

Supplementary Materials and Methods

Tissue Preparation

A pulse of BrdU (100 mg/kg) was administered intraperitoneally to 12-week-old male WT (n = 19), SERTKO (n = 11), TPH1KO (n = 10), TPH2KO (n = 7), and TPH2+/+ littermate control (n = 7) mice. After a 1-hour chase, animals were euthanized and distal small intestine was removed and flushed with phosphate-buffered saline. The most distal 2-cm segment of intestine was discarded. Two segments of ileum (1 cm in length) were fixed overnight, one with 2% and the other with 4% formaldehyde (from paraformaldehyde in 0.1 mol/L phosphate buffer) at 4°C. Tissue was dehydrated, cleared, embedded in paraffin, and sectioned at 5 μ m. An additional 1-cm segment of intestine was fixed overnight with a mixture of 4% formaldehyde and 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4). This tissue was postfixed for 1 hour with 1.0% OsO₄, dehydrated, cleared with propylene oxide, embedded in Epon 812, and semithin-sectioned at 0.7 μ m for light microscopic examination. The semithin sections, which avoided overlapping of cells, were used to quantify villus and cellular parameters. Longer (5-cm) segments of intestine were removed and used to scrape mucosa from the remainder of the bowel wall. The isolated mucosa and mucosa-free preparations were used to prepare homogenates for the analysis of activated cleaved caspase-3 (see later).

Cleaved Caspase-3 Immunocytochemistry

Heat-induced antigen retrieval was used. For antigen retrieval, sections on slides were heated in a citrate buffer (pH 6.0) for 20 minutes in a pressure cooker and cooled for 20 minutes. Endogenous peroxidase activity was inactivated with 3% hydrogen peroxide. Nonspecific antigens were blocked with 10% goat serum and 1% bovine serum albumin. Tissue was incubated with rabbit antibodies to cleaved caspase-3 (Cell Signaling), and diluted 1:200 for 90 minutes. Sites of bound antibody were visualized with 3, 3'-diaminobenzidine using an avidin-biotin immunoperoxidase technique (Vectastatin Elite ABC kit; Vector Labs, Burlingame, CA). Immunostained preparations were counterstained with hematoxylin.

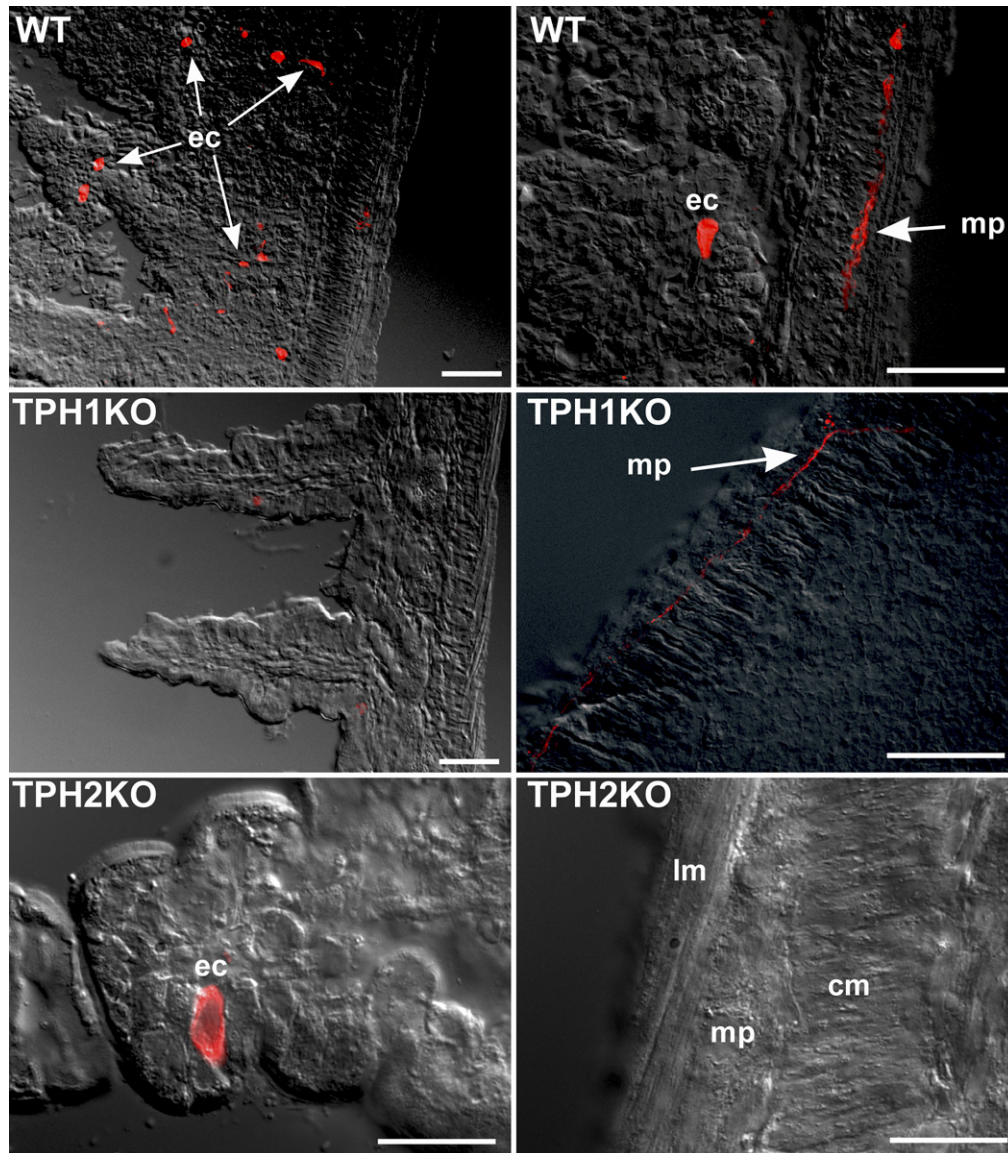
Cleaved Caspase-3 Western Blot Analysis

Tissue samples were homogenized for 15 seconds at 4°C in radioimmunoprecipitation assay buffer containing 1 mmol/L sodium vanadate and a protease inhibitor cocktail (Sigma-Aldrich). After centrifugation at

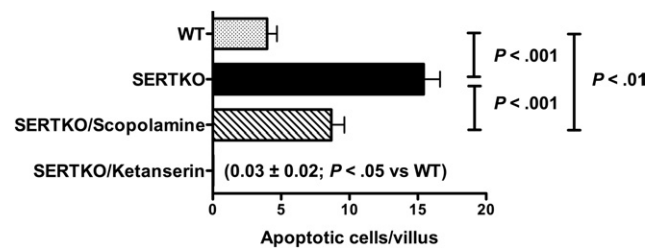
13,000 rpm at 4°C to remove tissue debris, aliquots of homogenate containing 50 μ g of protein were mixed with Laemmli buffer, boiled for 5 minutes, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to a nitrocellulose membrane (BioRad Laboratories, Hercules CA), incubated for 1 hour at room temperature in Tris-buffered saline containing 5% nonfat dry milk and 0.1% Tween 20, and then incubated overnight at 4°C with primary antibodies. Goat antibodies to rabbit IgG labeled with horseradish peroxidase were used to visualize immunoreactive proteins (Amersham Biosciences, Piscataway, NJ). Chemiluminescence was used to detect signals (SuperSignal ECL system; Pierce Biotechnology, Rockford, IL). Membranes were immunostained for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to confirm consistent protein loading in each lane. The primary antibody dilutions were as follows: 1:1000 for rabbit polyclonal antibody to cleaved caspase-3 (Cell Signaling Technology) and 1:2000 for GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA). Bands were quantified using ImageJ software (National Institutes of Health, Bethesda, MD). The analysis of GAPDH expression showed negligible variation in protein loading.

Triple Immunostaining

WT mice (C57BL/6) were euthanized with carbon dioxide inhalation. A 2-cm segment of terminal ileum was removed and fixed overnight with 4.0% formaldehyde (from paraformaldehyde) in 0.1 mol/L phosphate buffer. The fixed tissue was frozen in liquid N₂ and sectioned at 10 μ m in a cryostat-microtome. Sections were thaw-mounted onto Permafrost slides and incubated with primary antibodies to HuC/D (Invitrogen; biotin-conjugated mouse monoclonal, A21272, 1:20 dilution), 5HT_{2A} receptors (Abcam; ab16028, 1:100 dilution), and ChAT (Millipore; goat polyclonal, ab144P, 1:100 dilution). Antibody bound to HuC/D was detected with goat Alexa 680-labeled anti-mouse secondary antibodies coupled to streptavidin (1:200 dilution). Primary antibodies bound to 5-HT_{2A} receptors were detected with goat anti-rabbit secondary antibodies labeled with Alexa 488 (1:200 dilution). Primary antibodies bound to ChAT were detected with donkey secondary anti-goat antibodies labeled with Alexa 594 (1:200 dilution). The dichroic mirrors and emission filters were specific and did not permit cross-detection of fluorophores.



Supplementary Figure 1. The immunocytochemically shown 5-HT content of EC cells and neurons reflects the genomic status of mice. WT: 5-HT immunoreactivity is found both in EC (ec) cells and the myenteric plexus (mp) of WT mice. TPH1KO: 5-HT immunoreactivity is deficient in the mucosa but still present in nerve fibers of the myenteric plexus (mp). TPH2KO: 5-HT immunoreactivity is present in mucosal EC cells (ec) but is not seen in the myenteric plexus, a ganglion of which (mp) is located between the longitudinal (lm) and the circular (cm) muscle. Scale bars: 20 μ m.



Supplementary Figure 2. Serotonergic signaling affects the rate of apoptosis of enterocytes. The number of enterocytes in which apoptosis (TUNEL detection) is shown is significantly greater in SERTKO than in WT mice. Scopolamine antagonizes and ketanserin abolishes the SERTKO-driven enhancement of apoptosis. The mean number of apoptotic enterocytes in ketanserin-treated SERTKO mice is significantly less than in SERTKO mice treated with scopolamine and is significantly less than in WT animals.