Regional cytoarchitecture of the adult and developing mouse enteric nervous system

Highlights

- Myenteric neurons, but not submucosal neurons, form circumferential stripes
- Conditional intensity function plots quantify and reveal tissue organization
- Neuronal stripes arise from proximal to distal during embryonic development
- Neuronal marker expression differs by region but not among individual stripes

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In brief

The enteric nervous system spans the gastrointestinal (GI) tract and controls diverse GI functions, yet its structure is often characterized as a uniform meshwork. Hamnett et al. demonstrate that enteric neurons are organized into circumferential stripes that regionally differ in organization, development, and cellular composition.
Regional cytoarchitecture of the adult and developing mouse enteric nervous system

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SUMMARY

The organization and cellular composition of tissues are key determinants of their biological function. In the mammalian gastrointestinal (GI) tract, the enteric nervous system (ENS) intercalates between muscular and epithelial layers of the gut wall and can control GI function independent of central nervous system (CNS) input.¹ As in the CNS, distinct regions of the GI tract are highly specialized and support diverse functions, yet the regional and spatial organization of the ENS remains poorly characterized.² Cellular arrangements,³⁴ circuit connectivity patterns,⁵⁶ and diverse cell types⁷–⁹ are known to underpin ENS functional complexity and GI function, but enteric neurons are most typically described only as a uniform meshwork of interconnected ganglia. Here, we present a bird’s eye view of the mouse ENS, describing its previously underappreciated cytoarchitecture and regional variation. We visually and computationally demonstrate that enteric neurons are organized in circumferential neuronal stripes. This organization emerges gradually during the perinatal period, with neuronal stripe formation in the small intestine (SI) preceding that in the colon. The width of neuronal stripes varies throughout the length of the GI tract, and distinct neuronal subtypes differentially populate specific regions of the GI tract, with stark contrasts between SI and colon as well as within subregions of each. This characterization provides a blueprint for future understanding of region-specific GI function and identifying ENS structural correlates of diverse GI disorders.

RESULTS

Neuronal organization in the adult enteric nervous system differs by region and plexus

Based on morphology and function, the adult mouse intestines can be broadly divided into 5 regions: duodenum, jejunum, ileum, proximal colon (PC), and distal colon (DC; Figure 1A). In all regions, enteric neurons are located within the myenteric plexus (MP), which controls motility, or the submucosal plexus (SMP), which regulates secretion and absorption (Figure 1A). To understand the broad layout of enteric neurons, we imaged large areas (up to ~50 mm²) of both MP and SMP in each intestinal region of adult mice. Immunostaining neuron somas in the MP revealed circumferential orientation of ganglia, which loosely coalesced into a noncontinuous stripe pattern perpendicular to the longitudinal axis, herein referred to as neuronal stripes (Figures 1B, 1C, and S1A). Submucosal ganglia of the small intestine (SI) did not show this arrangement, nor did they correlate with epithelial crypt organization (Figures S1B and S1C). In contrast, submucosal ganglia of the PC were organized into diagonal stripes, converging opposite the mesenteric border and aligning with mucosal folds (Figure 1D). These diagonal stripes were absent from DC SMP (Figure S1C).

Other regions of the gastrointestinal (GI) tract also showed myenteric ganglia organization, though not as parallel stripes (Figures S1D–S1F). In forestomach and glandular stomach, ganglia converged around the stomach toward the lesser curvature (Figures S1E–S1H). The few esophageal neurons showed a weak diagonal orientation (Figure S1D).

Unlike neurons, glia are found throughout the muscle and epithelial layers in addition to neuronal plexuses (Figures S1I–S1L). Within plexuses, glia tended toward the same organization as neurons, albeit with more frequent extra-ganglionic glia than neurons. Glial projections in the circular muscle showed a strong
Figure 1. Regional organization of enteric neurons in the adult ENS

(A) Schematic illustrating the regions of the intestines (top) and the structure and orientation of the submucosal (SMP) and myenteric plexuses (MP) within the intestine wall (bottom). Black ovals represent neurons.

(B–D) Representative immunohistochemistry (IHC) images of adult whole-mount MP of ileum (B) and proximal-mid colon (C) for neuronal label HuC/D, and SMP of PC (D) for HuC/D (cyan) and epithelial label E-cadherin (yellow).

(E) Representative images of immunohistochemical staining of full-thickness whole-mount tissue of ileum (left) and PC (right) for HuC/D (MP, magenta; SMP, green).

(F) Smoothed profiles of HuC/D signal intensity along the longitudinal (x) axis of images in (E), highlighting stripes (peaks) in MP (magenta) and lack thereof in SMP (green).

(G) Representative X-ray tomography images of heavy metal staining of adult ileum (left) and PC (right). Colored boxes represent locations of magnified images. Cell bodies identified based on morphology and location, annotated with blue circles.
Circumferential orientation (Figures S1I–S1J’), likely because of close apposition to neuron fibers (Figure S1M). Submucosal glia and their projections followed pathways between crypts (Figures S1K–S1L’). In PC SMP, the presence of many more extra-ganglionic glia masked the diagonal stripes so evident for neurons (Figure S1L). However, this organizational difference between neurons and glia appeared limited to the PC SMP.

Full-thickness whole-mount preparations of the intestines did not suggest any structural interplexus relationship and highlighted the orientation difference between the MP and SMP in the PC (Figure 1E). MP stripe formations emerged as peaks in longitudinal axis signal intensity profiles of whole-mount images, which also confirmed the lack of circumferential structure in the SMP (Figure 1F). We confirmed this enteric nervous system (ENS) organization using a separate imaging approach, synchrotron source X-ray tomography that allows simultaneous visualization of multiple cell types of both MP and SMP (Figures 1G and 1H). Taken together, the MP, but not the SMP, displays a striped neuronal architecture.

We next sought to characterize and quantify this region- and plexus-specific ENS structure. While intensity profiles (Figure 1F) are useful visualizations, they cannot reveal any structure not perpendicular to the x axis, such as in PC SMP, and they are susceptible to tissue distortions. We therefore used conditional intensity function (CIF) plots to generate spatial probability maps of neuronal locations relative to a given neuron (Figure 1I; STAR Methods). An average neuron was always situated within a stripe flanked by higher-order stripes, clearly displayed when the CIF is collapsed into a two-dimensional (2D) graph (Figure 1L, bottom). The PC contained the thickest stripes (Figure 1J), while the largest interstripe distances existed in the SI (Figure 1K). Myenteric neurons were denser in the PC and sparsest in the duodenum (Figure 1L). We found no sex-related differences (Figures S1N–S1P). Neuronal density was also different between stomach regions, while the esophagus was the sparsest populated region overall (Figure S1Q). Glial density closely followed neuronal density (Figures S1R and S1S).

SMP neurons were sparser than myenteric neurons, and in contrast to the MP, the densest SMP region was the duodenum (Figure 1M). Further, individual SMP ganglia tended to have a circumferential orientation, but only the PC exhibited any macrostructure beyond ganglia organization in the SMP (Figure 1N). Therefore, organization of enteric neurons and glia quantitatively and qualitatively differs between regions and plexuses of the GI tract.

Myenteric organization emerges progressively in a regionally specific manner

To assess how neuronal stripes arise, we visualized myenteric neurons in whole-mount preparations from late embryonic through neonatal ages (Figures 2A and S2A). At embryonic day (E)14.5, when enteric neurons first populate the entire length of the mouse intestines,13 neurons were scattered throughout the SI and colon (Figure 2A). Beginning at E16.5 in the jejunum and E18.5 in the DC, scattered neurons reorganized into circumferential neuronal stripes, which resolved into individual ganglia postnatally (Figure 2A). CIF analysis confirmed the gradual emergence of neuronal stripes in the developing MP and differing organizational timelines between SI and colon (Figure 2B). To quantify this progressive organization, we compared nearest-neighbor distances of HuC/D-positive neurons to synthetically generated data with an imposed minimum nearest-neighbor distance of 10 μm, approximately the diameter of an enteric neuron (Figure 2C). At E14.5, the dispersion of neurons did not differ compared to random distributions; this dispersion transitioned to highly clustered at E16.5 in the duodenum and jejunum, at E18.5 in the ileum, and at early postnatal stages in the colon (Figure 2D). Thus, neuronal stripes arise from a gradual reorganization of myenteric neurons, which occurs embryonically in the more proximal intestines and neonatally in the more distal intestines.

We next assessed whether gut growth and neuronal cell death contribute to the emergence of neuronal stripes. Intestinal length increased 10-fold between E14.5 and postnatal day (P)21, which correlated with decreased neuronal density and increased interstripe distance (Figures 2E–2J and S2B–S2G). We used the apoptotic marker caspase-3 to identify apoptotic neurons but detected only sparse labeling in the developing MP across region and development (Figures 2K and 2L). Collectively, these results suggest that intestinal growth, but not neuronal cell death, may influence organization of the developing MP.

Neuronal subtype distribution varies by intestinal region

We next sought to assess regional distribution of neuronal subtypes, focusing on known determinants of subtype function, such as calcium-binding protein (CBP; Figures 3A–3C), neurotransmitter (Figures 3D–3I), and neuropeptide (Figures 3J–3N) expression.11–13 We observed differences in relative marker expression both between the SI and colon and between regions within each. We found that markers attributed to a particular neuronal functional subtype tended to have similar regional distributions. For instance, markers representing excitatory motor

(H) Schematic of MP (blue) and SMP (red) cell body locations from samples in (I). Yellow shading represents the SMP.
(I) Organizational analysis of whole-mount MP HuC/D IHC of all intestinal regions (top) using conditional intensity functions (CIFs; 2nd row), showing probability density of neuron locations. A value of 1 is expected density based on uniform neuron distribution. CIFs: yellow, high probability density; blue, low.
(J and K) Violin plots of stripe width (J) and interstripe distance (K) from MP whole-mount preparations analyzed by CIFs as in (I). n = 34 mice for all groups (J) and n = 21–29 (K).
(L and M) Neuronal density (mean ± SEM) in MP (L) and SMP (M) across intestinal regions. n = 34 (L) and 7 (M).
(N) Organizational analysis of whole-mount SMP HuC/D IHC of all intestinal regions (top) using CIFs (2nd row) as in (I). Lack of obvious stripe structure precluded further analysis.

All tests one-way ANOVA, other than (K), which used a mixed-effects model. Letters represent significant differences, such that two groups are not significantly different if they share a common letter. Full details of pairwise comparisons can be found in Table S1. Scale bars as indicated. AU, arbitrary units; D, duodenum; DC, distal colon; I, ileum; J, jejunum; PC, proximal colon. See also Figure S1.
neurons, including calretinin (Figures 3A and S3A), choline acetyltransferase (ChAT)-GFP (Figures 3D, S3D, and S3M), and tachykinin precursor 1 (Tac1)Cre-tdT (Figures 3Ja and S4F), tended to decrease in expression from proximal to distal, with highest expression in the duodenum and lowest in the colon, although this was not significant for Tac1Cre-tdT. ChAT-GFP had the highest expression of any marker, present in 42%–75% of neurons, depending on region (Figure 3D).

In contrast to excitatory motor neurons, expression of inhibitory motor neuron markers tended to increase distally. nNOS (Figures 3Ea and S3E) showed a non-significant trend of increasing expression from duodenum to DC, and vasoactive intestinal peptide (Vip)Cre-tdT (Figures 3L, S4C, and S4I) was highest in the DC. Secretagogin, a marker of a currently undefined subtype, showed a similarly shaped profile to VipCre-tdT (Figure 3C), with approximately twice as many secretagogin neurons in the
DC (~20%) compared to the SI (~11%) and PC (~9%) (Figures 3C and S3C). Secretagogin labeling also revealed qualitatively different fiber characteristics depending on region. In the SI, secretagogin fibers were restricted to interganglionic tracts, but fibers additionally innervated longitudinal and circular muscle in the PC and DC, respectively (Figures 3C and S3C). This may suggest as-yet uncharacterized regionally specific functions for secretagogin neurons.

Calbindin 1 (Calb1)Cre-tdT (Figures 3B, S3B, and S3J) and 5-hydroxytryptamine (5-HT; Figures 3H and S3H) both showed expression peaks in the duodenum and the PC. 5-HT and Calb1Cre-tdT fibers were restricted to the MP, consistent with their postulated interneuron and sensory neuron identities, respectively. This fiber localization was also observed for vesicular glutamate transporter 2 (VGLUT2)Cre-tdT (Figures 3F, S3F, and S3K), a potential interneuron or sensory neuron marker. VGLUT2Cre-tdT expression was highest in the PC (6%), almost 3-fold higher than in the ileum, though it lacked the duodenal peak of 5-HT or Calb1Cre-tdT (Figure 3F). VGLUT2Cre-tdT was the only marker more highly expressed in both colonic regions than any SI region. Somatostatin (Sst), another interneuron marker, also showed highly significant enrichment in the PC (~13%), ~4-fold higher than in the ileum and DC (~3%) (Figures 3M, 3M, and S4D).

Two markers showed pronounced enrichment in the ileum: glutamate decarboxylase 2 (Gad2)Cre-tdT (Figures 3G, S3G, and S3L) and pituitary adenylate-lysylcyclase-activating polypeptide (Pacap)Cre-tdT (Figures 3N and S4E). PacapCre-tdT expression was widespread, ranging from 20% (duodenum) to 40% (ileum). Despite its postulated function as a motor neuron marker, we observed few muscle-innervating fibers; thus, PACAP may have additional roles beyond its suggested motor neuron function. Tyrosine hydroxylase (TH; Figures 3I and S3I), labeling <1% of neurons, also peaked in the ileum, albeit with comparable levels in the jejenum and PC. Only proenkephalin (Penk)Cre-tdT (Figures 3K, S4B, and S4G), expressed in both excitatory motor and interneurons, showed enrichment in the jejenum, though this was not significant.

In addition to neuronal expression, several markers were found in non-neuronal cells. PenkCre-tdT was present in diverse cell types including suspected immune cells and fibroblasts, which was supported by immunohistochemistry (IHC) against enkephalin (Figure S4H). PacapCre-tdT was expressed in glia and could be observed alongside neuronal and sensory neuron expression within the same ganglion (Figure 3N, PC). Finally, Tac1Cre-tdT (Figure S4F) and Gad2Cre-tdT (Figure S3G, jejunum) also showed limited non-neuronal expression.

**Within-region organization of neuronal subtypes is regular across neuronal stripes**

We next examined neuronal subtype representation across myenteric neuronal stripes. We selected 4 markers covering a range of different cell types: motor neurons (VipCre-tdT and calretinin), sensory neurons (Calb1Cre-tdT), and interneurons (Sst) (Figures 4A–4D). Within enteric neuronal stripes, the proportion of neurons positive for a given marker broadly reflected that of the entire region, and these distributions shifted appropriately between regions. For instance, calretinin marked 35% of ileum neurons overall (Figure 3A), and individual stripes contained 15%–60% calretinin neurons, with a peak at ~35% (Figure 4A). Subtypes were well distributed across stripes, with few stripes containing zero neurons of a given subtype unless that marker was very sparsely expressed, such as ileal Sst (Figures 3M and 4D). This was further illustrated when comparing longitudinal axis intensity profiles of HuC/D signal with that of a given subtype (Figures 4E, S4J, and S4K). Only at very low subtype expression did the correlation between profiles vanish, such as in ileal Sst (Figures 3M and 4D).

**DISCUSSION**

By combining large-scale image analysis with computational methods over multiple regions and ages of the mouse intestines, we demonstrate that the mouse MP possesses a gross cytoarchitectural organization of circumferential neuronal stripes that can be used to classify subsets of neurons by their spatial distribution and quantitative pattern of expression. Further, our computational approach for spatial analysis provides a new tool to describe and quantify patternning across tissues and organs.

The role of individual neuronal stripes remains to be elucidated, though structural differences seen between regions likely contribute to the particular functions of SI and colon. Clonally related ENS cells inhabit overlapping domains and exhibit coordinated activity, whether these functional units map onto the macrostructure of neuronal stripes has yet to be explored. Further, neuronal stripes could control rings of circular muscle, which possess the same orientation as neuronal stripes, or coordinate multiple longitudinal interneuron projections perpendicular to the stripes.

Another outstanding question regards the mechanisms underlying stripe development. We demonstrate that neuronal stripes emerge earlier in the proximal than in the distal intestines, which correlates with the timing of neural crest cell (NCC) colonization. The ENS arises from two populations of NCCs. In mouse, vagal NCCs enter the proximal intestines at E8.5 and migrate distally;16 sacral NCCs enter the distal intestines at E13.5 and...
Figure 4. Structural distribution of neuronal subtypes
(A–D) Frequency distribution plots (mean ± SEM) of the proportion of neurons in a neuronal stripe positive for calretinin (A; 7–12 stripes per region, 12 mice), Calb1Cre-tdT (B; 7–10 stripes, 4 mice), VipCre-tdT (C; 7–12 stripes, 4 mice), and somatostatin (D; 6–12 stripes, 9 mice). KS, Kolmogorov-Smirnov.

(E) Representative smoothed profiles of HuC/D and somatostatin signal intensity along the longitudinal axis in ileum (top) and PC (bottom). r value indicates Pearson r correlation between the two profiles.

(F) Scatterplot showing the relationship between the proportion of total (HuC/D) neurons positive for a given marker (Calb1Cre-tdT, calretinin, somatostatin, or VipCre-tdT) in the ileum or PC and the Pearson r value for that sample, representing the strength of correlation between the HuC/D signal intensity profile and that of the marker (as in E). Note that the association is weaker for lowly expressed markers (e.g., somatostatin) but rises steeply when more than ~5% of neurons are positive for a given marker. See also Figure S4.
migrate proximally. Their final locations and extent of overlap remain unknown and may contribute to structural, developmental, and subtype distribution differences among GI regions. Both cell-autonomous and non-cell-autonomous mechanisms may influence ENS structural development. In chick embryos, reduction of vagal NCCs below a critical value results in aganglionosis, inhibition of NCC apoptosis increases ENS cell number. Additionally, work in mice has shown that genetic manipulation of enteric neuron precursors, including the removal of the cell adhesion molecule β1 integrin or the DNA-binding protein Hand2, disrupts MP structural development.

The role of non-neuronal tissues in ENS structural development is only partially understood. Studies in both chick and mouse suggest that intestinal vasculature is not required for normal intestine colonization by NCCs, and the organization of intestinal vasculature does not correlate with MP structure in the adult mouse ENS. However, emerging evidence suggests that smooth muscle may influence ENS structural development, also supported by our observations of neuron organization in the stomach and esophagus. In chick, the circular muscle differentiates just before the arrival of NCCs, and inhibition of circular muscle contractions changes the anisotropy of the embryonic chick ENS. Both mechanical forces and morphogens influence intestinal smooth muscle orientation during development, and whether these factors affect neuronal stripe development and patterning has yet to be explored. Such mechanistic insights will not only improve our understanding of ENS development in vivo but will also be useful for generating intestinal organoids, examples of which have two ENS plexuses but lack patterning along the longitudinal axis.

Our analysis of enteric neuron subtypes throughout the intestines reveals striking differences in distribution for many neurotransmitters, neuropeptides, and CBPs across intestinal regions and neuronal stripes. Regional differences in neuronal marker distribution have been observed previously, and such differences are perhaps unsurprising, considering the specific functions of the SI, colon, and subregions thereof, and regional differences in extrinsic ENS innervation. Despite this, few studies have specifically investigated regionality, with many focusing on the ileum or pooling entire organs. Only recently have studies begun to examine regional differences, particularly between the PC and DC, where we also see strongly contrasting cytoarchitecture and subtype distributions. The functional relevance of regional expression profiles has been determined for some markers, such as Sst, which is regulated by the microbiome and is more prevalent in areas of increased microbial load. The proportion of VGLUT2-positive cells is also sensitive to the ileal microbiome, but the importance of its increased levels down the length of the colon awaits determination. The functional significance of other distribution profiles, such as high 5-HT expression in the duodenum or peak VIP expression in the distal colon, remains to be elucidated.

Notwithstanding regionality, the proportion of enteric neurons that we observe expressing given markers, such as ChAT, nNOS, calretinin, TH, and Sst, are comparable with prior immunolabeling and RNA sequencing (RNA-seq) studies of enteric cell types. Genetic reporter lines allow for unambiguous counting of marker expression in cases where the native protein is not localized to the soma, including Substance P (Tac1Cre) and enkephalin (PenkCre), without the need for pharmacological agents such as colchicine. This allows clarification for populations found to have different expression levels in distinct studies. For example, reports of the proportion of enkephalin neurons range from 6% to 23%. In PenkCre tdT mice, we found that 21%–30% of neurons were Penk-positive, depending on intestinal region, values supported by RNA-seq studies, in which Penk is found across several subtype clusters. There are also diverging reports on the extent of calbindin expression, a putative sensory neuron marker. Calbindin has been observed in 30% of myenteric neurons in IHC studies while transcriptomic profiling finds more restricted expression. Our analysis found Calb1Cre-tdT in <10% of myenteric neurons in all regions, and as low as 2% in the DC, agreeing with transcriptomic studies. Subsequent studies should investigate regional variation in combinatorial expression or neurochemical coding of neuronal clusters defined in single-cell RNA-seq studies, particularly for markers such as Penk or ChAT that are expressed in many different subtypes.

Our characterization of regional enteric organization and composition can serve as a blueprint to understand how local enteric neurocircuitry underlies region-specific motility patterns and function. Further, this anatomical map and computational approach can help inform our understanding of the pathophysiology of a wide range of diseases. Assessing ENS structure may help identify anatomical changes in human GI diseases with no known correlates within the ENS, such as intestinal pseudo-obstruction. Additionally, neurodegenerative disorders such as Parkinson’s disease and Alzheimer’s disease have identified ENS pathologies, yet the regional differences, spatial organization, and subtype identities of affected enteric neurons are unknown. Comprehensive analyses of CNS structure have served as platforms to dissect basic physiology and disease, and our work represents a parallel step toward an intricate understanding of the ENS.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:
SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2022.08.030.

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AUTHOR CONTRIBUTIONS

Conceptualization, R.H., L.B.D., and J.A.K.; methodology, R.H., L.B.D., V.S., Z.W., J.G.-F., and S.D.; software, Z.W. and S.D.; analysis, R.H., L.B.D., V.S., Z.W., and S.D.; investigation, R.H., L.B.D., V.S., J.G.-F., and V.D.A.; writing – original draft, R.H., L.B.D., and J.A.K.; writing – review & editing, R.H., L.B.D., J.G.-F., and J.A.K.; supervision, N.K., S.D., and J.A.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Mouse anti-Calbindin, 1:100 | Swant | Cat# CB300; RRID: AB_10000347 |
| Rabbit anti-Calretinin, 1:200 | Millipore | Cat# AB5054; RRID: AB_2068506 |
| Rabbit anti-Caspase3, 1:500 | Abcam | Cat# AB2302; RRID: AB_302962 |
| Rabbit anti-Choline acetyltransferase (ChAT), 1:4000 | Gift from Thomas Jessell/Susan Morton | Cat# CU1574; RRID: AB_2750952 |
| Rat anti-E-cadherin, 1:2000 | ThermoFisher | Cat# 13-1900; RRID: AB_2533005 |
| Rabbit anti-GAD65, 1:50000 | Gift from Thomas Jessell/Susan Morton | Cat# CU1196; RRID: AB_2904512 |
| Sheep anti-GFP, 1:1000 | Biogenesis | Cat# 4745–1051; RRID: AB_619712 |
| Human anti-HuC/D, 1:75000 | Gift from Vanda Lennon | Cat# HuC/D_Lennon; RRID: AB_2813895 |
| Rabbit anti-met-Enkephalin, 1:1000 | Immunostar | Cat# 20065; RRID: AB_572250 |
| Sheep anti-nNOS, 1:1000 | Millipore | Cat# AB1529; RRID: AB_90743 |
| Rabbit anti-nNOS, 1:2000 | Sigma-Aldrich | Cat# N7280; RRID: AB_260796 |
| Chicken anti-Secretagogin, 1:3000 | EnCor Bio | Cat# CPCA-SCGN; RRID: AB_2744521 |
| Goat anti-Serotonin (5-HT), 1:2000 | Immunostar | Cat# 20079; RRID: AB_572262 |
| Rat anti-Somatostatin, 1:500 | Millipore | Cat# MAB354; RRID: AB_2255365 |
| Rabbit anti-Substance P, 1:4000 | Immunostar | Cat# 20064; RRID: AB_572266 |
| Rabbit anti-Tyrosine hydroxylase (TH), 1:2000 | Peli-Freez | Cat# P40101-150; RRID: AB_2617184 |
| Mouse anti-Tuj1, 1:2000 | Sigma | Cat# T8578; RRID: AB_1841228 |
| Guinea pig anti-VGLUT2, 1:3000 | Synaptic Systems | Cat# 135 404; RRID: AB_887884 |
| Donkey anti-Chicken Cy3 | Jackson ImmunoResearch | Cat#703-165-155; RRID: AB_2340363 |
| Donkey anti-Chicken AF 647 | Jackson ImmunoResearch | Cat#703-605-155; RRID: AB_2340379 |
| Donkey anti-Guinea pig AF 647 | Jackson ImmunoResearch | Cat#706-605-148; RRID: AB_2340476 |
| Donkey anti-Goat Cy3 | Jackson ImmunoResearch | Cat#705-165-147; RRID: AB_2307351 |
| Donkey anti-Goat AF 647 | Jackson ImmunoResearch | Cat#705-605-003; RRID: AB_2340436 |
| Donkey anti-Human AF 488 | Jackson ImmunoResearch | Cat#709-545-149; RRID: AB_2340566 |
| Donkey anti-Human AF 405 | Jackson ImmunoResearch | Cat#709-475-149; RRID: AB_2340553 |
| Donkey anti-Human AF 647 | Jackson ImmunoResearch | Cat#709-605-149; RRID: AB_2340578 |
| Goat anti-Mouse Cy3 | Jackson ImmunoResearch | Cat#115-165-205; RRID: AB_2338694 |
| Goat anti-Mouse AF 647 | Jackson ImmunoResearch | Cat#115-605-205; RRID: AB_2338916 |
| Donkey anti-Rabbit AF 488 | Invitrogen | Cat#A21206; RRID: AB_2535792 |
| Donkey anti-Rabbit Cy3 | Jackson ImmunoResearch | Cat#711-165-152; RRID: AB_2307443 |
| Donkey anti-Rabbit Cy5 | Jackson ImmunoResearch | Cat#711-175-152; RRID: AB_2340607 |
| Donkey anti-Rat AF 488 | Invitrogen | Cat#A21208; RRID: AB_141709 |
| Donkey anti-Rat Cy3 | Jackson ImmunoResearch | Cat#712-165-153; RRID: AB_2340667 |
| Donkey anti-Sheep AF 488 | Invitrogen | Cat#A11015; RRID: AB_141362 |
| Donkey anti-Sheep Cy5 | Jackson ImmunoResearch | Cat#713-175-147; RRID: AB_2340730 |
| FAb Fragment Donkey Anti-mouse | Jackson ImmunoResearch | Cat#715-007-003 |

### Chemicals, peptides, and recombinant proteins

| Chemicals, peptides, and recombinant proteins | Source | Identifier |
|-----------------------------------------------|--------|------------|
| Sodium Cacodylate Buffer, 0.2M, pH 7.4 | Electron Microscopy Sciences | Cat#11653 |
| Osmium tetroxide, 4% aqueous solution | Electron Microscopy Sciences | Cat#19190 |
| Potassium hexacyanoferrate(II) trihydrate | Sigma-Aldrich | Cat#P3289 |
| Pyrogallol | Sigma-Aldrich | Cat#P0381 |
| 4% Uranyl Acetate Solution | Electron Microscopy Sciences | Cat#22400-4 |

(Continued on next page)
**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Julia A. Kaltschmidt (jukalts@stanford.edu)

**Materials availability**
This study did not generate new unique reagents.

**Data and code availability**
- All data reported in this paper will be shared by the lead contact upon request.
- All original code has been deposited at Zenodo and is publicly available as of the date of publication. DOIs are listed in the key resources table.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Animals**
All procedures conformed to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Stanford University Administrative Panel on Laboratory Animal Care or the University of Chicago Institutional Animal Use and Care Committee. Mice were group housed up to a maximum of five adults per cage. Food and water were provided...
Marker selection
For experiments assessing neuronal subtype distribution, we selected a variety of markers (via either genetically encoded reporters, or antibodies (key resources table)) covering calcium binding proteins, neurotransmitters and neuropeptides, which are known determinants of enteric subtype function or which delineate functionally relevant populations. The markers included in the study were chosen based on several criteria: (i) they have been shown to be relevant to enteric neurobiology, (ii) they cover markers previously used to identify functional neuronal subtypes (e.g. as in Qu et al.), (iii) they delineate a limited number of clusters in recent RNAseq screens (such as VGLUT2 in ENC7 and ENC12, Sst in ENC5, Gad2 in ENC10 from Morarach et al.) and (iv) it was possible to count them unambiguously (i.e. there was strong staining within the soma), either through immunostaining or available Cre recombinase lines, facilitating automated counting.

Calretinin, ChAT, and Tac1 are all known to mark excitatory motor neurons. Penk also marks excitatory motor populations, in addition to some interneurons. Somatostatin has been suggested to delineate a population of interneurons, as does myenteric 5-HT. nNOS, VIP and PACAP have all been suggested to mediate inhibitory neuromuscular transmission. Calbindin is suggested to mark sensory neurons. The functions of VGLUT2 and Gad2 neurons are not known, but were included as they are well-known neurotransmitter markers in the nervous system and had been identified in the ENS by recent scRNAseq studies as belonging to specific subpopulations. The same applies to tyrosine hydroxylase (TH), known to be expressed in intrinsic enteric neurons, though its function remains elusive. Secretagogin, a CBP first identified in the pancreas, was similarly identified recently, and has not yet been described in the ENS.

Immunohistochemistry
For investigations into the structure of the adult ENS, the small intestine and colon were dissected out from 2-6 months-old mice culled by CO2 and cervical dislocation. Intestines were flushed of fecal contents using cold PBS before cutting the proximal, middle and distal 2-3 cm of the small intestine to isolate the duodenum, jejenum and ileum. The colon was cut in two, and all segments were pinned to Sylgard 170 under light tension in cold PBS on ice. The musentery was cut away, then each segment was cut open longitudinally along the mesenteric border. The tissue was then stretched flat and pinned to the Sylgard, muscularis facing upwards, and fixed in 4% PFA for 90 minutes at 4°C with shaking. For adult single-plexus wholemount preparations, the muscularis (both muscle layers and MP) was peeled away once fixed using fine forceps and a cotton bud. For neonatal ages (postnatal days 0, 10, 15, and 21), intestines were cut open along the mesenteric border, pinned mucosa-down on Sylgard 170 in ice-cold PBS, and the muscularis was peeled away with fine forceps. Muscularis tissue was stored in ice-cold PBS and then pinned onto Sylgard 170 in a flat sheet in 35 mm glass dishes using insect pins. Tissue was immediately fixed as described above. Residual PFA was removed via PBS washes before proceeding directly to immunostaining, or tissue was stored in PBS containing 0.1% sodium azide for up to 3 weeks at 4°C.

For swiss roll section collection, the intestines were dissected as above. The SI was divided into duodenum, jejenum and ileum, while the colon was kept intact. A glass rod was placed through each segment and placed on filter paper. A scalpel was used to open each segment, and the glass rod was used to gently flatten the opened tissue so that the mucosa was facing up and the muscularis was flat against the filter paper. The tissue and filter paper were stacked on top of each other and placed in 4% PFA at 4°C for 90 minutes. Following 3 PBS washes, the stack was placed in 30% sucrose overnight for cryopreservation. The following day, each segment was rolled up into a spiral from distal to proximal and embedded in OCT (Tissue-Tek) before being frozen on dry ice and stored at -80°C. To section, embedded tissue was allowed to equilibrate to -16°C in a cryostat (Leica CM3050 S), and 16 μm sections were collected on slides such that a single section contained the full length of a tissue segment.

For embryonic experiments, pregnant mice were culled by CO2 and cervical dislocation. The uterus was removed, and embryos were placed in ice-cold PBS. The intestines were dissected from each embryo, and the mesentry was carefully removed. To measure intestinal length, the cleaned intestine was laid adjacent to a ruler. In a Sylgard 170 plate, a pin was placed in the stomach and anus to keep the intestines taut and straight. Tissue was fixed in 4% PFA for 90 minutes at 4°C with shaking.

For immunohistochemistry, large intact pieces of tissue were cut into smaller pieces (size dependent on the experiment and age, but typically ~7x7 mm2) and transferred to WHO microtitration trays (International Scientific Supplies) containing PBS. Tissue was then put into PBT (PBS, 1% BSA, 0.1% Triton X-100) containing the primary antibodies overnight at 4°C with shaking. For mouse antibodies, tissue was pre-treated first with Affinipure FAb Fragment Donkey Anti-mouse (Jackson 715-007-003) diluted 1:50 in PBT, and then in 5% normal goat serum in PBT, for 2 h each prior to primary antibody incubation. The following day, tissue was washed 3 times in PBT for 30 minutes each before transferring to PBT containing secondary antibodies for 2 h at room temperature.
with shaking. Tissue was washed twice in PBT and twice in PBS, then mounted onto slides using a paintbrush. Any folds or bumps in mounted tissue were removed by gentle manipulation with paint brushes under a dissection microscope. The tissue was allowed to partly air-dry, then was rinsed in ddH₂O and coverslipped using Fluoromount-G (Southern Biotech).

For embryonic experiments, immunohistochemistry was done as in adults with the following changes. Intact intestines were placed in WHO microtiritation trays and treated as above. After immunohistochemistry, fixed intestines were cannulated with a cleaning wire for 33-gauge needles (Hamilton) and cut along the wire. Embryonic intestines were mounted full-thickness.

For swiss roll immunohistochemistry, sections on slides were stained as in adult wholemount tissue but staining was performed in a stationary incubation chamber to prevent drying out.

X-ray tomography
Mice were deeply anesthetized using pentobarbital (60 mg/kg intraperitoneal) to be non-responsive to toe pinch and transcardially perfused with 10 ml of 0.1 M sodium cacodylate buffer (Electron Microscopy Sciences (EMS)) to flush the vasculature followed by buffered fixative made of 2.5% glutaraldehyde (EMS), 2% paraformaldehyde (EMS) in 0.1 M sodium cacodylate buffer (EMS). The intestines were removed, and the mesentery was gently severed. The intestines were divided into segments (duodenum, jejunum, ileum, proximal and distal colon). Ileum and proximal colon were dissected out and gently flushed with 0.1 M sodium cacodylate buffer (EMS). The dissected segments were post-fixed overnight at 4°C in the fixative described above. After post-fixation, the intestines were cut open along the mesentery and dissected into smaller pieces before staining with heavy metals as described by Hua et al. Briefly, the tissues were extensively washed in 0.1 M sodium cacodylate buffer (EMS). This was followed by sequential staining with 2% buffered osmium tetroxide (EMS), 2.5% potassium ferrocyanide (Sigma-Aldrich) with no rinse in between followed by pyrogallol (Sigma-Aldrich), unbuffered 2% osmium tetroxide (EMS), 1% uranyl acetate (EMS), and 0.66% aspartic acid buffered lead (II) nitrate (Sigma-Aldrich) with extensive rinses in between each of the steps. The stained tissues were dehydrated in graded ethanol, propylene oxide and infiltrated with epon resin (EMS). The tissues were finally embedded in fresh epon and cured in an oven at 60°C for 48 h.

The epon embedded samples were imaged at 32-ID beamline at the Advanced Photon Source (APS) in Argonne National Laboratory. The sample was placed on a rotation stage and projection images were acquired at 600 nm/pixel resolution as the sample was rotated over 180 degrees. The acquired images were 3D-reconstructed using the TomoPy toolbox.

Image acquisition
Images were acquired using a 20x (NA 0.75) oil objective on a Leica SP8 confocal microscope. Regions to be imaged were identified, acquired and stitched using the Navigator mode within LASX (Leica). A z-stack of the entire region was taken, ensuring that the full depth of the area of interest (e.g. the myenteric plexus) was captured in all tiles. Z-stacks were acquired with 3 μm between each focal plane for adult tissue and with 2 μm between each focal plane for embryonic and neonatal tissue.

QUANTIFICATION AND STATISTICAL ANALYSIS

X-ray tomography analysis
3D-reconstructed data were manually annotated using Knossos, an open-source software (https://knossos.app/). We identified the myenteric ganglia in between the circular and longitudinal muscle layers and the submucosal ganglia in the submucosal space. All cell bodies were identified based on the circular outline and the presence of a nucleus within the cell.

Image analysis
Cell counting
Image analysis was primarily performed using ImageJ/FIJI (NIH, Bethesda, MD). For neuronal density analysis, HuC/D images (maximum intensity projections for adults, single plane for embryonic/neonatal) were blurred before thresholding and watersheding. Cells were counted using the Analyze Particles function, and density was calculated based on area of tissue measured in FIJI. To count neuronal subtypes, the Image Calculator function was used to combine thresholded HuC/D stacks (prior to maximum projection) with raw image stacks of a defined cell type. The result of this calculation was then maximally projected and counted in an automated fashion as described above. The exceptions to this were 5-HT and TH, which were counted manually after being combined with the thresholded HuC/D image stack, and were identified by their distinctive cytoplasmic expression pattern. To quantify apoptotic neurons in the embryonic/neonatal intestines, the Image Calculator function was used to combine thresholded HuC/D maximum projections with raw maximum projections of Caspase-3 labeling. The result was then counted as above.

Conditional intensity function (CIF) analysis
To visualize and evaluate spatial patterns, we calculated the conditional intensity function (CIF), which generates a spatial density map of neuron locations relative to a given neuron. Square images measuring ~1800x1800 μm for adult and ~400x400 μm for embryonic and neonatal tissue, respectively, were processed in FIJI as described above, and the XY coordinates of each neuron were obtained using Analyze Particles. We empirically estimated the CIF for a given sample by iterating over all neurons and calculating the number of neurons in a 2D grid around that neuron. Total image area was normalized to 1, divided into 100 bins per unit length. The 2D grid’s width and height were both 0.7 for adult data, while they were 0.8 and 0.5, respectively, for embryonic/neonatal data. Density
values were normalized to expected density based on a uniform distribution of neurons, given a value of 1. We excluded the center grid point from the resulting CIF plot, which included data from the neuron used for conditioning.

We then transformed the 2D grid into a one-dimensional line by averaging along the y-axis, using either the full y-axis (for interstripe distance calculations) or a smaller proportion (for stripe width calculations). For adult data, this smaller proportion was a length of 0.1 relative image length above and below the center, and for developmental data, we used a value of 0.2. For developmental data, which contained far fewer neurons, we smoothed with a Gaussian of 20 µm standard deviation. We then identified the first minima and first peak next to the center. Stripe width was taken as the width at the half height from minimum to center peak. Interstripe distance was taken as the distance between the left and center peaks. For interstripe distance analysis, samples in which secondary stripes could not be unambiguously identified were not included. All analyses were implemented in Python.

**Nearest-neighbor and empirical distribution function (EDF) analysis**

To assess enteric neuron organization across development, we tested each of the samples for deviation from a hypothesis of random neuron positions, known as complete spatial randomness. Our analysis was based on nearest-neighbor distances. The distribution of nearest-neighbor distances under complete spatial randomness can be calculated. Specifically, the mean of the distribution and its variance can be approximated by a normal distribution. Embryonic and neonatal images were processed as above to generate XY coordinates, and then for each sample we calculated the mean of the data and extracted a z-score for the deviation of the data from the expected value. In addition, to better visualize deviations, we generated synthetic samples under the assumption of complete spatial randomness. We then plotted the empirical distribution function for the data as well as an envelope defined by the maximum and minimum of the empirical distribution function over 500 synthetic samples.

We observed that some deviation from randomness was driven mostly by the fact that the synthetically generated samples exhibited overlap in space while our data, taken from a single-plane image, did not overlap. To make the analysis more robust to this property, we generated synthetic samples with a minimum distance imposed. We chose this minimum distance as 10 µm, approximately the average diameter of an enteric neuron across all ages examined. We then generated 500 random samples and calculated their z-score and defined a metric that is the difference between the z-score of the data and the mean of the z-score of the synthetic samples. This allowed better identification of deviations from other spatial properties (e.g., stripes). All analyses were implemented in Python.

**Longitudinal axis signal profile analysis**

Profiles of HuC/D and neuronal subtype signal intensity along the longitudinal tissue axis were generated in FIJI using the Plot Profile function, once the image had been adequately aligned such that the longitudinal axis of the tissue matched the x-axis. These profiles were then exported to Prism 9 (GraphPad) for correlation analysis (see general statistical analysis). After analysis, profiles were smoothed in Prism 9 by averaging 12.5 µm either side of a given point to aid in visualizing peaks in intensity. To calculate the number of HuC/D neurons and neurons of a given subtype present in stripes within an image, smoothed profiles were first exported to Microsoft Excel and peaks in the data were automatically identified. The locations of these peaks were then exported to FIJI, which created a box measuring the full y-axis and 50 µm either side of each peak location. This box would constitute a stripe, and Analyze Particles was used on thresholded images (as above) to count the number of objects within each stripe. This data was converted to frequency distributions in Prism 9.

**General statistical analysis**

Statistical tests and graphical representation of data were performed using Prism 9 software (GraphPad). Statistical comparisons were performed using one-way ANOVA to assess if age or intestinal region were significant factors for a variety of measurements (neuronal density, intestine length, etc.); repeated measures one-way ANOVAs were used for intestinal region. Mixed effects models were used in cases of missing data. Tukey’s or Dunnett’s correction for multiple comparisons were used where appropriate to determine significant differences (p < 0.05) between individual regions or ages. The results of these corrections are indicated on graphs as letters to indicate significant differences. Two-way ANOVAs were used to determine the effect of sex on neuronal cytoarchitecture, followed by Sidák’s multiple comparisons tests. Correlation was determined using Pearson’s correlation coefficient. The Kolmogorov-Smirnov (KS) test was used to compare the frequency distributions of neuron subtypes as a proportion of neurons in neuronal stripes. Full details of these statistical tests can be found in Table S1.