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Expression and function of 5-HT_3 receptors in the enteric neurons of mice lacking the serotonin transporter

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Liu, Min-Tsai, Stephen Rayport, Yan Jiang, Dennis L. Murphy, and Michael D. Gershon. Expression and function of 5-HT_3 receptors in the enteric neurons of mice lacking the serotonin transporter. *Am J Physiol Gastrointest Liver Physiol* 283: G1398–G1411, 2002. First published September 4, 2002; 10.1152/ajpgi.00203.2002.—The actions of enteric 5-HT are terminated by 5-HT transporter (SERT)-mediated uptake, and gastrointestinal motility is abnormal in SERT −/− mice. We tested the hypothesis that adaptive changes in enteric 5-HT_3 receptors help SERT −/− mice survive despite inefficient 5-HT inactivation. Expression of mRNA encoding enteric 5-HT_{3A} subunits was similar in SERT +/+ and −/− mice, but that of 5-HT_{3B} subunits was fourfold less in SERT −/− mice. 5-HT_{3B} mRNA was found, by in situ hybridization, in epithelial cells and enteric neurons. 5-HT evoked a fast inward current in myenteric neurons that was pharmacologically identified as 5-HT_3 mediated. The EC_{50} of the 5-HT response was lower in SERT +/+ (18 μM) than in SERT −/− (36 μM) mice and desensitized rapidly in a greater proportion of SERT −/− neurons; however, peak amplitudes, steady-state current, and decay time constants were not different. Adaptive changes thus occur in the subunit composition of enteric 5-HT_3 receptors of SERT −/− mice that are reflected in 5-HT_3 receptor affinity and desensitization.

serotonin receptors; small intestine; enteric nervous system; electrophysiology

5-HT IS UTILIZED IN THE BOWEL as a neurotransmitter (24) and a paracrine messenger from enterochromaffin (EC) cells (17) to initiate peristaltic and secretory reflexes (8, 14, 42, 43, 54). Released 5-HT must be inactivated to prevent its actions from becoming excessive and receptors from desensitizing. To be inactivated, 5-HT must enter cells, because its catabolic enzymes are intracellular (25, 26). 5-HT is charged at physiological pH; therefore, its uptake across plasma membranes requires a high-affinity 5-HT transporter (5-HTT; SERT), which is identical in the brain (3, 4, 10), enteric nervous system (ENS), and gastrointestinal mucosa (11, 12, 65).

Transgenic mice that lack SERT have been generated by deletion of the second exon by homologous recombination (1). High-affinity uptake of 5-HT is not detectable in either the brain (1) or the gut (12) of these animals. SERT −/− mice are viable into adulthood. In part, they survive because transporters other than SERT mediate 5-HT uptake (12, 58). In the gut, alternative transporters include the dopamine transporter and organic cation transporters-1 and -3 (12). The affinity of the dopamine transporter and the organic cation transporters for 5-HT is much lower than that of SERT; however, they have a high capacity and prevent 5-HT from accumulating to toxic levels. A number of presumably adaptive changes have been found in the brains of SERT −/− mice, including desensitization of presynaptic 5-HT_{1A} receptors (18, 28, 44) and a differential regulation of adenosine A_1 and A_{2A} receptors (50). The alternative transporters and other adaptive changes, however, compensate incompletely for the absence of SERT. Developmental defects occur in SERT −/− mice, including a lack of barrel formation by thalamocortical afferents in the cerebral cortex (55), increased stool water, and a colorectal motility that alternates between excessive (diarrhea) and inadequate (constipation) (12).

5-HT_3 receptors are expressed in the ENS (36, 47, 60) and play important roles in enteric physiology. They rapidly depolarize neurons (20, 47) by invoking a fast inward current (70, 71), which is responsible for 5-HT-mediated fast neurotransmission (71). A combination of 5-HT_3 and 5-HT_4 antagonists inhibits the peristaltic reflex (39, 51, 52). 5-HT_3 antagonists decrease the frequency of migrating motor complexes in the isolated murine terminal ileum and colon (9). 5-HT_3 receptors are also the physiological mediators of sensory nerve excitation by 5-HT from EC cells (2, 31, 34), which enables 5-HT_3 antagonists to prevent the nausea associated with cancer chemotherapy (30).

The many roles played by 5-HT_3 receptors in the bowel suggest that the ability of the gut to function in SERT −/− mice might involve adaptive changes in 5-HT_3 receptors. Experiments were therefore carried out to examine the expression and function of 5-HT_3 receptors in the ENS of SERT −/− mice. The transcrip-

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tion of mRNA encoding the 5-HT$_{3A}$ (46) and 5-HT$_{3B}$ (15) subunits was analyzed by RT-PCR and quantified. The previously unknown distribution of 5-HT$_{3B}$ subunits was studied by in situ hybridization (ISH). 5-HT$_3$ receptor functions were analyzed by whole cell patch-clamp studies in cultured myenteric neurons. The data indicate that the expression of 5-HT$_{3B}$, but not that of 5-HT$_{3A}$, is reduced in SERT −/− mice. This change is associated with adaptive changes in receptor affinity and resistance to desensitization that may help the ENS of SERT −/− mice to function despite their abnormal 5-HT inactivation.

MATERIALS AND METHODS

Animals. Experiments were carried out with adult mice of either sex (6–12 mo of age; 30–40 g body wt). The genotypes of the animals were SERT +/+ and SERT −/− mice on backgrounds that were either CD-1 (n = 20) or C57BL/6J (n = 16). Comparisons were always made between littermates. Genotypes of the mice were determined as described by Bengel et al. (1). All of the procedures involving animals and their care followed National Institutes of Health guidelines and were approved by the Animal Care and Use Committee of Columbia University.

RNA isolation and RT-PCR. Total RNA (2 μg) was extracted from the ileum, colon, brain, and spleen and was prepared by using a commercial kit (RNA STAT-60) according to the manufacturer's instructions (Tel-Test, Friendswood, TX). cDNA was prepared from this RNA by reverse transcription at 42°C (30 min) in the presence of random primers and murine leukemia virus reverse transcriptase (GeneAmp RNA PCR kit; Applied Biosystems, Foster City, CA). The reverse transcriptase was omitted in controls and permitted the detection of contamination of samples with genomic DNA. DNA contamination was removed by treating RNA with RNase-free DNase I (Promega, Madison, WI) for 15 min at 37°C before reverse transcription. All experiments were carried out with 0.1% diethyl pyrocarbonate (Sigma, St. Louis, MO)-treated distilled water. The cDNA was amplified by using the PCR with *Taq* DNA polymerase (Applied Biosystems). Initial denaturation was carried out at 95°C for 2 min, the denaturation that accompanied each cycle was carried out at 95°C for 45 s, and elongation was carried out at 72°C for 2 min (except in the case of β-actin, for which the time for elongation was 40 s). Primers, Mg^{2+} concentration, annealing time and temperature, and number of cycles are listed in Table 1. After the final PCR cycle, the reaction was extended for an additional 10 min at 72°C, and the reaction products were then cooled to 4°C. PCR products were resolved by electrophoresis through 1% agarose gels with ethidium bromide (0.3 μg/ml) in Tris-borate-EDTA electrophoresis buffer. The identity of the PCR products was verified by sequencing. For this purpose, PCR products from the ileum were subcloned into a pCR II vector digested with *Eco*R I by using a commercial kit (TA Cloning Kit; Invitrogen, Carlsbad, CA). Clones with inserts were further processed to isolate the plasmid DNA (Wizard Minipreps; Promega) and sequenced by dye termination (ABI Automated Sequencer; Perkin Elmer) in the core facility of Columbia University. The final cDNA sequences were compared with those in GenBank (BLAST search at National Center for Biotechnology Information, Bethesda, MD). All experiments were carried out with RNA prepared from three separate littermate pairs of SERT +/+ and −/− mice.

Competitive PCR. The method used to quantify mRNA encoding the 5-HT$_{3B}$ subunits was based on the technique of competitive PCR (63). Competitor DNA fragments for quantitative PCR were obtained by using mouse-specific primers to amplify DNA from chicks, a species that is a distant evolutionary relative of the mouse, under low-stringency annealing conditions (45°C for 1 min; 30 cycles). These artificially created fragments contain the mouse primer-specific ends and thus can be used to quantify mouse DNA amplified by these primers. The competitor DNA fragments differ in size from the corresponding mouse target DNA and are selected by agarose gel electrophoresis, which enables both fragments to be visually distinguished. After electrophoresis, fragments of appropriate size were excised and subcloned for sequencing as described above to verify that the competitor DNA did not overlap the PCR product from the target DNA. To compare constant amounts of cDNA from the tissues, the cDNAs were adjusted to equal amounts by using the β-actin DNA for calibration. Serial dilutions of competitor DNA were added to equal amounts of cDNA of each sample before applying PCR with primer pairs designed to amplify DNA encoding the 5-HT$_{3B}$ subunit (Table 1). For quantitative analysis, the ethidium bromide agarose gels were photographed by using a UV illuminator, scanned, and digitized. The optical density of the digitized images was analyzed by using Kodak Digital Science 1D Image Analysis v.1.51 software (Eastman Kodak, Rochester, NY).

Western blotting. The small intestines were rapidly removed, and the longitudinal muscle was removed with the adherent myenteric plexus (LMMP). The resulting LMMP sheets of tissue were homogenized in a cold lysis buffer (0.02 M Tris, 0.1% Triton, 1 mM EDTA) containing a commercial cocktail of protease inhibitors [diluted 1:10; a mixture of 4-(2-aminoethyl)benzenesulfonyl fluoride, aprotinin, leupeptin, bestatin, and pepstatin A; Sigma]. The lysate was centrifuged at a low speed to remove debris, and the protein concentration of the supernatant was measured by Bio-Rad

Table 1. *Primers and conditions used for PCR*

Product	Accession Number	Sense/ Antisense	Primer Pairs	Size, bp	[Mg^{2+}], mM	Annealing Condition	Cycles
5-HT$_{3A}$	M74425	S	5′-ACACCATCCAGGACATCAAC-3′	399	2.5	61°C, 1 min	30
		A	5′-CCATGCACACCACAAAGTAG-3′				
5-HT$_{3AL/S}$	M74425	S	5′-TGGTGTCTACTTTGTGGTGTG-3′	293	3.0	58°C, 1 min	35
		A	5′-TCTTCTCCAAGTCCTGAGGTC-3′				
5-HT$_{3B}$	AF155045	S	5′-GGCAGCTTCTTTCTGTGTCC-3′	567	3.0	64.7°C, 1 min	35
		A	5′-CCCGAGTTCGAAGAGAGTTG-3′				
β-actin	X03765	S	5′-GTGGGCCGCTCTAGGCACCAA-3′	540	2.5	65°C, 40 s	30
		A	5′-CTCTTTGATGTCACGCACGATTTC-3′				

S, sense; A, antisense.

protein assay (Bio-Rad Laboratories, Richmond, CA). The supernatant was then boiled for 5 min at 95°C in Laemmli solution (Bio-Rad) containing 0.35 M dithiothreitol. An aliquot containing 20 μg of protein was subjected to SDS-PAGE (10% polyacrylamide). The separated proteins were electrophoretically blotted onto a nitrocellulose sheet for Western blot analysis. The blots were bleached for 5 min with 6% H_2O_2 and blocked by incubation for 2 h in TBST buffer (0.05 M Tris, 0.15 M Tris, 0.05% Tween) containing 5% fat-free milk. The blots were probed with purified rabbit antibodies to the 5-HT$_3$ receptor (diluted 1:1,000; Oncogene, Boston, MA). Immunoreactivity was identified with goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase (diluted 1:5,000; Jackson Labs, West Grove, PA). Horseradish peroxidase activity was visualized with 4-chloro-1-naphthol (4-CN kit; Bio-Rad).

ISH. Small segments of ileum were fixed for 3 h with 4% (wt/vol) formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M PBS, pH 7.4. The tissue was cryoprotected by incubation overnight (at 4°C) in PBS containing 30% (wt/vol) sucrose. Tissues were then embedded in a sectioning compound (TissueTek OCT; Miles, Elkhart, IN), frozen in liquid N_2, and sectioned (10 μm) at −20°C by using a cryostat-microtome. Cultured neurons were fixed in the same fixative solution but kept for 1 h at room temperature. Fixed cultures were washed with PBS for 30 min and stored at 4°C until used. All experiments were carried out with 0.1% diethyl pyrocarbonate-treated distilled water.

Digoxigenin-labeled cRNA probes were prepared from mouse cDNA encoding the 5-HT$_{3B}$ subunits (Table 1). The cloned plasmid DNA was linearized with either *Bam*HI or *Xho*I and purified, and T7 and SP6 RNA polymerases were used to transcribe, respectively, sense and antisense cRNA probes. The cRNA probes were quantified by dot blotting by using a commercially supplied protocol (DIG-RNA labeling kit; Roche Molecular Biochemicals, Indianapolis, IN). ISH (13) was carried out by incubating tissue sections or neuronal cultures with sense or antisense probes (0.2–0.5 ng/μl in 100-μl wells) in a moist chamber at 60°C for 16 h. The hybridization buffer contained 50% formamide, 5× SSC (pH 4.5), 50 μg/ml yeast tRNA, 1% SDS, and 50 μg/ml heparin. Following hybridization, preparations were washed four times in 5× SSC (pH 4.5)-1% SDS-50% formamide at 60°C and three times in 5× SSC (pH 4.5)-50% formamide at 55°C. Bound digoxigenin was detected with antibodies to digoxigenin coupled to alkaline phosphatase (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 100 mM NaCl, 50 mM $MgCl_2$, 1% Tween-20, and 100 mM Tris·HCl at pH 9.5. The endogenous alkaline phosphatase activity of the intestines was inhibited by incubating tissue sections with 250 μg/ml levamisole (Sigma). The purple reaction product representing the sites in the tissue sections or cultured myenteric neurons containing mRNA encoding the 5-HT$_{3B}$ subunits were visualized by brightfield microscopy (Leica, Asbury, NJ).

Immunocytochemistry. For immunocytochemistry (ICC), fixed preparations were permeabilized and blocked with 0.5% Triton X-100 (vol/vol) and 4% normal horse serum in PBS for 30 min. The preparations were then incubated overnight (at 4°C) with rabbit antibodies (1 μg/ml) raised against the rat 5-HT$_3$ receptor (Oncogene). After being washed with PBS, the sites of bound primary antibody were detected by incubation with donkey anti-rabbit secondary antibodies coupled to FITC (diluted 1:400, 3 h at room temperature; Jackson Labs). The preparations were finally washed again with PBS and then were mounted in Vectashield (Vector Labs, Burlingame, CA). No immunostaining was observed when the primary antibody was omitted.

Combination of ISH and ICC. Double labeling was used to locate mRNA encoding the 5-HT$_{3B}$ subunit and 5-HT$_3$ receptor protein simultaneously. ISH was always carried out before ICC. The digoxigenin-labeled probe used for ISH was detected by using antibodies to digoxigenin coupled to 5-carboxy-tetramethyl-rhodamine-*N*-hydroxy-succinimide ester (TAMRA, diluted 1:20; Roche). The red fluorescence of TAMRA provided a good contrast for the green fluorescence of FITC to detect antibodies bound to 5-HT$_3$ receptors by ICC. Immunocytochemical preparations were examined by using a LSM 410 laser scanning confocal microscope (Zeiss, Thornwood, NY) equipped with a krypton/argon laser and attached to a Zeiss Axiovert 100TV inverted microscope. The fluorescence of FITC (excitation 488 nm, dichroic BP 515–540 nm) and TAMRA (excitation 568 nm, dichroic LP 590 nm) (Chroma optical filter sets) were viewed with dichroic mirrors and filters that permitted no cross-detection of fluorescence. Optical sections (10–15) were taken at 1.0-μm intervals. Images of 512 × 512 pixels were obtained with a confocal microscope and processed by using Adobe Photoshop 6.0.

Cell culture. Mice were anesthetized with CO_2 and decapitated, following procedures approved by the Columbia University Animal Care and Use Committee. The small intestine was removed, cleaned, and placed in iced sterile-filtered Krebs solution of the following composition (in mM): 121.3 NaCl, 5.95 KCl, 14.3 $NaHCO_3$, 1.34 NaH_2PO_4, 1.2 $MgCl_2$, 2.5 $CaCl_2$, and 12.7 glucose. The procedure used for the isolation of myenteric neurons for culture procedure has previously been described (41). Briefly, the LMMP was removed from the entire length of small intestine and minced into small pieces. The resulting tissue was suspended in 10 ml of oxygenated (95% O_2-5% CO_2) Krebs solution containing collagenase (type 1A; 1.3 mg/ml), protease (type IX; 1 mg/ml), DNase (type 1; 0.3 mg/ml), and bovine serum albumin fraction V (0.3 mg/ml). Tissue digestion was carried out with gentle stirring for 1 h at 37°C and terminated by centrifugation three times for 2 min at 2,000 rpm (Eppendorf microcentrifuge, model 5415C). The pellet was resuspended in culture medium consisting of DMEM and F-12K (Kaighn's modification) (1:1 mixture) supplemented with 10% heat-inactivated fetal bovine serum, gentamicin (50 μg/ml), penicillin-streptomycin (100 U/ml and 100 μg/ml), and amphotericin B (0.25 μg/ml). The suspended cells were then plated on glass coverslips coated with poly-L-ornithine (0.1 mg/ml; Sigma) and laminin (10 μg/ml) or Matrigel (1:4 dilution; Becton Dickinson Biosciences, Bedford, MA) and maintained in an incubator at 37°C in a humidified atmosphere of 5% CO_2 for up to 2 wk. After 2 days in culture, 10 μM cytosine arabinoside (Sigma) was added to the culture medium to limit the proliferation of nonneuronal cells, and the medium was changed twice weekly thereafter.

Whole cell patch-clamp recording and drug application. Recordings were carried out at room temperature on the stage of an inverted microscope (Axiovert IM35, Zeiss) 2–14 days after plating cells. The procedure was similar to that described previously (38). The culture medium was replaced with an external solution containing (in mM) 145 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 11 glucose, and 10 HEPES, pH 7.35, with NaOH. The concentrations of K^+ and Ca^{2+} were chosen to match those in the Krebs solution used in prior studies with sharp microelectrodes to make the data comparable. The recording chamber was continually perfused at a rate of 0.5 ml/min. Recording electrodes were made from borosilicate glass (inner diameter 0.86 mm, outer diameter

1.5 mm; Warner, Hamden, CT), pulled on a P-80/PC Brown-Flaming micropipette puller (Sutter, Novato, CA), and had tip resistances of 5–8 MΩ. The internal pipette solution contained (in mM) 140 potassium gluconate, 0.1 $CaCl_2$, 2 $MgCl_2$, 1 EGTA, 2 Na_2ATP, 0.2 Na_3GTP, and 10 HEPES, pH 7.25, with KOH. Whole cell currents were recorded using an Axoclamp 2A amplifier (Axon Instruments, Union City, CA) running in continuous, single-electrode, voltage-clamp mode; membrane potential was recorded in current-clamp bridge mode. The holding potential was set at −60 mV. Data for continuous gap-free recordings were digitized at 5 kHz by using a Digidata 1322A interface and AxoScope 8.1 (Axon Instruments). Data from the whole cell recordings were filtered at 2 kHz, digitized at 10 kHz (ITC-16; Instrutech, Port Washington, NY), and collected by using Pulse Control 5.0 (Instrutech) and IgorPro 3.1 (Wavemetrics, Lake Oswego, OR). Compensation for passive and leak conductance was made by adding a scaled average of four hyperpolarizing pulses (−5 mV) delivered following each episode of data acquisition (Subtraction Pulses Global).

Space-clamp problems were minimized by limiting application of drugs to the soma and proximal dendrites of recorded neurons. Although there may be some distortion in the records obtained, there is no reason to believe that this would be different in neurons from SERT +/+ and −/− mice. Drugs were applied by local perfusion using a gravity-fed, rapid-exchange U-tube system (29, 62); a drug application tube (~75 μm in diameter) that emerged from the U-tube was positioned ~100 μm from the recorded cell. Drugs were selected with a six-position rotary valve, and drug application was initiated by closing a solenoid in the outflow tube, reversing the flux in the drug application tube, directing the drug stream to the recorded cell. There was an ~1-s delay from when the solenoid was closed before the drug emerged, but the onset of drug application was rapid. In test experiments with 50 mM KCl, the rise time (10–90%) of the depolarizing response was 55.8 ± 4.2 ms (n = 4). In some experiments, fast green (Sigma) was used to monitor the application and removal of drugs; this showed that the drug emerged as a wave with a well-defined surface; when the solenoid was reopened, the fast green was completely removed within 3 s.

5-HT or 2-methyl-5-HT (2-Me-5-HT) was applied at 4-min intervals and 1-(*m*-chlorophenyl)-biguanide (mCPBG) at 6-min intervals to avoid desensitization. Because the 5-HT$_3$ receptor is desensitized more rapidly by high concentrations of agonists, all experiments were carried out with agonist concentrations that were submaximal, and compounds were never applied for longer than 10 s, unless otherwise indicated. When antagonists were studied, they were applied for 30 s before the coapplication of an agonist with the antagonist for 10 s. Current-voltage relationships were determined from a holding potential of −60 mV by applying 400-ms test pulses (−120 to +20 mV in 10-mV increments). Experiments were completed within 30 min of obtaining recordings to minimize time-dependent changes in response properties and cell deterioration. Offline data analysis was carried out with IgorPro 3.1 and AxoGraph 4.6 (Axon Instruments) software.

The time course of the 5-HT$_3$ receptor decay current was best fit with the equation of Chebyshev polynomials: $y = A_{fast}[\exp(-t/\tau_{fast})] + A_{slow}[\exp(-t/\tau_{slow})] + C_s$, where A is the amplitude, t is time, τ_{fast} and τ_{slow} are fast and slow time constants, and C_s is the steady-state current (AxoGraph). In some instances, the duration of desensitization of the 5-HT$_3$ receptors was shortened, and thus the rate of decay could be fit by a single exponential function. Concentration-effect curves for 5-HT were obtained by using an ascending sequence of 5-HT concentrations: 0.1, 0.5, 1, 5, 10, 50, 100, and 300 μM. Responses at each concentration were expressed as a fraction of the mean response to 300 μM 5-HT, and the composite curve was fit with the following equation: $y = A/[1+ (EC_{50}/x)^{nH}]$, where EC_{50} is the half-maximally effective concentration, n_H is the Hill coefficient and A is any number. Numerical data were expressed as means ± SE, and the significance of differences was evaluated by the Wilcoxon signed-rank test or unpaired Student's t-tests (StatView 4.5; Abacus, Berkeley, CA). Results were considered significant at the 1% level.

Compounds used. Enzymes for digestion of tissue were obtained from Sigma. Tissue culture reagents were obtained from GIBCO Life Technologies (Grand Island, NY). 5-HT creatinine sulfate, 2-Me-5-HT, mCPBG, tropisetron, TTX, hexamethonium, and cytosine arabinoside were purchased from Sigma/RBI (Natick, MA). Ondansetron and alosetron were supplied by Glaxo Wellcome (Research Triangle Park, NC).

RESULTS

mRNA encoding the 5-HT$_{3A}$ and 5-HT$_{3B}$ subunits is present in the mouse gut. mRNA encoding 5-HT$_3$ subunits was detected in the bowel of SERT +/+ and −/−

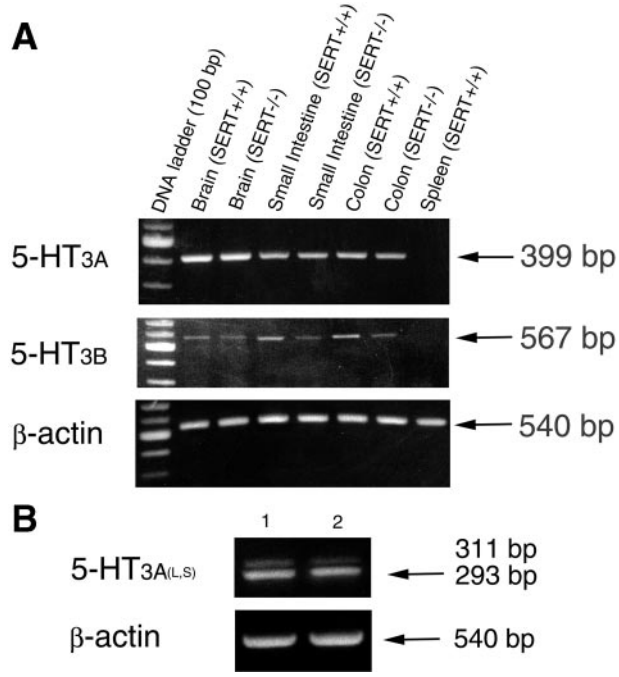

Fig. 1. mRNA encoding the 5-HT$_{3A}$ and 5-HT$_{3B}$ subunits is found in the bowel of 5-HT transporter (SERT) +/+ and −/− mice. *A*: mRNA encoding the 5-HT$_{3A}$ and 5-HT$_{3B}$ subunits demonstrated in various murine tissues by means of RT-PCR. The expected size of PCR products is shown at *right*. In wild-type (SERT +/+) mice, mRNAs encoding 5-HT$_{3A}$ (total) and 5-HT$_{3B}$ subunits are all found in the brain (positive control) but not the spleen (negative control). mRNA encoding each of these subunits are also found in the small intestine and colon of SERT +/+ and −/− mice. mRNA encoding β-actin was simultaneously demonstrated by RT-PCR to provide a semiquantitative reference. *B*: PCR products from the small intestines of SERT +/+ (*lane 1*) and −/− (*lane 2*) mice derived from mRNA encoding the long and short isoforms of the 5-HT$_{3A}$ subunit [5-HT$_{3A(L,S)}$] were separated on a 2% agarose gel. The fragments of 293 and 311 bp correspond to 5-HT$_{3AS}$ and 5-HT$_{3AL}$ subunits, respectively. mRNA encoding β-actin was simultaneously analyzed by RT-PCR to provide a semiquantitative frame of reference.

mice by using RT-PCR (Fig. 1). The mouse brain and spleen were studied at the same time as the gut as positive and negative controls, respectively. mRNA encoding β-actin was used to provide a semiquantitative reference to which to compare the products of amplification. PCR products of the expected size (Table 1) were obtained when cDNA from the small intestine and colon was amplified with primers corresponding to sequences found in the 5-HT$_{3A}$ and 5-HT$_{3B}$ receptor subunits (Fig. 1*A*). Subcloning and sequencing confirmed the identities of all of the PCR products. As expected, mRNA encoding 5-HT$_{3A}$ and 5-HT$_{3B}$ receptor subunits was also detected in the brain of SERT +/+ and −/− mice but not in the spleen.

Alternative splicing of mRNA encoding the 5-HT$_{3A}$ subunit is known to give rise to long and short isoforms, 5-HT$_{3AL}$ and 5-HT$_{3AS}$, which differ from one another by six amino acids in the cytoplasmic loop between the third and fourth transmembrane domains (45, 48, 66); furthermore, mRNA encoding the 5-HT$_{3AS}$ subunit has been found to be more abundant than that encoding 5-HT$_{3AL}$ in the mouse superior cervical ganglion (66) and guinea pig small intestine (45). To determine whether alternative splicing of mRNA encoding the 5-HT$_{3A}$ subunit occurs in the mouse gut and is affected by the knockout of SERT, cDNA was amplified with primers that flank the sequence that contains the amino acids that are deleted in 5-HT$_{3AS}$. PCR products were obtained (and resolved on a 2% agarose gel) from the small intestines of SERT +/+ and −/− mice corresponding to cDNA encoding both the 5-HT$_{3AS}$ and 5-HT$_{3AL}$ subunits (Fig. 1*B*). The nucleotide sequences of the splice variants were verified by sequencing. The apparent expression of mRNA encoding the 5-HT$_{3AL}$ subunit in both SERT +/+ and −/− mice was less than that of 5-HT$_{3AS}$. Semiquantitative RT-PCR did not reveal a difference between SERT +/+ and −/− mice in the expression of the 5-HT$_{3A}$ subunits or in its splice variants (5-HT$_{3AS}$ and 5-HT$_{3AL}$). In contrast, expression of the 5-HT$_{3B}$ subunits appeared to be reduced in both the small intestine and colon of SERT −/− mice (Fig. 1*A*).

mRNA encoding the 5-HT$_{3B}$ subunit is decreased in the gut of SERT −/− mice. The suggestion that expression of mRNA encoding 5-HT$_{3B}$ subunit might be reduced in SERT −/− mice was investigated by using competitive PCR to quantify 5-HT$_{3B}$ expression in the small intestine and colon of SERT +/+ and −/− mice. The cDNA obtained by reverse transcription from the tissues of SERT +/+ and −/− mice were compared (Fig. 2) by using β-actin as a standard to calibrate the amount of cDNA. Equal amounts of target DNA were then amplified simultaneously with known amounts of a competitor DNA fragment (5-HT$_{3B}$-c.f.), which was distinguished from that of murine DNA by size. This competitor (358 bp) did not overlap the PCR product from the mouse 5-HT$_{3B}$ subunit DNA (567 bp; Fig. 2, *insets*). The amount of amplified DNA encoding the 5-HT$_{3B}$ subunits (5-HT$_{3B}$-cDNA) was quantified by comparing it to that of the serially diluted competitor (5-HT$_{3B}$-c.f.) by optical density. The 5-HT$_{3B}$-c.f. (0.003–300 fmol/l) was coamplified with a fixed amount (1 μg) of target DNA from small intestine (Fig. 2*A*) or colon (Fig. 2*B*) of SERT +/+ and −/− mice. The small intestine (Fig. 2*A*) and the colon (Fig. 2*B*) of SERT +/+ mice each contained about fourfold more mRNA encoding the 5-HT$_{3B}$ subunit than did the small intestine and colon of their SERT −/− littermates.

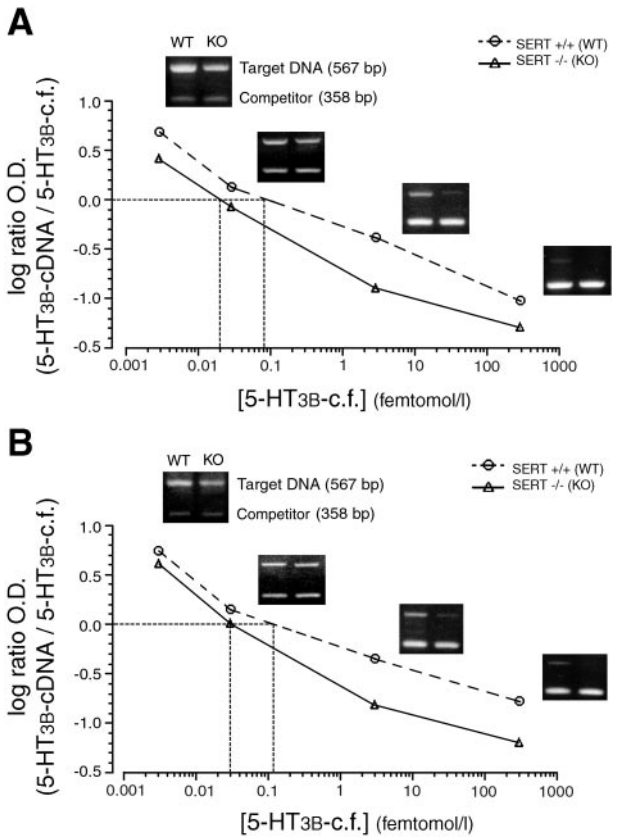

Fig. 2. mRNA encoding the 5-HT$_{3B}$ subunit is downregulated in the bowel of SERT −/− mice. Competitive PCR was used to quantify the relative expression of mRNA encoding the 5-HT$_{3B}$ subunits. The optical density (OD) of PCR products obtained by amplifying DNA with primers selective for the 5-HT$_{3B}$ subunits (5-HT$_{3B}$-cDNA or target DNA) was compared with that of the competitor cDNA fragment (5-HT$_{3B}$-c.f. or competitor). Serial dilutions of the competitor (0.003–300 fmol/l) were coamplified with a fixed amount of cDNA (1 μg) from the small intestine (*A*) or colon (*B*) of SERT +/+ (WT) and −/− (knockout; KO) mice. Fourfold more competitor was needed to compete out [to obtain a log ratio of (OD target DNA/OD competitor DNA) = 0] the DNA encoding the 5-HT$_{3B}$ subunits amplified from SERT +/+ mice than that amplified from SERT −/− animals. *Insets* depict representative gels obtained at each tested concentration of the competitor. Data points are mean values of 3 measurements.

mRNA encoding the 5-HT$_{3B}$ subunit is found in epithelial cells and enteric neurons. Although mRNA encoding the 5-HT$_{3A}$ receptor has been found to be present in both submucosal and myenteric ganglia of the rat small intestine (36), that encoding the 5-HT$_{3B}$ subunit of the receptor has not previously been located in the bowel of any species. The studies described above, however, indicated that it is the expression of the 5-HT$_{3B}$ subunit, and not that of the 5-HT$_{3A}$, that is affected by the absence of SERT in the gut of SERT −/− mice. ISH was thus used to identify the enteric

cells in which the 5-HT$_{3B}$ subunit is expressed. mRNA encoding the 5-HT$_{3B}$ subunit was observed in neurons of both the myenteric and submucosal plexuses in SERT +/+ mice (Fig. 3*A*). mRNA encoding the 5-HT$_{3B}$ subunit was also present in mucosal epithelial cells of the intestinal crypts of the SERT +/+ animals (Fig. 3*B*). These cells are most likely to be EC cells, which are known to contain 5-HT$_3$ receptors (23, 40, 57). mRNA encoding the 5-HT$_{3B}$ subunit was also found in myenteric and submucosal neurons in SERT −/− mice, and the degree to which the riboprobe hybridized with cells in these animals appeared to be much less than that in their SERT +/+ counterparts (Fig. 3*C*). Because the intensity of labeling was so severely reduced in the SERT −/− animals, few labeled cell bodies could be discerned in the ganglia of these animals, and no mRNA encoding the 5-HT$_{3B}$ subunit was detected in the epithelial cells of SERT −/− mice. No labeling of control tissue was observed when sections of gut from either SERT +/+ or −/− mice were hybridized with a sense riboprobe (Fig. 3*D*).

The 5-HT$_{3B}$ subunit is specifically expressed in a subset of enteric neurons in SERT +/+ mice. The polyclonal antibodies to the rat 5-HT$_3$ receptor used in the current study have previously been characterized in the rat central nervous system and the guinea pig bowel (40, 49). The sequence of amino acids (438–450) recognized by these antibodies is identical to that of the corresponding domain of the mouse 5-HT$_{3A}$ receptor (GenBank accession no. NP038589), with the exception of only a single mismatch. This sequence is not found in the 5-HT$_{3B}$ subunit. When probed with the 5-HT$_3$ antibodies, a single protein band (~58 kDa), corresponding in size to that of the 5-HT$_{3A}$ subunit, was found in extracts of the murine LMMP (Fig. 4*A*); therefore, we used this antibody to locate the 5-HT$_{3A}$ subunits. Myenteric neurons were isolated from the small intestine of SERT +/+ mice and cultured. Punctate 5-HT$_{3A}$ receptor immunoreactivity was found in a subset of neurons, labeling both varicose neurites (Fig. 4*B* and Fig. 5) and cell bodies (Fig. 5*A*). In similar cultures, mRNA encoding the 5-HT$_{3B}$ subunits was detected by ISH in a subset of 34.7 ± 7.8% of the total population of neurons (Fig. 4, *C* and *E*). No labeling was observed when control cells were hybridized with a sense riboprobe for the 5-HT$_{3B}$ subunit (Fig. 4, *D* and *F* and Fig. 5*B*). Double labeling to detect 5-HT$_{3A}$ receptor immunoreactivity and mRNA encoding the 5-HT$_{3B}$ subunit was carried out simultaneously to determine whether both subunits of the 5-HT$_3$ receptor are likely to be found in the same myenteric neurons (Fig. 5*A*). Antibodies to the 5-HT$_{3B}$ subunits are not available; therefore, mRNA encoding the 5-HT$_{3B}$ subunits was used as a surrogate marker for cells containing 5-HT$_{3B}$ subunits, although one cannot be certain that the 5-HT$_{3B}$ transcripts are translated. mRNA encoding the 5-HT$_{3B}$ subunit was restricted to nerve cell bodies, whereas the immunoreactivity of the 5-HT$_{3A}$ subunits was present both in cell bodies and varicose neurites. 5-HT$_{3A}$-immunoreactive neurons were found also to contain mRNA encoding the 5-HT$_{3B}$ subunit (Fig. 5*A*), a distribution consistent with the idea that both subunits are expressed by the same cells. No signal was detected in the cytoplasm when control cultures were hybridized under identical conditions with a sense riboprobe for the 5-HT$_{3B}$ subunit (Fig. 5*B*).

Current responses recorded from mouse enteric neurons. We evaluated the functional consequences of the downregulation of the 5-HT$_{3B}$ subunits in the myenteric neurons of SERT −/− mice by using whole cell patch-clamp recording. The myenteric neurons of mice have not previously been investigated by means of the patch-clamp technique; however, the properties of the murine myenteric neurons investigated in the current study were found to be similar to those reported for cultured myenteric neurons of guinea pigs (32, 64). In current-clamp mode, all of the neurons patched in this study had a resting membrane potential that was more negative than −40 mV, fired action potentials when

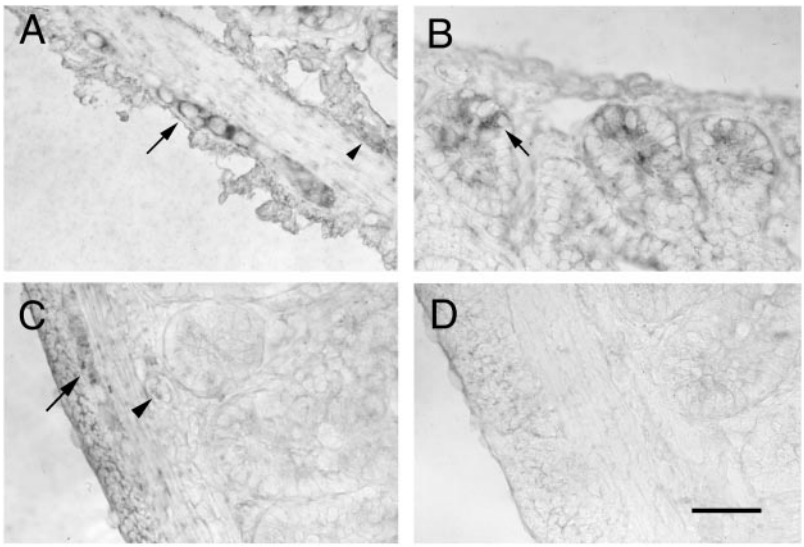

Fig. 3. mRNA encoding the 5-HT$_{3B}$ subunit is found in enteric neurons and epithelial cells. mRNA encoding the 5-HT$_{3B}$ subunit was located in frozen sections of mouse ileum by means of in situ hybridization (ISH). *A* and *B*: SERT +/+ mice. *C* and *D*: SERT −/− mice. *D*: sections hybridized with a sense riboprobes as negative control. There was no reactivity in any section. In SERT +/+ mice, mRNA encoding the 5-HT$_{3B}$ subunits is present in neurons of the myenteric (*A*, arrow) and submucosal (*A*, arrowhead) plexuses as well as in epithelial cells of some intestinal crypts (*B*, arrow). In SERT −/− mice, a reduced intensity of mRNA encoding the 5-HT$_{3B}$ subunits is also found in myenteric (*C*, arrow) and submucosal (*C*, arrowhead) neurons. Scale bar = 50 μm.

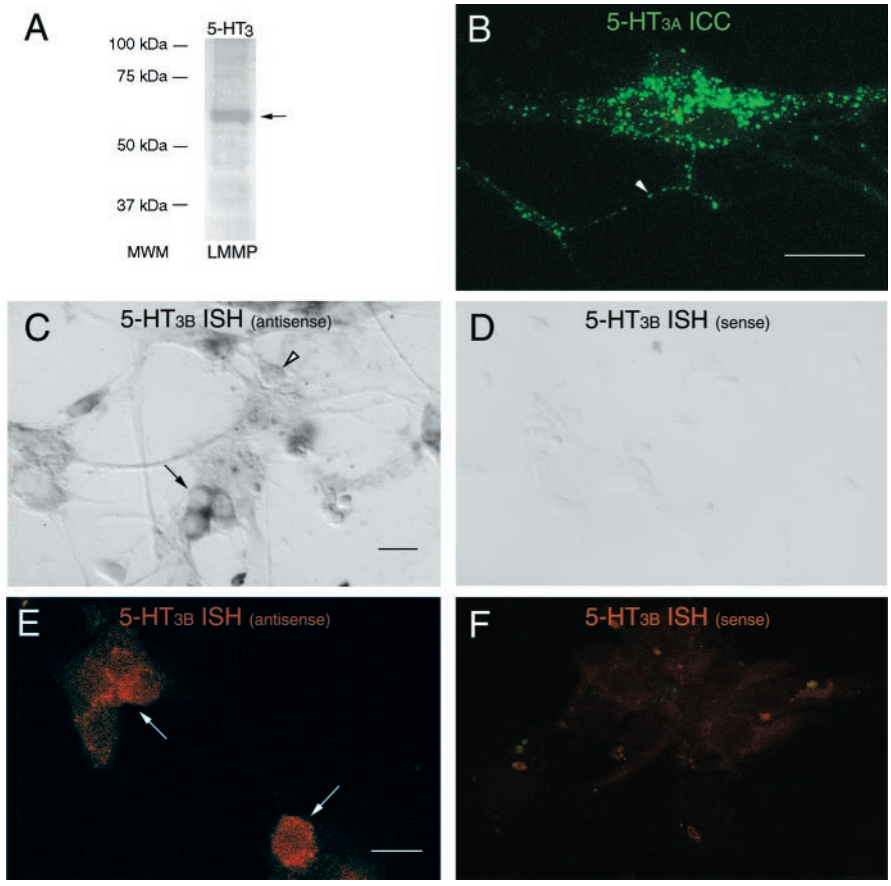

Fig. 4. Myenteric neurons that express 5-HT$_{3A}$ subunits can be detected by immunocytochemistry (ICC), and those that express 5-HT$_{3B}$ subunits can be visualized in similar preparations by ISH. Enteric neurons were isolated from SERT +/+ mice and cultured. *A*: a single major band at the expected molecular weight (arrow) of the 5-HT$_{3A}$ subunit reacts with antibodies to the 5-HT$_3$ receptor in an immunoblot prepared from the murine longitudinal muscle-myenteric plexus (LMMP). Markers for molecular weights (MWM) are shown at *left*. *B*: punctate 5-HT$_{3A}$ immunoreactivity is found in a subset of myenteric neuronal perikarya and varicose neurites (arrowhead). *C* and *E*: mRNA encoding the 5-HT$_{3B}$ subunits visualized in myenteric neurons by ISH. Bound probe was visualized with alkaline phosphatase (*C*, arrow) or immunofluorescence (*E*, arrows). Some neurons do not express hybridizing mRNA (*C*, arrowhead). *D* and *F*: control. No labeling is observed when cells are hybridized with a sense riboprobe encoding the 5-HT$_{3B}$ subunits. Scale bars = 20 μm.

injected with pulses of depolarizing current, and showed action potential frequency adaptation when injected with long (400 ms) depolarizing current pulses. The resting membrane potentials for SERT +/+ and −/− myenteric neurons, −48.69 ± 1.16 mV (n = 23) and −45.80 ± 1.05 mV (n = 20), respectively, did not differ significantly, nor did their input resistance of 160.8 ± 25.2 MΩ and 236.6 ± 58.8 MΩ for SERT +/+ and −/− enteric neurons (means ± SE, n = 8). In voltage-clamp mode, neurons were identified as those cells that generated Na^+-dependent fast inward currents at the onset of 400-ms depolarizing voltage steps (from a holding potential of −60 mV); TTX (1 μM) was used to block the Na^+-dependent fast inward currents, and hexamethonium (300 μM) was used to block cholinergic synapses in cultured myenteric neurons (data not shown). Prior studies of guinea pig myenteric neurons (32, 64) used KCl-containing patch pipettes instead of the potassium gluconate employed in the current study; therefore, we verified that the potassium gluconate in the internal solution did not affect the recorded current-voltage relationship by comparing the slopes of current responses to depolarizing voltage ramps that changed the holding potential from

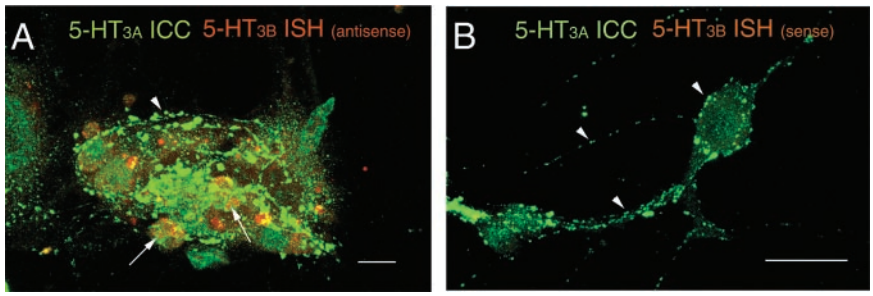

Fig. 5. 5-HT$_{3A}$ and 5-HT$_{3B}$ subunits are expressed by the same subset of myenteric neurons. Enteric neurons were isolated from SERT +/+ mice and cultured. mRNA encoding the 5-HT$_{3B}$ subunit, demonstrated by ISH, colocalized in enteric neurons with simultaneously demonstrated 5-HT$_{3A}$ immunoreactivity. *A*: mRNA encoding the 5-HT$_{3B}$ subunit is restricted to neuronal perikarya (arrows), whereas the immunoreactivity of the 5-HT$_{3A}$ subunit is present in both the perikarya and varicose neurites (*A* and *B*, arrowheads). *B*: control for ISH. Only 5-HT$_{3A}$ immunoreactivity is demonstrated when control cultures are hybridized with a sense riboprobe encoding the 5-HT$_{3B}$ subunits. Scale bars = 20 μm.

−80 to 0 mV in 160 ms. The input resistance was 156.79 ± 21.86 MΩ (n = 4) when recorded with pipettes filled with KCl and 173.88 ± 20.83 MΩ (n = 4) when recorded with pipettes filled with potassium gluconate. These values do not differ significantly.

Myenteric neurons have been classified physiologically as AH or S cells (21, 67). The AH neuron is characterized by a prolonged hyperpolarizing afterpotential lasting several seconds, with a Ca^{2+} shoulder on the repolarizing phase of its action potential. These cells also display a sustained hyperpolarization-activated cationic current (22, 61). When injected with a prolonged depolarizing current, S but not AH neurons fire repetitively and S but not AH neurons display anodal break excitation. These two cell types could not be distinguished in murine myenteric neurons, even when recordings were obtained in the current-clamp mode. In any case, no significant differences between SERT +/+ and −/− mice were observed in the current-voltage relationships of whole cell currents recorded.

5-HT and 5-HT$_3$ agonists evoke fast inward currents in myenteric neurons. Fast inward currents were evoked in myenteric neurons by local applications (10 s) of 50 μM 5-HT, mCPBG, or 2-Me-5-HT (Fig. 6*A*) in 66.7% (n = 42/63) of enteric neurons from SERT +/+ mice and in about the same proportion (63.5%; n = 33/52) of those from SERT −/− animals. The peak amplitude and the time to peak response of the fast inward current for all of the tested compounds were directly related to their concentrations. In contrast, the rate at which the response decayed was an inverse function of the concentrations of these agonists (Fig. 6*B*). In small subsets of neurons, 5-HT evoked a prolonged sustained inward current or an outward current (~5–20 pA; Fig. 6*C*). These responses appeared to be independent of the fast inward current elicited by 5-HT because they were observed in neurons that did or did not display the fast response and thus are likely mediated by other G protein-coupled subtypes of 5-HT receptors on subpopulations of myenteric neurons, which appeared to involve other subtypes of 5-HT receptors and were not analyzed with data derived from responses mediated by 5-HT$_3$ receptors.

5-HT$_3$ antagonists block 5-HT-induced fast inward currents. The 5-HT-evoked fast inward currents were abolished by the 5-HT$_3$-selective antagonists tropisetron (1 μM), ondansetron (1 μM), or alosetron (0.15–3 μM). Alosetron (0.15 μM) completely abolished the fast inward current, and, in the subset of neurons in which the fast inward current was followed by a sustained current, alosetron also abolished the sustained current (Fig. 7*A*). Ondansetron (1 μM) also antagonized the fast inward current evoked by 5-HT (Fig. 7*B*). Ondansetron (1 μM) reproducibly abolished the fast inward current, but, in contrast to alosetron, it did not block the small sustained currents in the subset of cells in which they were elicited; these cells were excluded from the final analysis (Fig. 7*C*). Recovery following the washout of antagonists was slow and usually incomplete during the 30-min recording period. Similar effects of ondansetron and alosetron have been obtained in studies of cultured myenteric neurons of guinea pigs (70, 71). Our observations confirm the identification of 5-HT-evoked fast inward currents as 5-HT$_3$-mediated events.

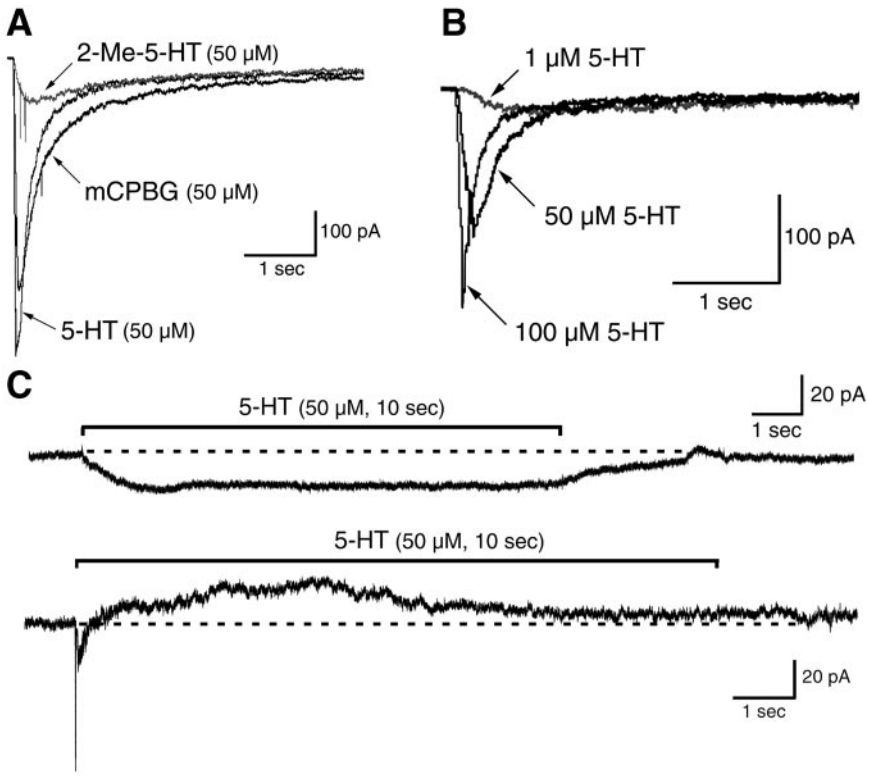

Fig. 6. 5-HT and 5-HT$_3$ agonists evoke fast inward currents in myenteric neurons. *A*: fast inward currents were evoked in enteric neurons by local applications (10 s) of 50 μM 5-HT, 1-(*m*-chlorophenyl)-biguanide (mCPBG), or 2-methyl-5-HT (2-Me-5-HT). The rank order of potencies of the 5-HT$_3$ receptor agonists is 5-HT > mCPBG > 2-Me-5-HT. *B*: 5-HT$_3$-evoked fast inward currents are concentration dependent. The peak amplitude and the time to peak response are directly related to 5-HT concentration. In contrast, the rate at which the response decayed is inversely related to its concentration. *C*: in small subsets of neurons, 5-HT evoked a prolonged sustained inward current (*C*, *top* trace) or an outward current (*C*, *bottom* trace), which was independent of the fast inward current elicited by 5-HT.

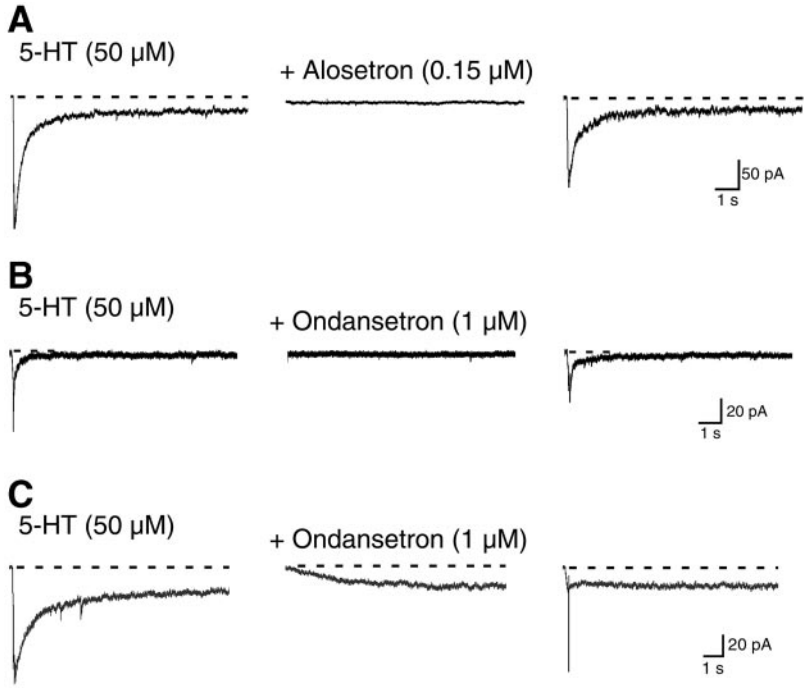

Fig. 7. 5-HT$_3$ antagonists block 5-HT$_3$-induced fast inward currents. *A*: alosetron (0.15 μM) abolished both the fast inward current evoked by 5-HT (50 μM; 10 s) and the small sustained inward current that followed the fast component. *B*: ondansetron (1 μM) abolished the fast inward current evoked by 5-HT (50 μM; 10 s). *C*: ondansetron (1 μM) abolished the fast inward current evoked by 5-HT (50 μM; 10 s), but a late sustained inward current (~20 pA) persisted despite the continued presence of ondansetron. Recovery following the washout of antagonists was slow and usually incomplete during the 30-min recording period (*right* traces; *A*, *B*, and *C*).

5-HT$_3$ receptor sensitivity is decreased in SERT −/− mice. To determine the effect of the knockout of SERT on the 5-HT$_3$ receptor sensitivity, the peak of the fast inward current induced by 5-HT was measured as a function of the 5-HT concentration (Fig. 8). In both SERT +/+ and −/− mice, the threshold response was obtained at ~1 μM 5-HT and then increased in a concentration-dependent manner. The Hill coefficient measured in neurons from SERT +/+ mice (n_H = 0.87) was similar to that measured in those from SERT −/− animals (n_H = 0.89) and in both was slightly less than unity; however, the concentration-effect curve for the 5-HT-induced fast inward current was shifted to the right in the SERT −/− animals ($P < 0.01$, Wilcoxon signed-rank test; n = 8). The EC_{50} was 17.65 μM in neurons from SERT +/+ mice vs. 35.88 μM in SERT −/− mice. The downregulation of 5-HT$_{3B}$ subunits in SERT −/− mice is thus associated with a reduction in the affinity of 5-HT$_3$ receptors for 5-HT.

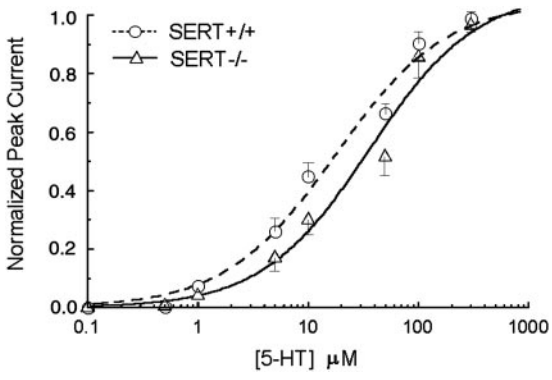

Fig. 8. The concentration-effect curve for 5-HT$_3$-mediated responses of mouse enteric neurons is shifted to the right in SERT −/− mice. Peak inward currents evoked by 5-HT are plotted as a function of the 5-HT concentration. Data are normalized to the peak current evoked in each neuron by 300 μM 5-HT. Curves are best fit with a logistic function and are sigmoid in both SERT +/+ and −/− mice. The EC_{50} of 5-HT$_3$-mediated responses is lower in SERT +/+ mice than in SERT −/− mice. Data points are mean values of 8 experiments. When not visible, error bars are smaller than symbols; the differences between SERT +/+ and −/− mice were significant.

Peak currents activated by 5-HT$_3$ agonists. The amplitudes of the fast inward currents evoked by a 50-μM concentration of three 5-HT$_3$ agonists, 5-HT, mCPBG, and 2-Me-5-HT, were compared in myenteric neurons from SERT +/+ and −/− mice. The amplitudes of the inward current evoked by these compounds were found to differ in neurons from both SERT +/+ and −/− mice so that 5-HT > mCPBG > 2-Me-5-HT (Table 2); however, differences between SERT +/+ and −/− mice were not significant.

Time course of 5-HT-mediated currents. The maximal amplitude of the 5-HT-induced inward current was reached quickly. The current decayed over a slower time course that could be defined by two exponential time constants (τ_{fast} and τ_{slow}). Three types of response, rapidly desensitizing, mixed, and slowly desensitizing, were distinguished on the basis of the time course of the decay (Fig. 9; Table 3). A rapidly desensitizing response was defined as one in which the transient inward current fully decayed within 1 s (Fig. 9*A*). A slowly desensitizing response was one in which the inward current did not fully decay during the 10-s application of 5-HT (50 μM; Fig. 9*C*); the steady-state current that followed the deactivation phase disappeared within 12 s. A mixed response was defined as one in which the time to decay of the inward current was >1 but <10 s (Fig. 9*B*). For each type of response (rapidly desensitizing, mixed, and slowly desensitizing), the peak amplitude, the decay time constants

Table 2. *Summary of peak currents mediated by 5-HT$_3$ agonists*

Compounds	Mouse Type	RMP, mV	Peak Amplitude, pA	Time to Peak, ms
5-HT	SERT +/+	−46.64 ± 1.90	−227.61 ± 54.76	251.7 ± 16.1
	SERT −/−	−45.00 ± 2.03	−175.44 ± 46.40	258.2 ± 15.6
mCPBG	SERT +/+	−44.50 ± 1.50	−160.84 ± 42.02	250.2 ± 16.8
	SERT −/−	−46.60 ± 2.44	−105.10 ± 36.83	249.9 ± 15.7
2-Me-5-HT	SERT +/+	−48.67 ± 2.35	−49.79 ± 11.61	451.9 ± 46.3
	SERT −/−	−47.50 ± 1.50	−50.79 ± 13.40	436.9 ± 46.7

Data are expressed as means ± SE; $n = 10$. Time to peak is the time measured between the onset of the application of 5-HT and occurrence of the peak current. No significant differences between 5-HT transporter (SERT) +/+ and SERT −/− mice were detected in any of the parameters shown. RMP, resting membrane potential; mCPBG, 1-(*m*-chlorophenyl)-biguanide; 2-Me-5-HT, 2-methyl-5-HT.

(τ_{fast} and τ_{slow}), and the steady-state current (C_s) were compared in myenteric neurons from SERT +/+ and −/− mice (Table 3).

Responses obtained from neurons in SERT +/+ and −/− mice did not differ significantly in peak amplitude, τ_{fast} and τ_{slow}, and C_s (Table 3). The knockout of SERT, therefore, does not appear to change the electrical properties of 5-HT$_3$-gated channels in individual neurons. In contrast, the absence of SERT did cause a significant change to occur in the relative incidence of the three 5-HT$_3$-mediated responses (Fig. 9*D*). The proportion of neurons exhibiting rapidly desensitizing responses was only 3/30 (10%) in SERT +/+ mice but increased about threefold in SERT −/− animals to 9/28 (32.1%). Correspondingly, the proportion of neurons in which the response to 5-HT decayed slowly was decreased in SERT −/− mice to 2/28 (7.1%), again, about threefold, from 6/30 (20%) in SERT +/+ controls. The majority of neurons in both SERT +/+ (21/30; 70%) and −/− (17/28; 60.7%) mice still exhibited a mixed response to 5-HT (Fig. 9*D*), in which the 5-HT$_3$-mediated fast inward currents decayed at an intermediated rate. The knockout of SERT was thus associated with an increase in cells exhibiting rapidly desensitizing 5-HT$_3$-mediated responses.

DISCUSSION

The current study was undertaken to determine whether adaptation occurs in 5-HT$_3$ receptors in SERT −/− mice to compensate for the inefficient inactivation of 5-HT in these animals. 5-HT receptors in SERT −/− mice are likely to be exposed to concentrations of 5-HT that are higher and more prolonged than normal. At the time its primary structure was first deduced (46), the 5-HT$_3$ receptor was thought to be composed of a single protein, now known as the 5-HT$_{3A}$ subunit. Since the properties of native 5-HT$_3$ receptors are largely mimicked by the functional expression of 5-HT$_{3A}$ subunits, the role of 5-HT$_{3B}$ subunits has not been entirely clear. The 5-HT$_{3B}$ subunit has now been cloned and sequenced from tissues of humans (15, 16), rats, and mice (33). Rodent neurons normally express heteromeric 5-HT$_{3A/B}$ subunits (33).The 5-HT$_{3B}$ subunit cannot, by itself, constitute an effective ligand-gated ion channel and thus functions only in conjunc-

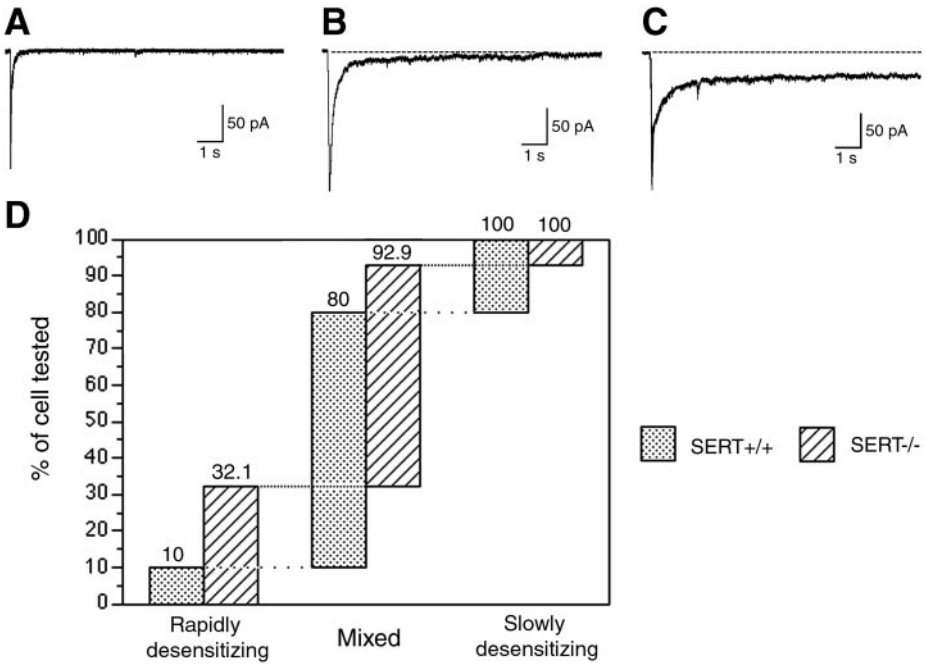

Fig. 9. 5-HT$_3$-mediated responses decay rapidly in a larger subset of the myenteric neurons of SERT −/− than +/+ mice. Enteric neurons were categorized according to the kinetics of the decay of 5-HT$_3$-mediated responses. *A*: rapidly desensitizing response. The 5-HT-evoked fast inward current deactivated rapidly within 1 s when applying 5-HT (50 μM) for 10 s. *B*: mixed response. The decay current mediated by 5-HT$_3$ receptors desensitized within 10 s. *C*: slowly desensitizing response. The fast inward current evoked by 5-HT decayed very slowly and did not completely desensitize during the application of 5-HT (50 μM; 10 s). *D*: proportions of neurons displaying each of the 3 categories of 5-HT$_3$-mediated response. Most neurons exhibit mixed responses in both SERT +/+ and −/− mice; however, the rapidly desensitizing proportion is increased and the slowly desensitizing proportion is decreased in SERT −/− animals.

Table 3. *Kinetics of 5-HT$_3$-evoked fast inward currents*

Category	Mouse Type	Peak Current, pA	τ_{fast}, ms	τ_{slow}, s	Steady-State Current, pA	*n*
Rapidly desensitizing	SERT +/+	−143.22 ± 58.58	91.2 ± 22.6	2.47 ± 1.48	−8.82 ± 3.26	3
	SERT −/−	−150.12 ± 43.21	106.0 ± 18.6	2.68 ± 0.60	−4.96 ± 1.50	9
Mixed	SERT +/+	−155.68 ± 33.19	183.3 ± 23.7	3.61 ± 0.45	−23.46 ± 3.57	21
	SERT −/−	−94.14 ± 20.58	165.5 ± 19.0	3.13 ± 0.40	−17.44 ± 3.68	17
Slowly desensitizing	SERT +/+	−383.29 ± 120.94	263.7 ± 51.1	16.08 ± 1.94	−55.11 ± 15.29	6
	SERT −/−	−287.55 ± 165.85	410.9 ± 95.0	22.39 ± 10.82	−59.72 ± 17.34	2

Data are expressed as means ± SE. No significant differences between SERT +/+ and SERT −/− mice were detected in any of the parameters shown. τ_{fast} and τ_{slow}, fast and slow time constants, respectively.

tion with 5-HT$_{3A}$ subunits (15, 16, 59). Although the pharmacology of expressed homomeric and heteromeric 5-HT$_3$ receptors is similar (6), biophysical properties of the two types of 5-HT$_3$ receptor complexes differ (15, 16, 33). The single-channel conductance of activated heteromeric 5-HT$_{3A/B}$ receptors is greater than that of homomeric 5-HT$_{3A}$ receptors; moreover, heteromeric receptor complexes are less permeable than homomeric 5-HT$_{3A}$ receptors to Ca^{2+} and less sensitive to inhibition by tubocurarine. Adaptive changes in expression of 5-HT$_{3B}$ subunits in SERT −/− mice, therefore, might allow 5-HT$_3$-mediated responses to be modulated.

Both the A and the B subunits of the 5-HT$_3$ receptors were found to be expressed in the murine bowel by RT-PCR, but the level of expression of the 5-HT$_{3B}$ subunit was higher in SERT +/+ than in SERT −/− animals. In contrast, no difference between SERT +/+ and −/− mice was detected by means of semiquantitative RT-PCR in the expression of the 5-HT$_{3A}$ subunits (5-HT$_{3A}$ and 5-HT$_{3AL/S}$). In SERT +/+ mice, mRNA encoding the 5-HT$_{3B}$ subunit was found, by ISH, to be located in epithelial cells and in both submucosal and myenteric neurons. This mRNA was also detectable in the SERT −/− animals, although quantitative measurements (competitive PCR) indicated that the level of 5-HT$_{3B}$ expression was about fourfold higher in the gut of SERT +/+ mice than in their SERT −/− littermates. These observations suggest that there is probably more 5-HT$_{3B}$ subunit protein available for incorporation into enteric 5-HT$_3$ receptors in SERT +/+ than in SERT −/− animals. If so, then the ratio of heteromeric (5-HT$_{3A/B}$) to homomeric (5-HT$_{3A}$) receptor complexes would be greater in SERT +/+ mice.

The concentration-effect curve for 5-HT-induced fast inward currents was found, in whole cell patch-clamp studies of cultured myenteric neurons, to shift to the right in neurons of SERT −/− mice. The observations that the 5-HT$_3$ antagonists tropisetron, ondansetron, and alosetron blocked the 5-HT-induced fast inward currents confirmed that they were 5-HT$_3$ mediated. The rightward shift of the concentration-effect curve suggests that the affinities of the 5-HT$_3$ receptors of SERT −/− mice for 5-HT are lower than those of SERT +/+ animals. Other investigators have also reported that the affinities of homomeric and heteromeric 5-HT$_3$ receptors expressed differ, although the nature of the difference appears to depend on intrinsic properties of the heterologous cells in which the receptors are expressed (15, 16, 33); nevertheless, the whole cell recordings and measurements of Ca^{2+} influx have revealed that coexpression of 5-HT$_{3B}$ subunits with 5-HT$_{3A}$ subunits can enhance the affinity of 5-HT$_3$ receptor complexes for 5-HT in heterologous cells (16). The decreased sensitivity of the enteric neuronal 5-HT$_3$ receptors of SERT −/− mice for 5-HT may thus be due to the associated decrease in expression of 5-HT$_{3B}$ subunits that occurs in these animals.

The proportion of neurons that exhibited rapidly desensitizing 5-HT$_3$-mediated fast inward currents increased in SERT −/− mice. The rate of desensitization of homomeric (5-HT$_{3A}$) receptor complexes has been reported to be accelerated by the extracellular presence of Ca^{2+} (37, 69). Homomeric (5-HT$_{3A}$) subunits are both Ca^{2+} permeant and inhibited by Ca^{2+} (7, 53, 56). In contrast, heteromeric (5-HT$_{3A/B}$) receptors are relatively impermeable to Ca^{2+}; therefore, native 5-HT$_3$ receptors, which have much lower permeabilities to divalent cations than those reported for 5-HT$_{3A}$ homomeric receptors, are likely to contain 5-HT$_{3B}$ subunits (15, 16). In the current study, 5-HT$_3$-mediated currents were recorded with the extracellular [Ca^{2+}] set at 2.5 mM; therefore, Ca^{2+} could play a role in the desensitization of 5-HT$_3$ receptors if those of SERT −/− mice were to be hyperpermeable to Ca^{2+}. The relative increase in the proportion of 5-HT$_{3A}$ subunits that occurs in enteric neurons of SERT −/− mice may be associated with an increase in the permeability of 5-HT$_3$ receptor complexes to Ca^{2+}, which in turn could contribute to the acceleration of desensitization of the receptors in affected neurons. Conceivably, the rapid desensitization of 5-HT$_3$ receptors might also be regulated by receptor phosphorylation. Phosphorylation has been reported to occur in studies of transfected cells that express 5-HT$_{3A}$ receptors (5, 27, 35, 68). There is no reason, however, to assume that the mechanism by which 5-HT$_3$ receptor desensitization is regulated in cell lines is the same as that in the native myenteric neurons of SERT −/− mice confronted with slow inactivation of 5-HT. Cells that express only 5-HT$_{3A}$ subunits, moreover, obviously cannot downregulate 5-HT$_{3B}$ expression. The increase in the proportion of rapidly desensitizing neurons, however achieved, would be expected to protect against overstimulation by the prolonged presence of 5-HT in contact with 5-HT$_3$ receptors in SERT −/− mice.

It is not clear why the change in rate of desensitization is seen in only some neurons and not all of those studied. The most likely explanation is that the partial nature of the phenomenon is a reflection of the means by which the rate of desensitization is measured. Obviously, for any given neuron, it is impossible to record the rate of desensitization of 5-HT_3 receptors before and after the knockout of SERT. Only populations of neurons can be examined in mice that do or do not express SERT. A small change in the rate of desensitization, therefore, is evident only at the extremes of the population, an increase in the proportion of rapidly desensitizing neurons at the expense of those which desensitize slowly. Changes in rates of 5-HT_3 receptor desensitization in the mass of neurons in between are missed because there is no way to determine how any single neuron of a SERT −/− mouse might have behaved if SERT had been present. Still to be determined, moreover, is the mechanism that links the presumed excess of 5-HT in the environment of neurons of SERT −/− mice to the observed alteration in the expression of the gene encoding the 5-HT_{3B} subunit.

The electrophysiological properties of the inward current evoked by 5-HT in enteric neurons were found to vary from cell to cell in both SERT +/+ and −/− mice even though an effort was made to maintain constant recording conditions. Conceivably, the proportions of 5-HT_{3A} and 5-HT_{3B} subunits may vary between cells and even within heteromeric receptor complexes. Because the respective sensitivities of the techniques of ISH and ICC are unknown, the proportions of 5-HT_{3A} and 5-HT_{3B} subunits in individual receptor complexes and cells cannot be determined by ICC or ISH. An attempt was made to assay mRNA encoding the 5-HT_{3B} subunits in cytoplasm aspirated from cells used for patch-clamp recording; however, consistent measurements could not be obtained in each cell from which a recording was obtained. It thus remains possible that a varying subunit composition of 5-HT_3 receptor complexes contributes to the electrophysiological differences between 5-HT_3-mediated responses recorded in different neurons. In addition, distortions might have been encountered in individual neurons due to their extension of neurites and consequent space-clamp problems. We compared the kinetic responses of the whole-cell currents from two populations of cultured neurons (SERT +/+ and −/−). Differences between populations of neurons in response to 5-HT_3 agonists were studied rather than change in Ca^{2+} or K^+ currents in individual neurons. Distortions due to uncontrolled space-clamp effects, therefore, are likely to contribute to the overall variability but are unlikely to systematically affect either population and thus should not affect significant differences between the two populations.

Prior patch-clamp studies of enteric 5-HT_3 receptors used intracellular solutions that contained Cs^+ or high concentrations of EGTA (70, 71). These additives block the Ca^{2+}-activated K^+ conductance modulated by the activity of G protein-coupled 5-HT receptors, which were thus not detected. We used an intracellular solution containing potassium gluconate to permit K^+ channels to contribute to the recorded 5-HT-induced currents; therefore, we were able to test voltage responses in the current-clamp mode at the beginning of each experiment to compare the responses we obtained with patch electrodes with those obtained in prior studies in current-clamp mode with sharp microelectrodes. Very few previous current-clamp studies of murine myenteric neurons with sharp microelectrodes have been reported, and this is the first report of whole cell patch-clamp recordings from myenteric neurons. Small steady-state currents during the desensitizing phase of 5-HT_3 receptor-mediated responses have previously been observed in guinea pig myenteric neurons with CsCl-containing patch pipettes that would have masked the effects of G protein-coupled receptors on K^+ channels (71). We excluded responses to 5-HT or agonists that were associated with a delayed onset or a sustained inward and/or outward current in our final data analysis to be certain that the responses we measured were exclusively those mediated by 5-HT_3 receptors. No hyperpolarization-activated cationic currents were observed in either voltage- or current-clamp mode, and 5-HT-evoked fast inward currents were invariably abolished by the 5-HT_3-selective antagonists tropisetron, ondansetron, and alosetron; therefore, the small steady-state current recorded during the desensitizing phase of the fast inward current mediated by 5-HT could not have been influenced by metabotropic G protein-coupled 5-HT receptors, such as the 5-HT_{1P} receptor (24). None of the G protein-coupled subtypes of 5-HT receptors are antagonized by ondansetron or alosetron in the concentrations of these drugs that were utilized.

Colorectal motility is most often greater than normal in animals that lack SERT, leading to excessive stool water; however, some knockout mice also undergo transient episodes of extremely slow colorectal motility (12). These periods of constipation are thought to be associated with 5-HT receptor desensitization. It is possible that the increased tendency of 5-HT_3 receptors to desensitize rapidly in SERT −/− mice contributes to the constipation. Chronic inhibition of 5-HT_3 receptors in human subjects with alosetron was also associated with an increased incidence of constipation (19). It is possible that adaptive changes in 5-HT_3 receptors similar to those found in SERT −/− mice might also occur in the chronic administration of SERT inhibitors, such as serotonin-selective reuptake inhibitors, tricyclic antidepressants, and cocaine, for the treatment of depression or for recreational use. Gastrointestinal side effects of these compounds are frequent and often vexatious.

In summary, adaptive compensation occurs in enteric 5-HT_3 receptors of SERT −/− mice. These changes consist of the downregulation of 5-HT_{3B} subunits, which is associated electrophysiologically with an increase in the EC_{50} for receptor sensitivity and an increase in the proportion of neurons that desensitize rapidly. Major electrical properties of 5-HT_3-mediated

fast inward currents, including the activation peak amplitude, the exponential time constants of the decay curve, and the amplitude of the steady-state current are unchanged by the knockout of SERT. The adaptive changes are thus unlikely to interfere with 5-HT$_3$-gated channel functions; however, their nature is such that the changes should be protective against excessive stimulation of neurons via 5-HT$_3$ receptors in animals in which extracellular 5-HT after release from stimulated EC cells or enteric neurons is likely to be persistent. The mechanism by which SERT knockout feeds back to alter 5-HT$_{3B}$ subunit expressions remains to be determined.

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