4e or 5-HT4b, 5-HT4e and 5-HT4f expression was myenteric plexus specific. The 5-HT4a and 5-HT4f isoforms have previously been considered to be restricted to the brain (24). 5-HT4e and 5-HT4f expression in the bowel was probably missed in prior studies because the whole gut, in which these transcripts are rare, and not dissected layers, in which they are more concentrated, was examined; nevertheless, although not brain specific, 5-HT4e and 5-HT4f are probably neuron specific, and their restriction to the myenteric plexus illustrates the resemblance of this plexus to the CNS.

Transcripts encoding the 5-HT4 receptor were identified by in situ hybridization in subsets of enteric neurons of both plexuses, ICCs, and smooth muscle cells of the muscularis externa. Enteric 5-HT4 expression, therefore, is not restricted to the ENS. These data are consistent with prior pharmacological studies that have reported direct 5-HT4-mediated effects on gastrointestinal muscle as well as nerve. Reported neuronal 5-HT4 actions have all been excitatory. For example, 5-HT4 stimulation enhances the amplitude of electrically driven twitches of longitudinal smooth muscle because it increases the release of ACh from motoneurons (27, 59, 86, 92, 94). 5-HT4 stimulation, within the ENS is also excitatory (87) but has been analyzed mainly by using microelectrodes, which have detected an increase in the amplitude of fast EPSPs (41, 65, 79, 93). 5-HT4 receptor stimulation has also been reported to facilitate peristaltic reflexes by increasing the release of ACh and CGRP from activated submucosal intrinsic primary afferent neurons (IPANs) (37, 47–49, 55). In contrast to the excitatory, prokinetic responses mediated by the neuronal actions of the 5-HT4 receptor, those that are mediated by 5-HT4 receptors on muscle cells, in rat esophagus, rat ileum, and human colon have all been reported to be inhibitory (5, 18, 63, 91, 95).

It is not surprising that the Gs-coupled 5-HT4 receptor, which increases intracellular cAMP, should relax intestinal muscle cells. Virtually anything that increases the concentration of cAMP in smooth muscle relaxes these cells (17, 71, 100). The surprise is that the neuronal and muscular effects of the 5-HT4 receptor should be functionally antagonistic. The function of 5-HT4 receptors on ICCs and especially IC-MY, in which they are most numerous, is difficult to predict, because it has yet to be investigated. The ICC 5-HT4 receptors are probably coupled to Gs and associated with a rise in cAMP, as in other cells, but the consequences of increasing cAMP in ICC remain to be determined. There is evidence that ICCs are innervated by excitatory motor nerves (54); therefore, it is conceivable that 5-HT4 receptors on ICCs amplify excitatory signals and thus synergize with those on axons terminal. It is possible that smooth muscle (and ICC) 5-HT4 receptors are...
5-HT4 receptors in mouse enteric nervous system

5-HT4 immunoreactivity was also seen in nerve fibers within the submucosal and myenteric plexuses. The immunogen was a synthetic peptide with a high degree of sequence homology to all 5-HT4 receptor isoforms. The antibodies were specific for 5-HT4 receptor protein. A subset of living cells without prior permeabilization. Western blots of cell preparations, such as those used to measure bicarbonate secretion, stripped mucosal preparations, such as those used to measure bicarbonate secretion, probably contain submucosal ganglia, which in turn might have been the locus of the 5-HT4 activity that affects bicarbonate secretion. Inhibitory interneurons in the enteric nervous system (ENS) are often sites where receptors and other signaling molecules congregate; 5-HT2B receptors are also concentrated in caveolae of smooth muscle (35). No 5-HT4 immunoreactivity was detected in mucosal nerves.

The presence of 5-HT4 immunoreactivity on smooth muscle cell plasma membranes is consistent with the prior pharmacological studies of smooth muscle, a cell type that has never been claimed to express 5-HT1P activity. 5-HT4 receptors have been reported to evoke duodenal bicarbonate secretion in vitro, an effect that has been thought to be mucosal. The investigation of bicarbonate secretion, however, was not accompanied by a demonstration of 5-HT4 immunoreactivity or mRNA by in situ hybridization. Stripped mucosal preparations, such as those used to measure bicarbonate secretion, probably contain submucosal ganglia, which in turn might have been the locus of the 5-HT4 activity that affects bicarbonate secretion.

Some investigators have speculated that 5-HT1P activity in the bowel is actually due to stimulation of 5-HT4 receptors (49, 62, 63). The evidence for this speculation is largely derived from pharmacological studies of smooth muscle, a cell type that has never been claimed to express 5-HT1P activity. 5-HT4 agonists presynaptically increase the release of ACh from
electrically stimulated preparations of bowel; this effect is not blocked by the 5-HT<sub>1P</sub> antagonist 5-HTP-DP (26–28, 59). 5-HT<sub>4</sub> receptors also act presynaptically to increase the amplitude of fast EPSPs evoked by stimulating inputs to myenteric neurons (41, 65, 79, 80), an effect that is not shared by 5-HT<sub>1P</sub> agonists. Finally, 5-HT<sub>1P</sub> activity has been found to stimulate submucosal IPANs (81); moreover, this effect is blocked by 5-HTP-DP and resistant to 5-HT<sub>4</sub> antagonists. Mucosal application of 5-HT and 5-HT<sub>1P</sub> agonists activate submucosal IPANs, whereas 5-HT<sub>4</sub> agonists do not. If 5-HT<sub>4</sub> receptor activity is not, as proposed, identical to the 5-HT<sub>4</sub> receptor (49, found but they could not be attributed to stimulation of 5-HT<sub>4</sub> agonists. Interestingly, in some cells, exposure to 5-HT ap-

The slow inward current, therefore, was 5-HT<sub>1P</sub> dependent. In contrast, 5-HT and 5-HT<sub>4</sub> agonists (tsegaser and renzapride) acted presynaptically to enhance the amplitude of fast EPSCs. These EPSCs were blocked by hexamethonium and thus were cholinergic (nico-
tinic). 5-HT<sub>4</sub> antagonism by GR113808 did not affect the evoked EPSCs but prevented their enhancement by 5-HT<sub>4</sub> agonists. Interestingly, in some cells, exposure to 5-HT appeared to elicit low-amplitude transient fast currents, which could be superimposed on the 5-HT<sub>1P</sub>-mediated slow inward current. The low-amplitude transient fast currents resembled fast EPSCs. When this phenomenon occurred, the low-amplitude transient fast currents, but not the slow inward current, were blocked by GR113808. Presynaptic activation of 5-HT<sub>4</sub> receptors may therefore sometimes be able to evoke the release of transmitter to give rise to an EPSC-like activity. These observations, taken as a whole, suggest that 5-HT<sub>H</sub> receptors are predominantly, if not exclusively, presynaptic. Their stimulation enhances and may even provoke cholinergic neuro-
transmission.

5-HT<sub>4</sub> receptors belong to the group of seven-transmembrane G protein-coupled receptors and have been reported to mediate a number of postsynaptic responses in the CNS and the heart. For example, 5-HT<sub>4</sub> receptors activate cardiac L-type Ca<sup>2+</sup> channels or pacemaker currents in atrial myocytes (58, 78, 84), facilitate the hyperpolarization-activated cation current and a cyclic nucleotide-gated channel in hippocampal neurons (7, 22, 52), reduce the Ca<sup>2+</sup>-activated K<sup>+</sup> current responsible for the slow afterhyperpolarization in hippocampal neurons (1), and inhibit a voltage-activated K<sup>+</sup> current in collicular neurons (2, 10, 34). It would thus not have been surprising to find postsynaptic 5-HT<sub>4</sub>-mediated effects in the ENS. The 5-HT<sub>4</sub> receptor, however, is clearly not identical to 5-HT<sub>1P</sub> activity, which is postsynaptic. In intestinal muscle cells, 5-HT<sub>4</sub> receptor stimulation increases inositol 1,4,5-trisphosphate, intracellular Ca<sup>2+</sup> concentration, and cAMP by a mecha-
nism that is insensitive to pertussis toxin (62, 63). The 5-HT<sub>1P</sub> effect on enteric neurons is due to the antagonism of a Ca<sup>2+</sup>- activated K<sup>+</sup> current; therefore, it would be counterproductive for the 5-HT<sub>1P</sub>-initiated action to be associated with an increase in intracellular Ca<sup>2+</sup> concentration. The signal-trans-
duction pathway of the 5-HT<sub>1P</sub> receptor is pertussis toxin sensitive and blocked by intracellular injection of antibodies to G<sub>α<sub>o</sub></sub> (81, 82). 5-HT<sub>1P</sub> activity involves the generation of diacyl-
glycerol, which stimulates PKC to inactivate the Ca<sup>2+</sup>-acti-
vated K<sup>+</sup> current and, in type II/AH neurons, inhibits the afterhyperpolarization (76, 81). PKC can also activate type II adenylate cyclase, which increases intracellular cAMP and thus stimulates PKA, which acts with PKC to close Ca<sup>2+</sup>- activated K<sup>+</sup> channels (81). The coupling of 5-HT<sub>4</sub> receptors in enteric neurons to G<sub>α<sub>o</sub></sub> is not inhibited by pertussis toxin (41). In the current study, intracellular antibodies to G<sub>α<sub>o</sub></sub> were more effective than antibodies to G<sub>α<sub>o</sub></sub> in blocking the slow inward current evoked by 5-HT. These data are thus consistent with the idea that the slow inward current is 5-HT<sub>1P</sub> and not 5-HT<sub>4</sub> mediated, although the modest effect of intracellular antibodies to G<sub>α<sub>o</sub></sub> raises the possibility that 5-HT stimulates another receptor G<sub>α<sub>o</sub></sub>-coupled 5-HT receptor isoform, such as 5-HT<sub>7</sub> (74), which may contribute to the increase in cAMP elicited in enteric neurons by 5-HT. It is not necessary for 5-HT<sub>4</sub> agonists to evoke a postsynaptic effect on IPANs or other enteric neurons to exert a prokinetic action. They could do so nicely by increasing the strength of neurotransmission at cholinergic synapses, which our data and those of others have demonstrated that they do. Such an action is compatible with the observations that 5-HT<sub>4</sub> agonists promote the release of neurotransmitters from mucosally activated submucosal IPANs and are necessary to evoke a normal peristaltic reflex (47). 5-HT<sub>4</sub> receptors thus are not likely to be the initiators of the peristaltic reflex, but they would strengthen those reflexes when they are elicited by natural stimuli.

ACKNOWLEDGMENTS

We thank Drs. S. Rayport, R. Ambron, and Y. Sung for advice.

GRANTS

This work was supported by National Institutes of Health Grant NS-12969 and Novartis (to M. D. Gershon).

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