Supplementary Information

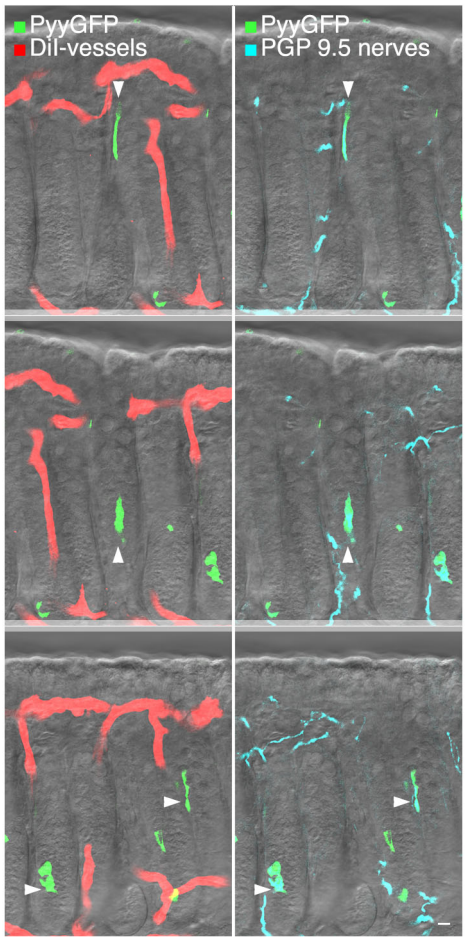

Figure S1. Neuropods contact nerves but not blood vessels. We used a vessel painting technique with the lipophilic dye DiI to determine the relationship of enteroendocrine cells with vessels and nerves. These three frames of tissue from the mid colon show that labeled vessels do not contact enteroendocrine cells. However, PGP 9.5-positive nerve fibers contact PyyGFP enteroendocrine cells at their neuropods. Arrowheads show cells of interest and their relationship to vessels versus nerves. Bar = 10μm

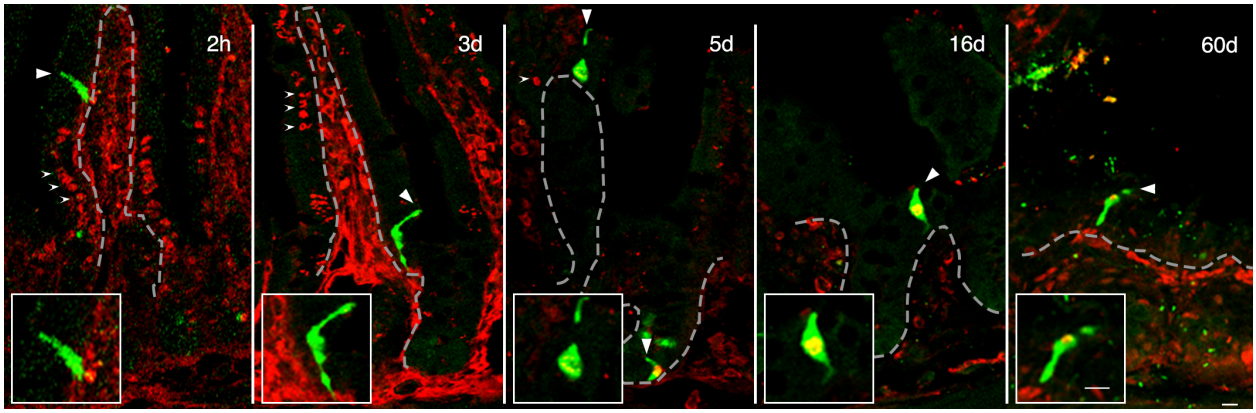

Figure S2. Long-lived enteroendocrine cells. We injected BrDU into PyyGFP mice to determine the enteroendocrine cell lifespan. At 2h-post injection, labeled epithelial cells were observed above the crypt-villus junction. Within two hours, 6.3% ± 1.2 (n=3) of PyyGFP cells were labeled with BrDU and at 3d, 27.6% ± 5.6 (n=3) PyyGFP cells were labeled. By 5d most labeled epithelial cells had disappeared. However, 23.6% ± 4.3 (n=3) of PyyGFP cells were labeled. These PyyGFP cells were not only observed both at the tip of the villus but also at the base of the crypts (arrow heads). Most important, 60d after initial injection, 15.8% ± 4.6 (n=3) of counted PyyGFP enteroendocrine cells were labeled indicating that a population of enteroendocrine cells is long-lived. Dotted lines separate epithelium from lamina propria. Bars = 10μm

Supplemental Table 1. Synaptic-transmission genes expressed in PyyGFP enteroendocrine cells.

Symbol	Function	Cat. No.	Tissue	Fold expression†	SEM‡	Pvalue	Tissue	Fold expression	SEM	Pvalue
Pyy	Neuropeptide	Mm00520716_g1	Colon	2.263	1.853	0.005	Ileum	2.500	2.139	0.005
Cck	Neuropeptide	Mm00446170_m1	Colon	1.166	0.520	0.004	Ileum	1.930	1.302	0.007
Gcg	Neuropeptide	Mm01269055_m1	Colon	2.005	1.313	0.001	Ileum	2.969	2.802	0.036
Th	Dopa synthesis	Mm00447557_m1	Colon	1.618	1.321	0.048	Ileum	2.873	2.471	0.015
Ddc	Dopa synthesis	Mm00516688_m1	Colon	0.817	0.289	0.012	Ileum	0.886	0.418	0.007
Dbh	Dopa synthesis	Mm00460472_m1	Colon	n.a.*			Ileum	n.a.		
Dat	Dopa synthesis	Mm00438388_m1	Colon	1.793	1.648	0.104	Ileum	1.931	1.930	0.603
Syn1	Presynaptic	Mm00449772_m1	Colon	2.431	2.127	0.174	Ileum	2.773	2.163	0.012
Pclo	Presynaptic	Mm00465330_m1	Colon	2.912	2.645	0.001	Ileum	3.873	3.537	0.048
Bsn	Presynaptic	Mm00464452_m1	Colon	2.191	1.862	0.006	Ileum	1.906	1.661	0.011
Lphn1	Presynaptic	Mm01247150_m1	Colon	1.902	1.703	0.109	Ileum	0.841	0.201	0.005
Nrx1	Presynaptic	Mm00660298_m1	Colon	2.312	2.031	0.013	Ileum	2.687	2.452	0.127
Nrx2	Presynaptic	Mm01236851_m1	Colon	1.911	1.337	0.000	Ileum	1.627	1.222	0.006
Nrxn3	Presynaptic	Mm00553213_m1	Colon	n.a.			Ileum	n.a.		
Cask	Presynaptic	Mm00438005_m1	Colon	1.906	1.901	0.761	Ileum	0.143	0.709	0.371
Ribeye	Presynaptic	Mm00515572_m1	Colon	0.282	0.122	0.869	Ileum	0.401	0.480	0.012
Munc13b	Presynaptic	Mm00498847_m1	Colon	1.724	1.513	0.025	Ileum	0.634	0.115	0.019
Rims1	Presynaptic	Mm01225747_m1	Colon	n.a.			Ileum	n.a.		
Rims2	Presynaptic	Mm00453603_m1	Colon	2.331	2.056	0.027	Ileum	3.122	2.875	0.028
Snap25	Presynaptic	Mm00456921_m1	Colon	2.060	2.019	0.858	Ileum	2.044	2.040	0.356
Dlg2	Postsynaptic	Mm00457160_m1	Colon	1.305	1.258	0.987	Ileum	1.532	1.332	0.688
Dlg3	Postsynaptic	Mm01225036_m1	Colon	1.772	1.749	0.001	Ileum	0.088	1.249	0.158
Psd95	Postsynaptic	Mm00492193_m1	Colon	3.789	3.772	0.018	Ileum	3.236	3.040	0.004
Nlgn1	Postsynaptic	Mm02344305_m1	Colon	-0.244	0.628	0.034	Ileum	2.511	2.316	0.208
Nlgn2	Postsynaptic	Mm01703404_m1	Colon	1.719	1.469	0.197	Ileum	1.044	0.365	0.006
Nlgn3	Postsynaptic	Mm00556834_m1	Colon	1.631	0.808	0.002	Ileum	1.597	1.418	0.039
Homer 1	Postsynaptic	Mm00516275_m1	Colon	1.499	0.976	0.366	Ileum	-0.037	0.873	0.404
Homer 2	Postsynaptic	Mm01314936_m1	Colon	1.791	1.639	0.910	Ileum	0.244	0.193	0.246
Homer 3	Postsynaptic	Mm00803747_m1	Colon	0.822	0.478	0.829	Ileum	2.142	1.955	0.246
Syncam1	Synaptic	Mm00457556_m1	Colon	n.a.			Ileum	1.649	1.352	0.155
Syncam2	Synaptic	Mm00618780_m1	Colon	-0.746	1.102	0.021	Ileum	-0.416	0.726	0.250
Syncam3	Synaptic	Mm01290177_m1	Colon	n.a.			Ileum	n.a.		
Syncam4	Synaptic	Mm00462221_m1	Colon	2.181	1.849	0.128	Ileum	2.302	1.889	0.257
Ncam1	Synaptic	Mm01149710_m1	Colon	1.823	1.470	0.028	Ileum	2.355	2.245	0.192
Vglut1	Glut trans.	Mm00812886_m1	Colon	n.a.			Ileum	n.a.		
Vglut2	Glut trans.	Mm00499876_m1	Colon	n.a.			Ileum	n.a.		
Vglut3	Glut trans.	Mm00805413_m1	Colon	n.a.			Ileum	0.971	0.735	0.659
Vgat	Gaba trans.	Mm00494138_m1	Colon	n.a.			Ileum	n.a.		

†Fold expression and SEM were calculated from $Log_{10}2^{-[(\Delta Ct\ PyyGFP\ positive\ cells) - (\Delta Ct\ GFP\ negative\ epithelial\ cells)]}$. ‡SEM means standard error of the mean with n=5. § $2^{-(\Delta Ct)}$ was used to separate means that differed significantly with Student's t-test. *n.a. means no amplification observed after 40 cycles.

Supplementary Video 1. A purified enteroendocrine cell and trigeminal neuron connect. This time-lapse footage shows the complete process through which a CckGFP enteroendocrine cell connects to a trigeminal (TG) neuron. At plating, the CckGFP cell lies next to the TG neuron. After 12:04 hours, the neuron extends a neurite (white arrow) towards the enteroendocrine cell, and the enteroendocrine cell responds by extending a cytoplasmic process that connects to TG neuron (~14.5 hours). The connection remained stable for 88 hours at which point the experiment was terminated. Bar = 10 μm

Supplementary Video 2. CckGFP enteroendocrine cell seeks to connect to putative axon of TG neuron. This time-lapse footage shows a CckGFP enteroendocrine cell at a distance from two TG neurons. The TG neuron extends a putative axon to which the CckGFP cell seeks to connect. Noteworthy is the motility of the CckGFP cells compared to other surrounding nonGFP epithelial cells. At the end, the dotted line shows the path of the putative axon to the connecting CckGFP enteroendocrine cell. The axonal fiber is 400mm in length. Bar = 10μm

Supplemental Methods

Mice. Animal care and experiments were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee of Duke University Medical Center. CckGFP, PyyGFP, PyyCre and RϕGT were previously developed and described (10, 14, 27, 28). The PyyCre mouse line was a generous gift of Dr. Andrew Leiter from University of Massachusetts Medical School (Worcester, MA). All PyyCre:RϕGT mice used here carried one PyyCre allele and one RϕGT allele. The CckGFP line was acquired and kept on a Swiss Webster background and the remaining lines on a C57BL6/J background. Animals were between 6 and 12 weeks of age unless otherwise stated.

Co-culture of enteroendocrine cells and sensory neurons. Intestinal epithelial cells were isolated by enzymatic digestion from PyyGFP or CckGFP mice and FAC sorted according to previous reports (14). The trigeminal and dorsal root ganglia served as sources of sensory neurons, as these are more numerous and easier to access in a mouse than those from the vagal nodose ganglia. For enteroendocrine cells, we used CckGFP mice because the yield of FACS cells per mouse is about 40-fold higher than that of PyyGFP mice (87166 ± 19030 CckGFP vs. 2146 ± 1290 PyyGFP; n = 6). This is important because the survival rate over time of enteroendocrine cells is rather low (0.50% ± 0.17% at D1 to 0.10% ± 0.02% at D6; average of 3 wells per n, n = 6). Thus, by using CckGFP cells, there were enough viable cells for time-lapse imaging. Primary sensory neurons were dissociated by enzymatic digestion from the trigeminal or dorsal root ganglia of wild-type C57BL6/J mice according to previous reports (15). Neurons were labeled with the Vybrant® DiI cell-labeling solution (Molecular Probes®). GFP-enteroendocrine cells and DiI-labeled neurons were mixed and plated on glass-bottom 35mm dishes (MatTek Corporation) coated with BD growth factor-reduced Matrigel™ (Becton, Dickinson and Company). The plating density was about 10,000 enteroendocrine cells and 5,000 neurons per dish. The culture medium is the following: advanced DMEM/F-12 (GIBCO®), 200 mM L-glutamine, 100 U/mL Penicillin/Streptomycin, 10 mM Hepes, 1X N-2 (GIBCO®), 1X B27 - No Vit A (GIBCO®), and 50 ng/mL EGF (R&D Systems Inc.). Cultures were then imaged over time in an Olympus Vivaview fluorescence incubator with a 20x/0.75 DIC Olympus UPlanSAPO objective. Images were collected using MetaMorph® software (Molecular Devices, LLC) every 10 minutes for endogenous GFP, DiI, and DIC channels at 1024 pixel resolution. Time-lapse videos were composed in MetaMorph®, and necessary labels and captions were added in Adobe Premiere Pro CC (Adobe Systems Inc.).

Monosynaptic rabies neurotracing. The virus was obtained from the Duke Viral Vector Core, and initial titer was 2.1x10^13 ffu/ml. Mice were infected with 1:10 dilution in PBS of B19G SADΔG-GFP rabies virus. The virus was delivered by enema into the distal colon of P3 wild type or PyyCre;RϕGT mice. Colon tissue collected for immunofluorescence 7 days after infection.

BrDU pulse labeling. Four week-old PyyGFP mice were injected once intra-peritoneally with 100 mg/kg BrDU. Colon and ileum tissues were collected for immunohistochemistry at 2 hours and 3, 5, 16, and 60 days post-injection. Prior to the immunofluorescence procedure, DNA from tissue sections was denatured in 2M HCL at 37°C for 30 minutes and tissues washed in boric acid. Subsequently immunofluorescence was performed as previously described (9).

Blood vessel painting and immunofluorescence. Blood vessel painting was done according to previous reports (11). We perfused PyyGFP mice using the lipophilic dye DiI and processed the vessel painted tissue for immunohistochemistry with the pan-neuronal marker PGP 9.5. The tissue sections for this purpose were between 60 and 100 microns thick. We cleared the tissue with FocusClear™ (Cedarlane Labs) to allow deep labeling and imaging. For additional immunofluorescence to determine the type of nerves interacting with enteroendocrine cells we used the following antibodies: goat anti-CGRP (Abcam, Cat#. 36001), rabbit anti-Calbindin (Swant, Cat#. CB38a), rabbit anti-NPY (Abcam, Cat#. Ab10980-50), rabbit anti-SYN1 (Cell Signaling, Cat#. 5297), rabbit anti-PSD95 (Cell Signaling, Cat#. 3450), rabbit anti-PGP 9.5 (Ultraclone, Cat#. RA95101), and rat anti-BrDU (Abcam, Cat#. 6326). Immunofluorescence procedures and some additional antibodies are described in reference (9).

Confocal microscopy and 3D data visualization. Image acquisition and 3D visualization software settings are described in detail in reference (9). Optical z-stacks of images were acquired using either a Zeiss 510 or a Zeiss 780 inverted confocal microscope. Optical z-stacks were volume rendered at automatic threshold using Imaris® (Bitplane Inc.). Some images have been rotated/flipped and cropped to fit in figure panels. Besides brightness/contrast, no additional adjustments have been performed.

Cell isolation, FACS sorting and qRT-PCR analysis of gene expression. The methods and statistical analysis of gene expression are detailed in reference (9, 14, 20). Fold expression represents $\log_{10} 2^{-[(\Delta Ct\ PyyGFP\ positive\ cells) - (\Delta Ct\ GFP\ negative\ epithelial\ cells)]}$ and error bars represent the SEM. Significant differences were separated by Student's t-test using $2^{-(\Delta Ct)}$ values at $\alpha = 0.05$.

Statistics. The relationship between enteroendocrine cells and blood vessels or nerves was quantified in three or five biological replicates (individual mice) respectively. Each biological replicate represented the average analysis of 20-30 GFP positive cells. The distance between blood vessels and a GFP positive cell was determined using the "measure" option of Fiji software. The number of GFP cells connected to nerves was expressed as a percentage of all of the cells counted. Quantification of BrDU labeled cells was done in three biological replicates and each represented the average of at least 15 PyyGFP cells counted. All values were expressed as the average ± SEM.

Study approval. The work with animals was approved by the Duke University Institutional Animal Care and Use Committee under the protocol A050-13-02. The Institutional Biosafety Committee approved the use of mutant rabies virus at the Animal Biosafety Level 2 and the registration number is 12-0024-08.