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Supplementary Information for

THE GUT MICROBIOTA REGULATES MATURATION OF THE ADULT ENTERIC NERVOUS SYSTEM VIA ENTERIC SEROTONIN NETWORKS

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Supplementary Information Text

Methods

Colonization experiments

Cecum content from age-matched CONV-R female mice was dissolved in 5 mL sterile, O_2-free reduced PBS (solution containing 90% PBS and 10% reducing solution with 0.5% Na_2S, $9H_2O$; 8% $NaHCO_3$ and 1% cysteine). GF female mice were then administered a single oral gavage of 200 µL of the cecum content solution. Colonized (CONV-D) mice were then housed in the same room as CONV-R mice.

Antibiotic treatment

CONV-R female mice were administered a cocktail of broad-spectrum antibiotics in their drinking water for three weeks. The cocktail consisted of 1 g/L ampicillin, 1 g/L metronidazole, 0.5 g/L vancomycin and 0.5 g/L neomycin (Sigma). Drinking water was sweetened with 10 g/L sucrose and fresh antibiotics were administered every four days. Fresh feces were collected at the beginning of the experiment, and then on days 7, 14 and 21. DNA was extracted from the feces using Macherey-Nagel Nucleospin® Soil kit, following the manufacturer's instructions. 500 pg of DNA were used to perform qPCR of the 16S rDNA and quantify the number of bacterial copies, using *E. coli* purified 16S rDNA as a standard. Primers used for qPCR were universal primers UniF GTGSTGCAYGGYYGTCGTCA and UniR ACGTCRTCCMCNCCTTCCTC (1).

Whole-gut transit studies

Mice were fasted for 4 hours before being given oral gavage of 100 µL of 1.5% methylcellulose containing 5% Evans Blue dye (Sigma). Intestinal transit was assessed 90 min after gavage. The total length of the intestine and the length covered by Evans Blue were measured, and transit was expressed as the percent of intestinal length covered by Evans Blue.

Pharmacological modulation of the 5-HT_4 receptor and neuronal serotonin depletion

All compounds were dissolved in sterile 0.9% NaCl, filter-sterilized and autoclaved. Vehicle injections consisted of 0.9% NaCl. 12-week-old female GF mice were given two daily i.p. injections of 5-HT_4 agonist sc-53116 hydrochloride hydrate (1 mg/kg; Sigma) for three days. For 5-HT_4 antagonist treatment and depletion of endogenous 5-HT in the ENS, mice were colonized as described above, and given two daily i.p. injections of

either GR-125487 sulfamate (1 mg/kg; Santa Cruz Biotechnology), PCPA (150mg/kg; Sigma) or reserpine (0.5 mg/kg; Sigma). The first injections were administrated one day prior to colonization, followed by injections for three days after colonization.

Immunohistochemistry and imaging

Whole-mount studies

3 cm of proximal colon obtained from mice in a fed state were cut longitudinally and soaked in a dark silicon-coated plate filled with cold PBS. The mucosal layer was removed and the LMMP was dissected and fixed with 4% paraformaldehyde overnight at 4°C. Then, the tissues were washed three times with cold PBS and kept in a sodium azide solution (0.1% in PBS) at 4°C. Samples were blocked for 1h at RT with 0.1 % sodium azide-PBS containing Triton X100 (0.5%), donkey serum (4%) and bovine serum albumin (4%). The samples were incubated overnight at room temperature with the primary antibodies diluted in the blocking solution (see Table S2), washed 3 times, and incubated 90 min at RT with the secondary antibodies (1:400, see Table S3). Finally, the tissues were washed twice, incubated with 0.1 µg/mL Hoechst 33342 solution during 5 min at RT, and washed twice. Samples were mounted with fluorescent mounting medium (Dako). The tissues were imaged by confocal microscopy using a Zeiss Laser Scanning Inverted Microscope LSM-700 equipped with 20x/0.8 and 40X/1.3 Oil NA objectives and Black Zen software (Carl Zeiss).

Immunohistochemistry in paraffin slides

The proximal colon was excised and fixed overnight in 4% PFA fixative. 10-µm-thick paraffin-embedded slides were prepared, paraffin was removed, and the slides were placed in citrate buffer solution in the 2100 Antigen Retriever (Aptum Biologics). After several rinses in PBS, immunohistochemistry was performed as described above for the whole-mount studies. After blinding the samples, 5-6 images were randomly generated per animal, and quantification was performed as described below.

Image analysis

All images were analyzed using Fiji (2), after blinding of samples. Three-dimensional reconstructions were obtained by deconvolution of images photographed in a *Z*-stack of focal planes (0.8 to 1.5 µm). For LMMP analysis, adjacent fields that were imaged were stitched together using the stitching tool available with the software (3), and a global field of 1 mm^2 was analyzed.

Cell and area counts

Numbers of HuC/D^+ and $Nestin^+$ cells were counted manually in the field using the multi-point click tool. Quantification of $Sox10^+$ cells was performed by converting images to binary using the automatic threshold tool followed by watershed segmentation, then particles were analyzed using the following criteria: size 15-200 μm^2, circularity 0.20-1.00. Count outlines were displayed, and false-positive cells were discarded after

superposition of the images. For Tuj1, S100β, 5-HT and 5-HT_4R analysis, the same threshold procedure was applied, and total area was measured. Images corresponding to the procedures are provided in Fig. S8.

RT-qPCR

Tissue was flash-frozen in liquid nitrogen and kept at -80°C. RNA was extracted using RNeasy mini kit (Qiagen), and cDNA was synthetized using High Capacity Reverse Transcription kit (Applied Biosystems). Quantitative PCR was then performed using iQ™ SYBR® Green supermix (BioRad) with the CFX96 Touch™ Real-Time PCR Detection System (BioRad). After validation of stability with qbase+ software (Biogazelle), *Rpl32*, *Gusb* and *Rps18* were chosen as housekeeping genes. Analysis was performed using CFX manager software (BioRad). Primers for qPCR are provided in Table S4.

Serotonin measurement in serum

Serum was collected from blood from vena cava using Microvette® 500 Z-Gel (Sarstedt) tubes, after centrifugation 5 minutes 10,000 g at room temperature. Serotonin was assessed using ELISA kit ADI-900-175 (Enzo Life Sciences), according to the manufacturer's instructions.

Fig. S1.

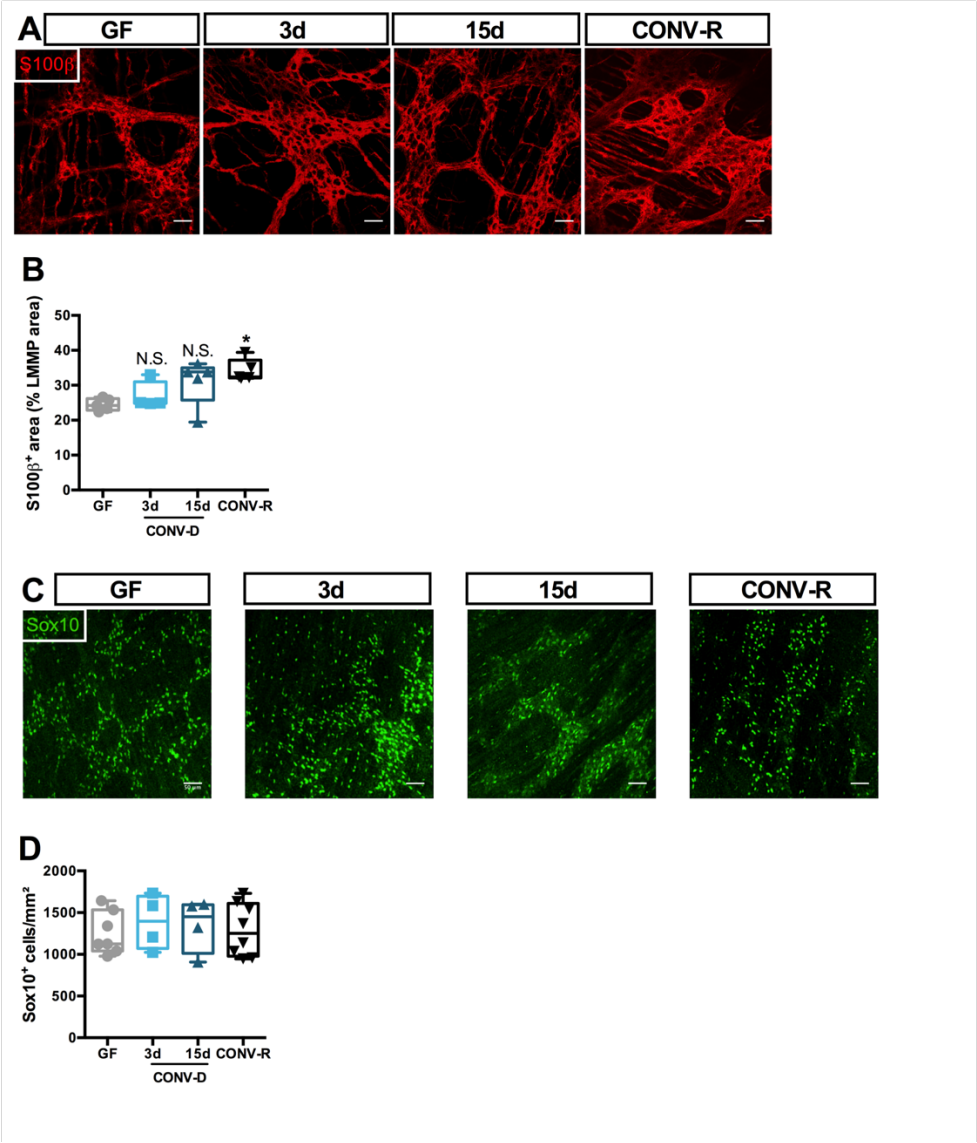

Fig. S1. The gut microbiota regulates anatomy of the enteric glial network. *(A)* Representative images of the colonic LMMP of GF mice, GF mice that were colonized (CONV-D) with microbiota from a CONV-R donor for 3 or 15 days, and CONV-R mice, showing glial marker S100β (red); quantification shown in *(B)*. * $P < 0.05$ vs. GF; *N.S.*: not significant; Kruskal-Wallis test followed by Dunn's post-hoc test. *(C)* Representative images of the LMMP of the mice, showing glial marker Sox10 (green); quantification shown in *(D)*. Scale bar: 50 µm.

Fig. S2.

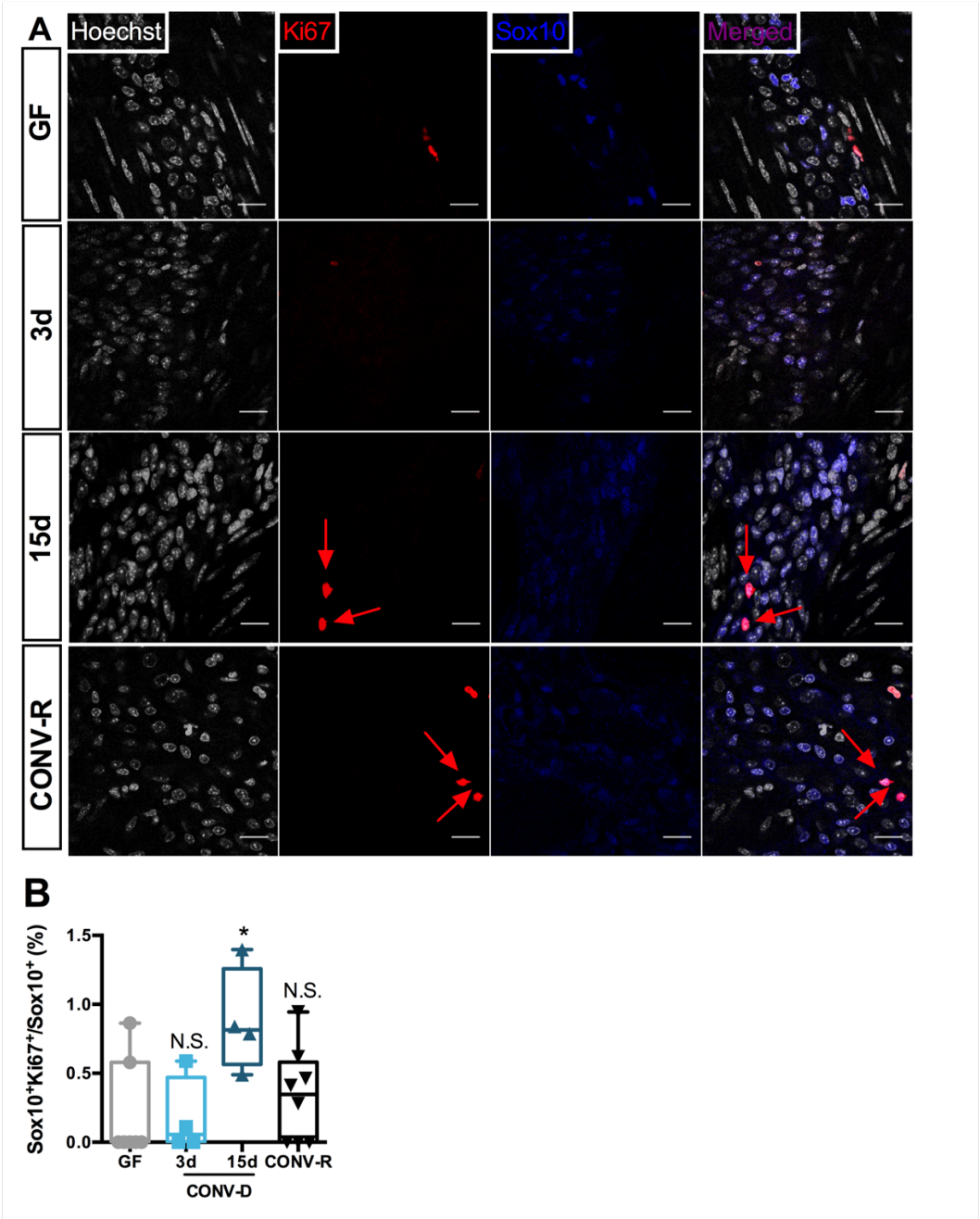

Fig. S2. Colonization of adult GF mice with a gut microbiota results in cycling of $Sox10^+$ cells. *(A)* Representative images of a colonic myenteric ganglion of the mice stained with cycling cell marker Ki67 (red arrows), neuronal and glial precursor marker Sox10 (blue), with nuclei counterstained with Hoechst (gray). *(B)* Quantification of double-positive Sox10/Ki67 cells. * $P < 0.05$ vs. GF; *N.S.*: not significant; Kruskal-Wallis test followed by Dunn's post-hoc test. Scale bar: 20 µm.

Fig. S3.

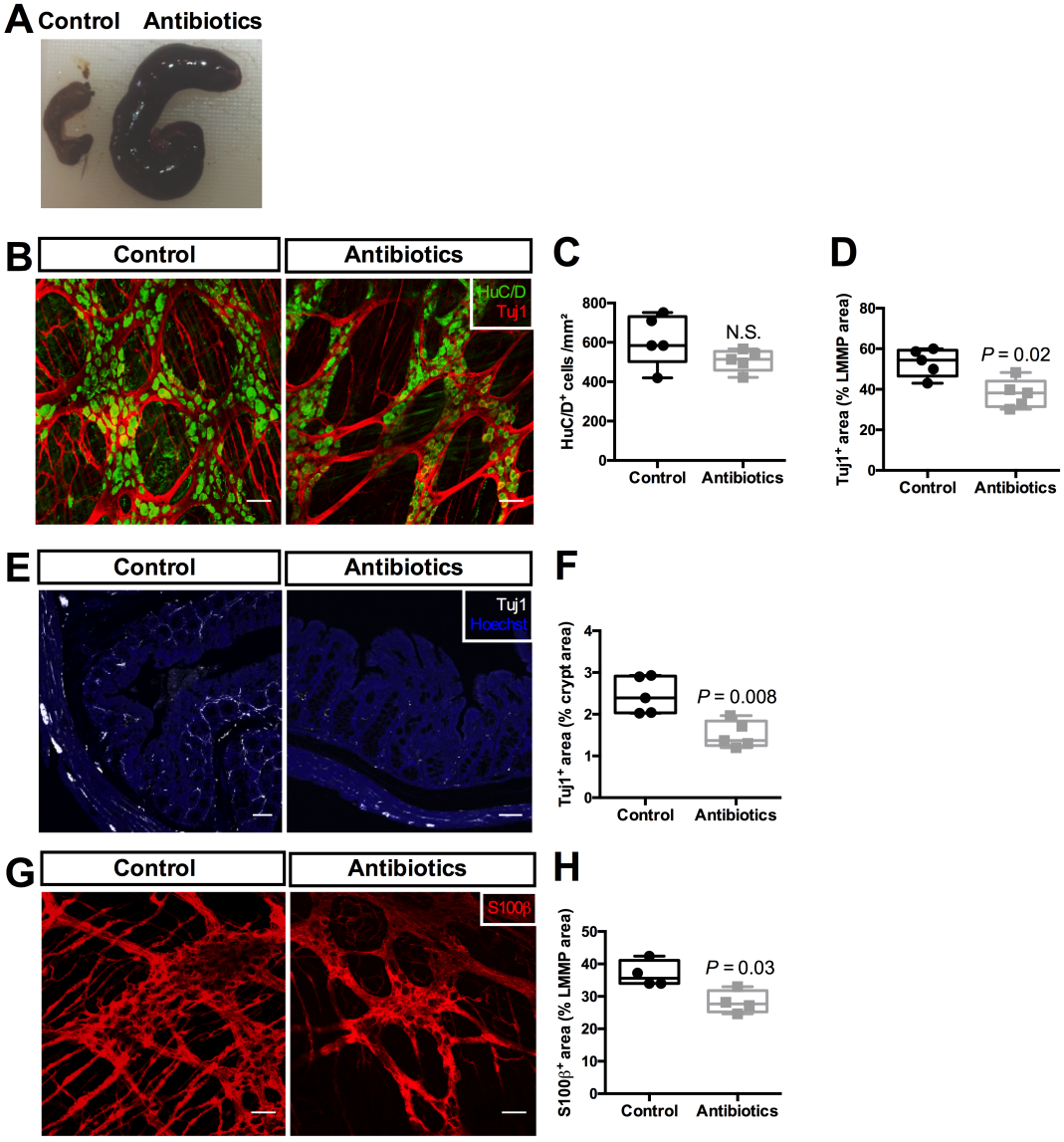

Fig. S3. Antibiotic treatment impairs innervation of the adult colon. *(A)* Cecum of a mice treated with antibiotics (right) or a control solution (left) in drinking water for three weeks. *(B)* Representative images of the colonic LMMP showing pan-neuronal marker HuC/D (green) and neuron-specific beta-III tubulin (Tuj1, red); quantification shown in *(C)* and *(D)*. *(E)* Representative images of the innervation of the colonic crypts of the mice using peripheral neuronal marker Tuj1; quantification shown in *(F)*. *(G)* LMMP of the mice stained with glial marker S100β (red); quantification shown in *(H)*. *P*-values reported after Mann-Whitney's test. *N.S.*: not significant. Scale bar: 50 µm.

Fig. S4.

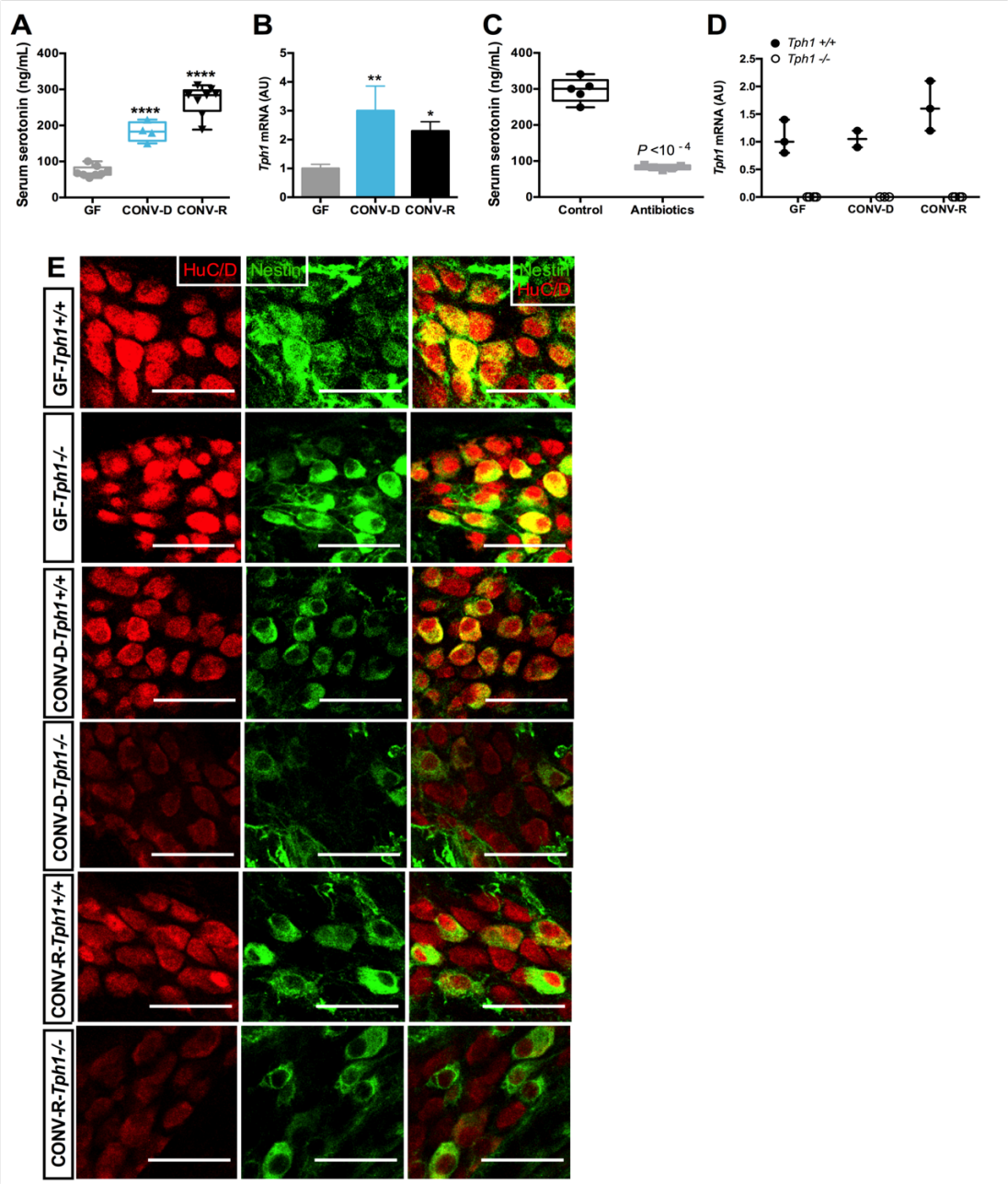

Fig. S4. Mucosal serotonin secretion is regulated by the gut microbiota. *(A)* Serum concentration of serotonin in germ-free, CONV-D and CONV-R mice. *(B)* Relative mRNA levels of *Tph1* in the colon. * $P < 0.05$ vs. GF, ** $P < 0.01$; **** $P < 10^{-4}$; Kruskal-Wallis non-parametric test followed by Dunn's multiple comparisons test. *(C)* Antibiotics significantly reduce circulating 5-HT. *(D)* *Tph1*$^{-/-}$ mice had no expression of *Tph1* in the colon, unlike their wild-type littermates. *(E)* Split panels of the images shown in Fig. 4D. Scale bar: 50 µm.

Fig. S5.

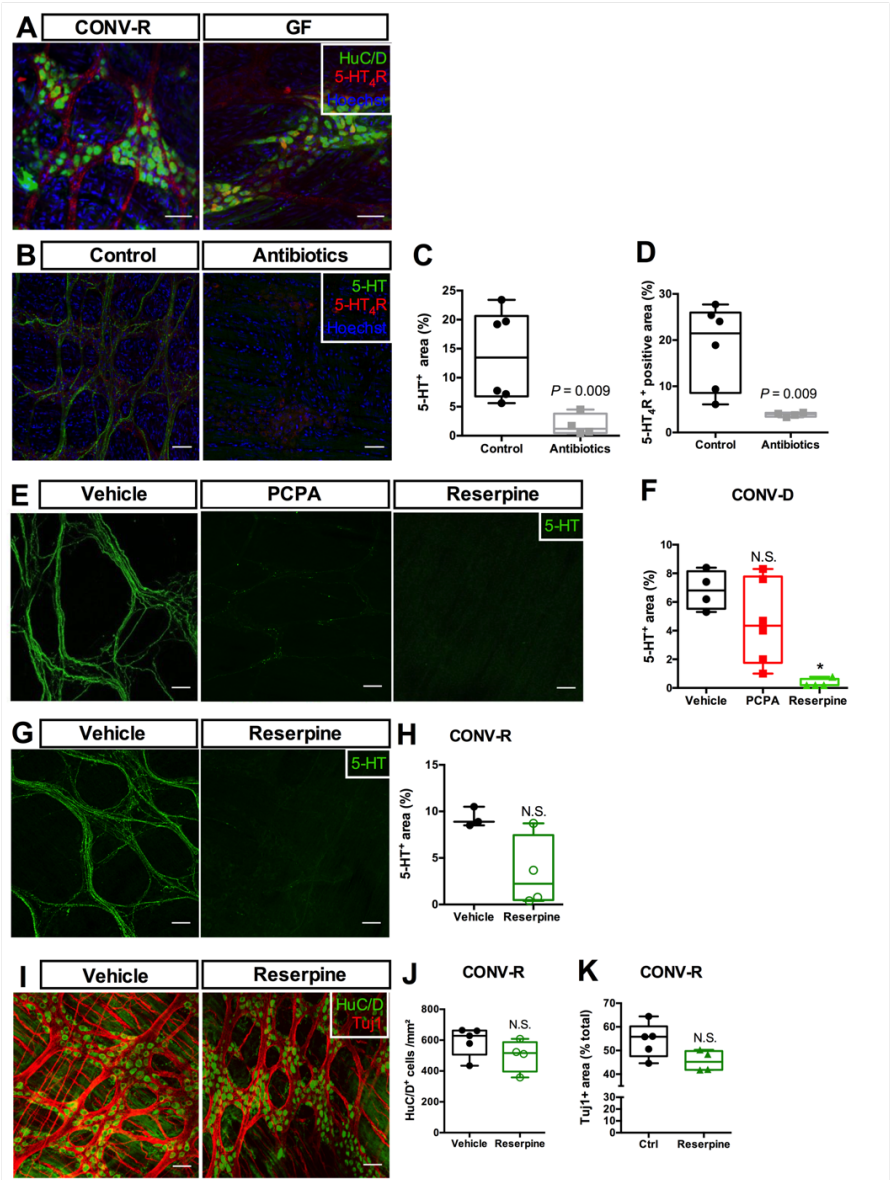

Fig. S5. Effect of microbiota or endogenous neuronal 5-HT depletion on the anatomy of the ENS. *(A)* Localization of 5-HT$_4$R in GF and CONV-R mice revealed that 5-HT$_4$R was expressed in the colonic myenteric plexus, and specifically in myenteric neurons. *(B)* Representative images of the colonic LMMP stained for 5-HT, showing that antibiotic-mediated depletion of the microbiota also induced depletion of 5-HT *(C)* and 5-HT$_4$R *(D)*; *P*-value expressed after Mann-Whitney's test. *(E-H)* Treatment with PCPA and reserpine effectively depleted endogenous 5-HT in the myenteric plexus of CONV-D *(E-F)* and CONV-R *(G-H)* mice. *(I)* Representative images of the LMMP of control and reserpine-treated CONV-R mice, showing pan-neuronal marker HuC/D (green) and neuron-specific beta-III tubulin (Tuj1, red); quantification shown in *(J-L)*. *P*-value expressed after Mann-Whitney's test. *N.S.*: not significant. Scale bar: 50 µm.

Fig. S6.

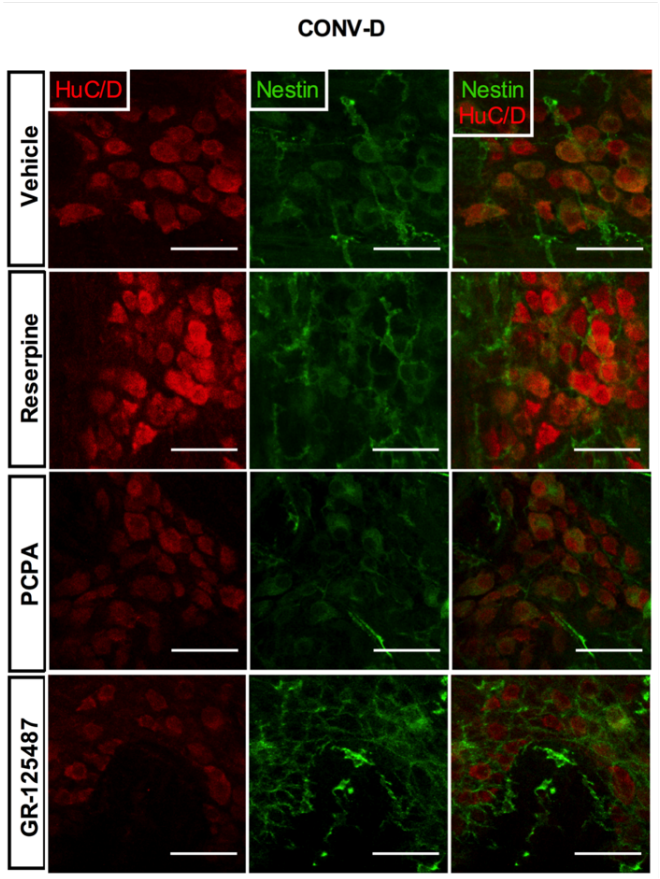

Fig. S6. Split panels of images shown in Fig. 5G. Scale bar: 50 µm.

Fig. S7.

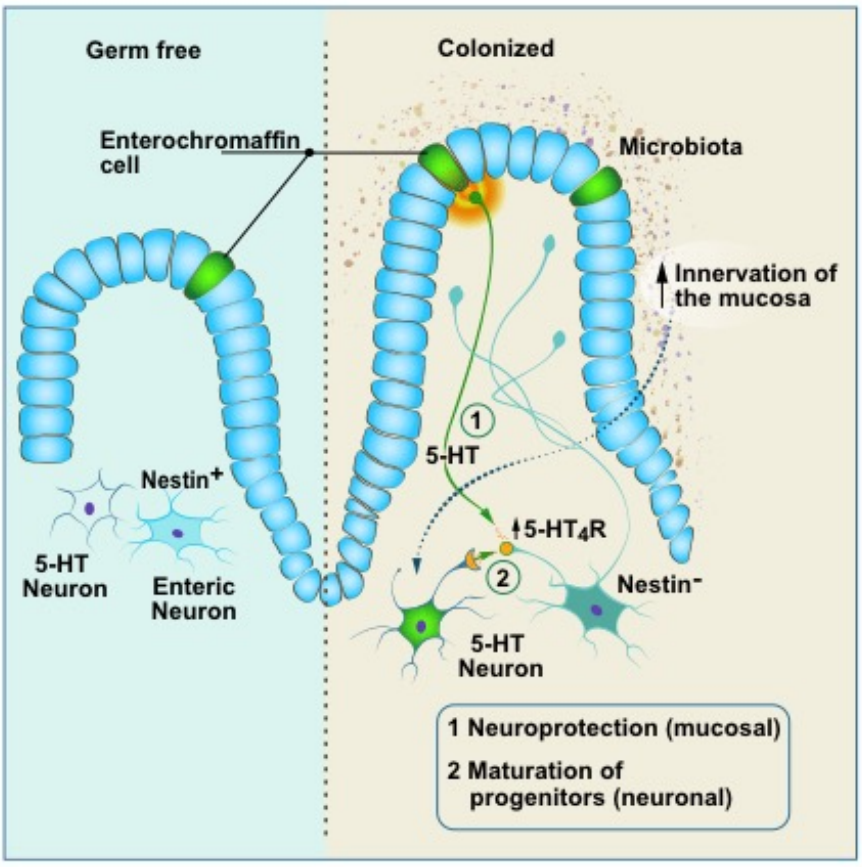

Fig. S7. Schematic representation of the roles of mucosal and neuronal 5-HT in the early phases of colonization. Germ-free mice have reduced 5-HT in both the mucosal (enterochromaffin cells,) and neuronal pools. Moreover, they have increased frequency of $Nestin^{+}$ myenteric neurons, i.e. neuronal precursors. After colonization, the gut microbiota induces production of 5-HT in enterochromaffin cells as well as in the neurons, potentially activating $5\text{-}HT_4$ receptors. Activation of $5\text{-}HT_4$ receptors by mucosal 5-HT is neuroprotective in the early stages of colonization, while neuronal 5-HT induces maturation of $Nestin^{+}$ progenitors. These changes occur concomitantly with increased innervation of the mucosa.

Fig. S8.

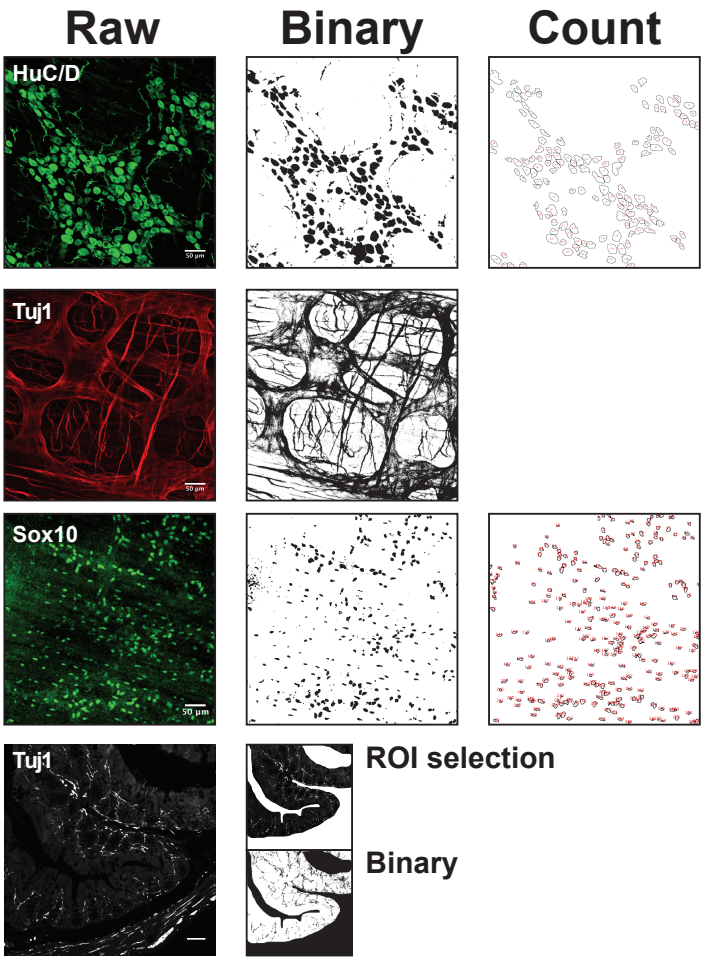

Fig. S8. Image analysis was performed using Fiji software, by conversion of raw images to binary and automated counting.

Table S1. Quantification of bacterial DNA in the feces of mice (expressed as copies of bacterial 16S rDNA per 500 pg of total DNA in feces).

	Control	Antibiotics
0 days	$7.05 \times 10^7 \pm 4.81 \times 10^6$	$5.51 \times 10^7 \pm 3.22 \times 10^6$
7 days	$6.99 \times 10^7 \pm 5.57 \times 10^6$	<1
14 days	$5.41 \times 10^7 \pm 4.27 \times 10^6$	<1
21 days	$8.01 \times 10^7 \pm 3.90 \times 10^6$	<1

Table S2. List of primary antibodies used for immunohistochemistry.

Primary antibody	Manufacturer	Reference	RRID	Dilution
Rabbit anti-Tuj1	Abcam	ab18207	AB_444319	1:500
Mouse anti-HuC/D	Life Technologies	A21271	AB_221448	1:500
Rabbit anti-HuC/D	Abcam	ab210554	N/A	1:300
Goat anti-Sox10	Santa Cruz	sc-17342	AB_2195374	1:100
Rabbit anti-S100β	Abcam	ab52642	AB_882426	1:500
Mouse anti-Nestin	Abcam	ab6142	AB_305313	1:100
Rabbit anti-Ki67	Abcam	ab16667	AB_302459	1:100
Rat anti-5HT	Abcam	ab6336	AB_449517	1:250
Rabbit anti-$5HT_4$	Abcam	ab60359	AB_2122438	1:500

Table S3. List of secondary antibodies used for immunohistochemistry (all from Life Technologies).

Secondary antibody	Fluorochrome	Reference	RRID	Dilution
Donkey anti-rabbit IgG	Alexa 568	A10042	AB_2534017	1:400
	Alexa 647	A31573	AB_2536183	
Donkey anti-mouse IgG	Alexa 488	A21202	AB_141607	
	Alexa 568	A10037	AB_2534013	
Donkey anti-goat IgG	Alexa 647	A21447	AB_141844	
Donkey anti-rat IgG	Alexa 488	A21208	AB_141709	

Table S4. Primer sequences for qPCR analysis.

Gene	Primer	Sequence
Gusb	Forward	GGCTGGTGACCTACTGGATTT
	Reverse	GGCACTGGGAACCTGAAGT
Rps18	Forward	ACCACAACCGCTATGGCTC
	Reverse	GCAAGGATTCCCAGCAACTTT
Rpl32	Forward	CCTCTGGTGAAGCCCAAGATC
	Reverse	TCTGGGTTTCCGCCAGTTT
Tph1	Forward	AACAAAGACCATTCCTCCGAAAG
	Reverse	TGTAACAGGCTCACATGATTCTC

References

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