

Enteric glial hub cells coordinate intestinal motility

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Abstract

Enteric glia are the predominant cell type in the enteric nervous system yet their identities and roles in gastrointestinal function are not well classified. Using our optimized single nucleus RNA-sequencing method, we identified distinct molecular classes of enteric glia and defined their morphological and spatial diversity. Our findings revealed a functionally specialized biosensor subtype of enteric glia that we call “hub cells.” Deletion of the mechanosensory ion channel PIEZO2 from adult enteric glial hub cells, but not other subtypes of enteric glia, led to defects in intestinal motility and gastric emptying in mice. These results provide insight into the multifaceted functions of different enteric glial cell subtypes in gut health and emphasize that therapies targeting enteric glia could advance the treatment of gastrointestinal diseases.

Main Text

The enteric nervous system (ENS) is a poorly characterized tissue in the gut often referred to as the 'second brain'. The ENS is a complex network of neurons and glia spanning the entire length of the gastrointestinal tract from the esophagus to the rectum. The ENS develops from neural crest cells that invade the gut wall and migrate inward to populate every layer of the tissue (**Fig. 1A**). In many other organs, single cell technologies including single nucleus RNA-sequencing (snRNA-seq) have increased our understanding of cellular diversity in complex tissues and enabled the identification of specialized cell types with unique roles in tissue function (1-8). However, efforts to understand the cellular diversity of the ENS have thus far focused on early development or neuronal diversity (9-12), and given technical challenges, have fallen short of achieving comprehensive transcriptional analysis of the adult whole duodenum at the single cell level.

Enteric glia are a major constituent of the digestive system, vastly outnumbering neurons, and they intermingle with neurons, smooth muscle cells, enterocytes, Paneth cells, goblet cells, and neuroendocrine cells. The large population size and close associations with different cell types position enteric glia as key players in a wide range of digestive functions. Whether enteric glia are functionally analogous to other glial cells in the body is contested, and their diversity and physiological roles are just beginning to be explored. A comprehensive classification of enteric glial identity and diversity is vital to delineate how glial cells impact intestinal health and physiology.

A global atlas of the adult mouse duodenum

The proximal region of the small intestine, the duodenum, sits at the epicenter of digestion. The stomach, gallbladder, and pancreas dump a deluge of chyme, bile, and enzymes into the lumen of the duodenum to regulate gastric emptying and promote the breakdown of food. The physiology inherent to the adult mouse duodenum has prevented global transcriptional analysis at the single cell level due to an abundance of RNases, ions, and variable pH. Here, we successfully overcome these obstacles with an optimized iodixanol gradient and citric acid-based method we call *CitraPrep* that enabled us to obtain high quality nuclear RNA from single cells in the adult mouse duodenum (**Fig. S1A-H**). Using *CitraPrep*, we profiled the transcriptomes of 57,033 nuclei and generated high quality, low contamination molecular blueprints of cell types in the proximal small intestine (**Figs. 1B-D, S2A-E**). We found differential expression of cell type specific genes across unsupervised clusters that spanned all major intestinal cell types including the ENS (**Fig. 1E, Table S1**) (13-16).

Enteric glial cells are transcriptionally unique from other glia in the body

To determine whether enteric glia are analogous to any of the glial cell types in the central or peripheral nervous system, we performed snRNA-seq on mouse duodenal and cortical tissues and analyzed publicly available snRNA-seq data from all three nervous systems (**Fig. 1F**) (17-21). When we computationally separated ectodermal-derived glial cell types from all other cells, we identified 7 glial cell types across 9 tissue regions, extending throughout the body from the cortex (head) to the sural nerve (feet) (**Figs. S3A-C, S4A-D**). After merging central and peripheral nervous system glial cells with enteric glia and controlling for batch effects, we identified 19 transcriptionally unique clusters through unsupervised clustering (**Figs. 1F, S4E**). Importantly, clusters were not defined by sample or tissue origin, and nuclei from the same cell types but originating from separate studies overlapped in identity (**Figs. 1F, S4A-D**). As expected, expression of many individual transcripts was conserved in glia across the three nervous systems. Strikingly however, enteric glia clustered mostly apart while closely related glial cell types like ependymal cells and tanycytes in the ventromedial hypothalamus mostly populated the same clusters (**Fig. 1G-H**). We noticed that enteric glia express many known glial cell genes, but in unusual combinations that conferred an identity distinct from other glia in the body. Analysis of enriched transcripts between cell types showed genetic programs related to known functions in defined glial cell types (**Fig. S4F-G**). Enteric glia were enriched

for processes associated not only with known enteric glial functions such as maintenance of intestinal epithelium (22), purine metabolism (23), and smooth muscle contraction (24-30), but also with previously undescribed functions such as lipid absorption, cholesterol metabolism (31, 32), and regulation of hormone levels. We further identified and validated highly enriched, differentially expressed glial cell type specific markers (**Fig. S4H-I, Table S2**). Interestingly, enteric glia express genes that are also expressed in the brain and have been linked to neurological diseases and disorders that harbor gut comorbidities of undefined origin (33). This includes Parkinson's disease (*SCNA*, *LRRK2*, and *PRKN*), amyotrophic lateral sclerosis (*C9ORF72*, *SOD1*, *TARDBP*, and *FUS*), leukodystrophies (*GFAP* and *PLP1*), schizophrenia (*TRIO*, *RB1CC1*, *XPO7*, *CUL1*, *HERC1*, and *GRIA3*) and autism spectrum disorder (including but not limited to *CHD2*, *SETBP1*, *KCNQ3*, *SOX5*, *DYRK1A*, and *TCF4*, high confidence SFARI score 1/1S) (34-37). Our data support that enteric glia are not a peripheral equivalent of astrocytes or other known glial cell types in the body but instead are a molecularly distinct glial cell type.

Beyond their distinction from central nervous system glia, there is evidence of further diversity within enteric glia. For example, enteric glia elicit calcium responses to different stimuli specific to the muscle layer of the duodenum when compared to the colon (38). Hence, we next sought to understand if enteric glial gene signatures diverge between the duodenum and the colon. We merged our data sets containing enteric glial cells from the whole duodenum with two sources: enteric glial cells from sorted ENS cells from the myenteric plexus of the colon and from mechanically isolated colonic mucosa (**Fig. S5A-E**) (9, 39). Indeed, we found that the majority of cells were separated by regional identity, and that the overall molecular profiles between duodenal and colonic enteric glia were distinct (**Fig. S5F-L, Table S3**).

Decoding the molecular, morphological, and spatial diversity of enteric glia

Enteric glia outnumber neurons in the mammalian intestine but their subtypes are not well defined (40). Upon selection and re-clustering of ENS cells, we identified 7 molecularly distinct enteric glia subpopulations (**Figs. 2A-B, S6A**). Our analysis of these clusters revealed molecular blueprints of enteric glia that were previously not described. We found a cluster of enteric glia with enriched expression of the PPAR nuclear hormone receptors, which are key regulators of lipid metabolism and inflammation (**Fig. S6B**). A single cluster had enriched expression of *Sox6*, *Nrp1*, *Vim*, *Nfia*, and other genes indicative of a less differentiated state while concurrently expressing transcripts of differentiated glial cells like *Gfap*. This transcriptionally poised state resembles that of pancreatic hub cells, a subpopulation of beta cells that transcriptionally display features of both mature and immature cells and act as pacemaker cells to synchronize release of insulin upon glucose stimulation (41). We named these cells enteric glial "hub cells". Enteric glia are known to secrete WNT ligands like WNT6 to promote regeneration of damaged intestinal epithelium (22). Consistently, we found high expression of *Wnt6* specifically in EGC6, while *Wnt4* and *Wnt5a* were highly expressed and specifically enriched in enteric glial hub cells (**Fig. S6B**).

We visualized the entire enteric glia population through labeling the ENS and immunostaining of *Sox10*-cre; RCL-tdTomato mouse tissue, where tdTomato marks all neural crest derived cells. When we then performed immunostaining using markers of non-canonical enteric glial cell subpopulations identified through snRNAseq, we were able to discern the broader heterogeneity of enteric glial cells (**Fig. S6C-D**). These data provide a foundational overview of enteric glia populations in the adult gut and resolve the molecular profiles of enteric glial subtypes (**Fig. S6E, Table S4**).

Four morphologically diverse enteric glial cells have been described (42, 43), but it is not known whether these identities correspond to specific molecular subtypes. Here, we designed an *in vitro* cell culture system optimized to grow primary enteric glial cells at scale. With this platform, we demonstrated the presence of the four previously defined morphological enteric glia subtypes and identified two additional subpopulations we call 'triad' and 'bipolar' in respect to cell shape (**Figs. 2C, S6F**). We then

assessed the morphology of the molecularly defined enteric glial subpopulations and quantified the proportion of cells with different morphological identities in culture by immunostaining. Focusing on enteric glial hub cells, we found a striking enrichment of high NFIA expression, an enteric glial hub cell enriched marker, with type 1 intraganglionic morphology (**Fig. 2D-F**). We next conducted immunostaining of *Sox10*-cre; RCL-tdTomato mouse tissue for markers of different enteric glia subtypes and detected widespread compartmentalization of enteric glial cells with some enriched in the submucosa, others in the mucosa, and a significant enrichment of glia expressing enteric glial hub cell markers in the muscle layer (**Figs. 2G-H, S6G**). This finding places enteric glial hub cells in a location where they have the opportunity to impact peristalsis by interacting with neighboring neurons and muscle cells. In summary, we show that the molecular profiles of enteric glial cells diverge in different spatial compartments. This transcriptional compartmentalization suggests that enteric glial cells in different layers of the intestine are functionally specialized.

Enteric glial hub cells are predicted to interact with enteric neurons

Equipped with 57,033 high-quality single nuclear transcriptomes from the entire adult duodenum, we mapped the connections between enteric glia and surrounding cells. Using receptor-ligand interactome analysis, we outlined predicted cell-cell interaction networks from each enteric glial subpopulation (44-46) (**Fig. S7A, Table S5**). This analysis predicted that enteric glial hub cells interact with interstitial cells of Cajal (ICCs), telocytes, and enteric neurons (**Figs. 3A-B, S7B-D**). As pancreatic hub cells act as specialized pacemaker cells to respond to glucose fluctuations in their environment and orchestrate islet oscillations (41), we hypothesized that enteric glial hub cells respond to signals to orchestrate function of cells in the muscle layer. To determine how enteric glial hub cells become activated to potentially influence these specific cell types, we assessed expression of genes ontologically associated with monoatomic ion channel activity and filtered for the most highly significant subtype-enriched transcripts (**Fig. S7E**). We noted significant expression of the mechanosensitive ion channel *Piezo2* in enteric glial hub cells (**Fig. 3C**). From these data, and given the spatial location of enteric glial hub cells, we hypothesized that enteric glial hub cells can influence the function of enteric neurons to regulate intestinal contractions and gut physiology through PIEZO2.

Enteric glial hub cells act as biomechanical sensors to regulate intestinal motility

Humans with *PIEZO2* loss-of-function mutations report a wide range of bowel dysfunctions including constipation and hardened stools (47, 48). It is known that PIEZO2 channels have a slight preference for Ca^{2+} ions (47); interestingly, activated enteric glia elicit dynamic Ca^{2+} responses (49-51). Genetic disruption of Ca^{2+} transients in *Gfap*⁺ enteric glia also leads to impaired gut motility (25), while forced influx of Ca^{2+} in *Gfap*⁺ enteric glia promotes gut motility (26). With this in mind, we set out to determine whether enteric glial hub cells directly sense force through the PIEZO2- Ca^{2+} axis to regulate gut motility.

We first confirmed transcript and protein expression of PIEZO2 in myenteric glial cells by its co-expression with SOX10 (marking all adult myenteric glia) and GFAP (marking a subset of myenteric glia including enteric glial hub cells) in both mouse and human tissue (**Fig. S8A-I**). Every PIEZO2⁺ enteric glial cell that we observed co-expressed the enteric glia marker GFAP (**Fig. S8A**), and *Piezo2* transcript itself was highly enriched in enteric glial hub cells (**Figs. 2D, S8B**); thus, targeting PIEZO2 via *Sox10* (all myenteric glia) or *Gfap* (all PIEZO2⁺ glia) would selectively disrupt PIEZO2 in enteric glial hub cells.

We next wanted to determine if enteric glia were competent to respond to mechanical force. We engineered a microfluidic biochip to mechanically stimulate tissue while simultaneously performing high-resolution calcium imaging (**Figs. 4A, S8J**). By selectively inducing expression of the genetically encoded calcium-sensitive indicator GCaMP6f in adult *Sox10*-cre^{ERT2} mice, we specifically recorded enteric glia activity in our intestine-on-a-chip platform. We generated *ex vivo* preparations of longitudinal muscle with

the myenteric plexus by microdissection and visualized changes in enteric glia activity via GCaMP6f before and after 1 dyn/cm² shear stimulation (52). As expected, we observed differential calcium responses across enteric glia (38), with some cells spontaneously active without any stimuli. We also identified a subset of myenteric glial cells that were mechanosensitive, responding only after stimulation with shear stress. In the majority of mechanoresponsive enteric glia, activation was reversed upon treatment with D-GsMTx4, which inhibits PIEZO2 (**Fig. 4B, Movie S1**). Immunostaining of these mechanosensory cells post-hoc showed that they expressed PIEZO2 and GFAP (**Fig. S8K**). These results support that a specialized mechanosensory subpopulation of enteric glia exists in the muscle layer of the small intestine.

To determine whether PIEZO2 conferred functional specialization to mechanically sensitive enteric glial hub cells, we crossed *Piezo2*^{fl/fl} mice to the *Sox10*-cre^{ERT2} driver line. Treatment of mice with tamoxifen at three weeks of age allowed us to circumvent perinatal lethality of *Piezo2* loss (53) and, importantly, enabled us to selectively target myenteric glial cells in the gut as they are the only cells in the adult gastrointestinal tract that express *Sox10* (54). Three weeks after tamoxifen administration, we confirmed *Piezo2* deletion by immunostaining, which showed loss of PIEZO2 from SOX10+ enteric glia in the adult mouse duodenum (**Fig. 4C**).

To understand how PIEZO2 loss in enteric glia affects gastrointestinal functions, we gavaged mice with a non-absorbable dye and measured the length of time for the colored fecal pellet to appear (**Fig. 4D**). In mice lacking PIEZO2 in all myenteric glia in the gut, we observed a significant delay in overall transit time compared to co-housed, littermate controls (**Fig. 4E**), along with a decrease in fecal water content likely due to slower gut movement (**Fig. S9A**). PIEZO2 deficient mice did not exhibit any differences in epithelial permeability, weight, or intestinal length compared to control littermates (**Fig. S9B-E**). To identify the gastrointestinal segment where luminal transit was delayed, we gavaged mice with a non-absorbable fluorescent dye and collected the luminal contents from equally sized segments of the gut after 30 minutes (**Fig. 4F, S9F**). We found a delay in transit early in the gastrointestinal tract of *Piezo2*^{fl/fl}; *Sox10*-cre^{ERT2} mice compared to littermate controls; most notably we observed a significant defect in gastric emptying demonstrated by more dye sequestered in the stomach (**Fig. 4G-H**). The defects observed after loss of PIEZO2 from enteric glia resemble a condition where gastric emptying is delayed in individuals called gastroparesis, a common comorbidity in neurological diseases (55, 56).

Harnessing our intestine-on-a-chip platform, we performed *ex vivo* contractility assays to assess neuron and muscle activity in tissue after loss of PIEZO2. In response to shear stress, we observed a regular oscillating contractile pattern that was completely abolished upon treatment with the voltage-dependent L-type calcium channel inhibitor nifedipine (**Figs. 4I-J, S9G-H**). In contrast, while *Piezo2*^{fl/fl}; *Sox10*-cre^{ERT2} tissue had comparable amplitude or strength of contractions, these tissues exhibited arrhythmicity compared to littermate controls (**Fig. 4I-J**).

The wave-like, rhythmic movements of the intestine called peristalsis are at least partially controlled through release of acetylcholine and nitric oxide by enteric neurons. Acetylcholine promotes contraction while nitric oxide induces relaxation of muscle; this relay of signaling must be tightly controlled for regulated, unidirectional movement of contents through the gastrointestinal tract. As enteric glial hub cells are predicted to interact with enteric neurons (**Fig. 3**), and loss of *Piezo2* from enteric glia leads to defects in peristalsis, we analyzed the levels of acetylcholine and nitric oxide in the muscle layer of the small intestine in *Piezo2*^{fl/fl}; *Sox10*-cre^{ERT2}. Indeed, neurotransmission in the myenteric plexus was disrupted, with a decrease in levels of nitric oxide and elevated levels of acetylcholine (**Fig. S9I-J**). Together, these data show that enteric glia regulate gut physiology by fine-tuning neurotransmission to control the oscillatory contractions in muscle tissue.

Expression of *Piezo2* is highly enriched in enteric glial hub cells while *Sox10* drives PIEZO2 deletion in all adult myenteric glia. While many enteric glial cells co-express glial markers SOX10, GFAP, and PLP1, subsets of enteric glia differentially expressed these markers (22, 43, 57). Our analyses show

that all PIEZO2⁺ enteric glia co-express GFAP (**Fig. S8A**). Computational selection of *Plp1*⁺ and *Gfap*⁺ cells revealed at the transcriptional level higher expression of *Piezo2* in cells co-expressing *Gfap* compared to *Plp1* (**Fig. 4K**). With this in mind, we used genetic drivers *Plp1*-cre^{ERT2} and *Gfap*-cre^{ERT2} crossed with *Piezo2*^{fl/fl} to specifically delete *Piezo2* in subsets of adult enteric glial cells. In mice with loss of PIEZO2 from *Gfap*⁺ cells, we detected a significant delay in transit time, and significant retention of dye in the stomach, phenocopying the global myenteric glia knockout of PIEZO2 (**Fig. 4L-M**). In contrast, *Plp1*-driven loss of PIEZO2 had no phenotypic difference from littermate controls (**Fig. 4N-O**). These data show that enteric glial hub cells are a functionally specialized subtype of enteric glia that express PIEZO2 and fine-tune intestinal physiology.

Discussion

Here, we report the existence of previously unknown subtypes of enteric glia including a molecularly and functionally distinct enteric glial cell subtype that we call enteric glial hub cells. Enteric glial hub cells reside in the muscle layer and directly sense force through PIEZO2 to regulate intestinal physiology, demonstrating that these cells may act as a center for coordinating intestinal motility.

Our data show that neurotransmission is dysregulated after loss of PIEZO2 in myenteric glia, with elevated acetylcholine and decreased nitric oxide. The regulated release of acetylcholine upstream and nitric oxide downstream of a bolus is essential for matter to move through the gastrointestinal tract in a coordinated, unidirectional manner. Accordingly, this process hinges on multiple cell types that initiate a relay of signals that cascade across different specialized cell types to tightly regulate constriction and opening of tissue. Our data suggests that enteric glial hub cells specifically modulate contraction and relaxation to synchronize peristalsis through the direct sensation of force via PIEZO2. Enteric glia have been shown to release ATP after calcium influx (23, 25, 58, 59); it will be interesting to determine what signals are changing downstream of PIEZO2 activation in enteric glial hub cells to impact enteric neurons and fine-tune peristalsis in the gut.

We demonstrated that loss of PIEZO2 in myenteric glia leads to disruptions in gastrointestinal physiology. Interestingly, gut motility, along with the number of responsive enteric glial cells and their subsequent calcium influx, is reduced with age. Here we see that loss of PIEZO2 in 2 month old mice results in gut motility most similar to aged, 12 month old mice, suggesting that PIEZO2-expressing enteric glial hub cells may be responsible for maintaining gut motility throughout aging (25). Whether enteric glial hub cell proportions or function change over time could provide insight into the functional decline of the aged gut.

We found that analogous to glia in the brain, enteric glia in the gut are diverse, suggesting that even beyond enteric glial hub cells, distinct subsets of enteric glia may perform defined functions in a manner that parallels the varied roles of glia in the brain. Although derived from neural crest cells, enteric glia express many markers of CNS glia and may respond to pathological cues such as genetic mutations or inflammation in a similar manner. Indeed, gut disturbances are unusually common in individuals with conditions ranging from multiple sclerosis, schizophrenia, Parkinson's disease, and autism (36, 60, 61). In fact, individuals with Parkinson's disease commonly develop gastroparesis (56), a defect in gastric emptying, which we show here is regulated by enteric glial hub cells. In spite of these overlaps, enteric glia express a unique combination of genes and develop from a different lineage, setting them apart from any other cell type in the body.

Single cell transcriptional profiling provides the opportunity to generate informed hypotheses about cell type diversity, proportion, interaction networks, and functionality across numerous tissues, organs, and species. Given major technical limitations preventing profiling of whole tissue, this type of analysis has remained outstanding in enteric glial biology. Previous work, while elegant and informative, was restricted to mechanical dissection and/or sorting-based methods for enteric glial molecular profiling. In this work,

we developed a method that overcomes major technical challenges, permitting us to successfully generate high quality nuclear transcriptomes from the whole adult duodenum. Through analysis of our datasets, we were able to predict subtype-specific functions across all enteric glia subpopulations defined, including proof-of-concept of functional specialization in enteric glial hub cells. While we show one function of enteric glial hub cells (mechanotransduction), these cells likely perform multiple functions that have yet to be discovered. Enteric glia are highly plastic; thus while we define different enteric glial subpopulations, these cellular subsets may represent either fluid, modular, shifting states of enteric glial cells or static identities. Lineage analyses will be essential to determine the modularity of enteric glia subpopulations. Overall, these data lay the foundation for future research on enteric glial cell function in health and disease.

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 Biochip generation: WJW
 Intestine-on-a-chip experiments and quantifications: MAS, WJW, and YMH
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Supplementary Materials

Figs. S1 to S9
 Tables S1 to S5
 Movie S1

Materials and Methods

Animals

Animal studies were approved by the Case Western Reserve University School of Medicine Institutional Animal Care and Use Committee. Mice were housed at 22–24°C with a 12 hour light/12 hour dark cycle with standard chow (Lab Diet Pico Lab 5V5R, 14.7% calories from fat, 63.3% calories from carbohydrate, 22.0% calories from protein) and water provided ad libitum unless otherwise indicated. The generation of *Plp1*-eGFP(1), *Plp1*-cre^{ERT2}(2), *GFAP*-cre^{ERT2}(3), *Sox10*-cre^{ERT2}(4), *Sox10*-cre(5), Ai14(RCL-tdT)(6), Ai95(RCL-GCaMP6f)-D(7), *Piezo2*-eGFP-IRES-cre(8), *Rosa26iDTR*(9), and floxed *Piezo2* (*Piezo2*^{loxP/loxP})(8) mice have been described previously. All mice were of C57Bl/6 background. With the exception of *Plp1*-eGFP mice obtained from Dr. Wendy Macklin, all other strains were obtained from The Jackson Laboratory. Animals were bred for mixed litters to compare phenotypes between littermates co-housed in the same cage. For all analyses, the date of birth was assigned as postnatal day 0. All genotyping was performed by Transnetyx.

Administration of tamoxifen

4-hydroxytamoxifen was dissolved in ethanol (100 mg/mL) at 65 °C for approximately 10 minutes with intermittent vortexing. Once the 4-hydroxytamoxifen solution was clear, corn oil was added (final concentration 20 mg/mL) and solution was either incubated overnight at 37 °C shaking or for approximately 15 minutes at 65 °C with intermittent vortexing to promote reconstitution. Solution was stored for up to 2 weeks at 4 °C and restricted to three cycles of heating to prevent loss of 4-hydroxytamoxifen activity. 4-hydroxytamoxifen was delivered to *Piezo2*^{loxP/loxP}, *Plp1*-cre^{ERT2}, *Piezo2*^{loxP/loxP}, *GFAP*-cre^{ERT2}, and *Piezo2*^{loxP/loxP}, *Sox10*-cre^{ERT2} mice on postnatal day 21 (3 weeks) at 75 mg/kg by intraperitoneal injection once daily for 5 days and collected 3 weeks after first injection for analysis. *Rosa26iDTR*; *Plp1*-cre^{ERT2}, *Plp1*-eGFP and *Rosa26iDTR*; *GFAP*-cre^{ERT2} mice were injected between 2 and 4 months at 75 mg/kg by intraperitoneal injection once daily for 5 days. All control mice were littermates, co-housed, and injected with the same dosage and regimen of 4-hydroxytamoxifen.

Diphtheria toxin (DTX) mediated ablation

After injection with 4-hydroxytamoxifen to induce recombination and a 3 week recovery period, *Rosa26iDTR*; *Plp1*-creERT2; *Plp1*-eGFP and *Rosa26iDTR*; *GFAP*-creERT2 mice were treated with intraperitoneal injections of DTX (50 ug/kg diluted in 0.9% saline) once daily for 5 days. Animals were tested in physiological assays and immunostaining 5 days after completion of DTX injections. Tissues were collected and analyzed by immunostaining for loss of eGFP or GFAP. All control mice were littermates, co-housed, and injected with the same dosage and regimen of DTX.

Data resources

The accession number for the raw data reported in this manuscript is GSE232703. The following publicly available datasets were used in this manuscript: GSE103892 for spinal cord, GSE172204 for ventromedial hypothalamus, GSE182098 for vagal, sural, sciatic, and peroneal nerves, GSE175421 for sensory and sympathetic ganglia, GSE132044 for cortex, GSE142431 for colon mucosa, and SCP1038 in the single cell portal at the Broad Institute for colon myenteric plexus.

Nuclei isolation protocol for brain tissue

Mouse cortex was removed from the brain of a PBS perfused animal prior to snap freezing and storage at -80°C until nuclei isolation. Glass dounce homogenizers were placed on ice (Wheaton Science Products, 357542). Frozen tissue was placed into a petri dish on ice and cut into 0.2cm cubed pieces or smaller using clean scissors and forceps in 1mL of HB (0.25M sucrose, 25mM KCl, 5mM MgCl₂, 20mM Tricine-KOH pH 7.8, 0.1U/uL RNasin (Promega, N2615), 0.5U/uL SUPERase RNase inhibitor (Thermo Fisher, AM2696), 1mM DTT (Sigma, D0632), 0.15mM spermine tetrahydrochloride (Sigma, S1141), 0.5mM spermidine trihydrochloride (Sigma, S2501), and cOmplete, EDTA-free protease inhibitor (Sigma,

11836170001). Chopped tissue (totaling less than 1.5cm cubed) was transferred to the glass dounce homogenizer on ice and 4mL of HB was added. Tissue was homogenized 20x with loose pestle before adding 320uL of NP-40 and homogenizing 40x with tight pestle. Nuclei were observed under the microscope for quality control before proceeding.

Tissue lysate was filtered through a 40um strainer into a 50mL conical tube. Next, 5mL of 50% iodixanol solution (5 volumes OptiPrep and 1 volume of Diluent composed of 150mM KCl, 30mM MgCl₂, 120mM Tricine-KOH pH 7.8 mixed, plus 0.1U/uL RNasin, 0.1U/uL SUPERase RNase inhibitor, 1mM DTT, 0.15mM spermine, and 0.5mM spermidine) was added to the filtered tissue lysate to make a 25% iodixanol solution and vortexed to mix. The tissue lysate was slowly underlaid with 30% iodixanol solution (30% OptiPrep in HB with 0.1U/uL RNasin, 0.1U/uL SUPERase RNase inhibitor, 1mM DTT, 0.15mM spermine, and 0.5mM spermidine), then slowly underlaid with 40% iodixanol solution (40% OptiPrep in HB with 0.1U/uL RNasin, 0.1U/uL SUPERase RNase inhibitor, 1mM DTT, 0.15mM spermine, and 0.5mM spermidine). Without disturbing the layers, the 50mL tube was weighed and balanced before centrifugation at 10,000g for 18 minutes at 4°C with no brake.

Nuclei were collected from the interface layer with a 1000mL pipette and placed into a new tube with 10mL nuclei wash buffer (1x PBS, 1% BSA, 0.1U/uL RNasin, and 0.1U/uL SUPERase RNase inhibitor) and vortexed before filtering through a 20um filter. Nuclei were pelleted by centrifugation at 500g for 5 minutes at 4°C. Supernatant was removed and tissue was washed a second time. The pellet was resuspended in 250uL of nuclei wash buffer and nuclei were counted using a hemocytometer.

CitraPrep nuclei isolation for adult small intestine

Mouse duodenal tissue was dissected from the junction of the stomach to approximately 3cm distal at the turn of the intestine, flushed with PBS to remove luminal contents, and snap frozen in liquid nitrogen.

Snap frozen tissues were stored at -80°C until nuclei isolation. Glass dounce homogenizers were placed on ice (Wheaton Science Products, 357542). We tested over a dozen iterations of extraction methods (dounced, crushed, homogenized), incubation times (5 minutes and 7 minutes), washing conditions (single, double, or triple washed), wash volumes (5mL, 10mL, and 50mL), chelating agent concentrations (10mM, 25mM, 30mM, and 50mM citric acid), RNase inhibitor concentrations (0.2 U/uL, 0.5 U/uL, and 1 U/uL), and RNase inhibitor enzyme mixes (SUPERase, RNasin, Protector, and in varying combination). We scored each condition by 28S:18S ribosomal RNA band ratios and percent ambient RNA, naming the top condition *CitraPrep* (**Fig. S1**). For *CitraPrep*, frozen tissue was placed into a petri dish on ice and cut into 0.2cm cubed pieces or smaller using clean scissors and forceps in 1mL of CitraHB (0.25M sucrose, 25mM KCl, 5mM MgCl₂, 20mM Tricine-KOH pH 7.8, 10mM citric acid, 0.5U/uL RNasin (Promega, N2615), 0.5U/uL SUPERase RNase inhibitor (Thermo Fisher, AM2696), 1mM DTT (Sigma, D0632), 0.15mM spermine tetrahydrochloride (Sigma, S1141), 0.5mM spermidine trihydrochloride (Sigma, S2501), and cOmplete, EDTA-free protease inhibitor (Sigma, 11836170001)). Chopped tissue (totaling less than 1.5cm cubed) was transferred to the glass dounce homogenizer on ice and 4mL of CitraHB was added. Tissue was incubated on ice for 7 minutes. Tissue was homogenized 20x with loose pestle before adding 320uL of NP-40 and homogenizing 40x with tight pestle. If tissue does not homogenize, an electric homogenizer with short bursts may be used. Nuclei were observed under the microscope for quality control before proceeding.

Tissue lysate was filtered through a 40um strainer into a 50mL conical tube. Next, 5mL of 50% iodixanol solution (5 volumes OptiPrep and 1 volume of Diluent composed of 150mM KCl, 30mM MgCl₂, 120mM Tricine-KOH pH 7.8 mixed, plus 10mM citric acid, 0.5U/uL RNasin, 0.5U/uL SUPERase RNase inhibitor, 1mM DTT, 0.15mM spermine, and 0.5mM spermidine) was added to the filtered tissue lysate to make a 25% iodixanol solution and vortexed to mix. The tissue lysate was slowly underlaid with 30% iodixanol solution (30% OptiPrep in CitraHB with 0.5U/uL RNasin, 0.5U/uL SUPERase RNase inhibitor, 1mM DTT, 0.15mM spermine, and 0.5mM spermidine), then slowly underlaid with 40% iodixanol solution (40% OptiPrep in CitraHB with 0.5U/uL RNasin, 0.5U/uL SUPERase RNase inhibitor, 1mM DTT, 0.15mM spermine, and 0.5mM spermidine). Without disturbing the layers, the 50mL tube was weighed and balanced before centrifugation at 10,000g for 18 minutes at 4°C with no brake.

Nuclei were collected from the interface layer with a 1000mL pipette and placed into a new tube with 10mL nuclei wash buffer (1x PBS, 1% BSA, 0.5U/uL RNasin, and 0.5U/uL SUPERase RNase inhibitor) and vortexed before filtering through a 20um filter. Nuclei were pelleted by centrifugation at 500g for 5 minutes at 4°C. Supernatant was removed and tissue was washed a second time. The pellet was resuspended in 250uL of nuclei wash buffer and nuclei were counted using a hemocytometer.

RNA isolation

RNA isolation was performed as described(10). Briefly, nuclear samples and tissues were placed in 1 mL of TRIzol (Ambion, R0278), with lysis of nuclei by vortexing and tissues by electric homogenizer (TissueRupter II, Qiagen, 9002755). Once homogenized, 200 uL of chloroform was added and samples were vortexed until uniformly opaque pink in color. Samples were centrifuged at 12,000 g for 20 minutes at 4°C to separate the aqueous layer. A p200 pipette was used to remove and transfer the aqueous layer to a new tube with 500 uL isopropanol. Samples were inverted and incubated at -20°C overnight for precipitation. Samples were centrifuged at 12,000 g for 20 minutes at 4°C to pellet RNA, supernatant was removed, and 1 mL of cold 75% ethanol was added. Samples were centrifuged at 8,000 g for 10 minutes at 4°C before removal of supernatant. After a final 5 minute spin, the remaining liquid was removed using a p200 pipette and pellets were air dried for approximately 10 minutes (adjusted to pellet size). RNase free water was added to reconstitute the pellet and samples were heated at 55°C for 5 minutes before immediately transferring to ice and storing at -80°C.

Analysis of RNA integrity

RNA quality was checked using RNA formaldehyde electrophoresis to assess ribosomal RNA band integrity. 1% agarose was prepared by heating water and adding 1x MOPS (Quality Biological, 351-059-101) and 2% formaldehyde in a chemical hood before pouring into a gel unit to solidify. 1-2ug of RNA was processed by adding 10uL of RNA loading dye (60 uL 10x MOPS, 120 uL 37% formaldehyde, 300 uL formamide, 30 uL bromophenol blue dye, 10 uL GelRed) and heating at 95 degrees for 2 minutes to denature RNA before immediate transfer to ice to prevent formaldehyde from evaporating. The gel was loaded at ran at 50-60 V in 1x MOPS buffer in water. The gel was visualized using GelRed with the BioRad GelDoc EZ Imager and the ratio of 28S and 18S bands were determined using ImageJ.

Single-nucleus RNA-sequencing

Single-nucleus suspensions for each sample was loaded into a separate well of a Chromium 10X Genomics single cell 3' library chip as per the manufacturer's protocol (10X Genomics: 3' GEM Library and Gel Bead Kit v3.1 1000128, Chromium Next GEM Chip G Single Cell Kit 1000127), aiming to recover 15,000 nuclei. All libraries were sequenced paired-end, single index following 10X Genomics guidelines on an Illumina NovaSEQ at University of Chicago Genomics Facility, aiming to sequence 50,000 reads per nuclei.

Processing single-nucleus RNA-sequencing

Sequencing data were preprocessed using CellBender v3 with low counts set at thresholded 200 and a training dataset of 150 epochs. The filtered dataset was then processed with an nUMI cutoff of >500 and <8000. Nuclei with fewer than 5% mitochondrial gene contamination were retained, albeit nearly all nuclei prepped with *CitraPrep* fell within this boundary (**Fig. S1**). Prior to normalization, cells expressing >2% of *Kcnqlot1* transcripts, a previously identified marker of low quality cells were removed from the analysis(11). A total of 57,033 nuclei passed quality control filtering with a mean detection of 1585.84 genes per nucleus.

The 4 mouse datasets were integrated using SCTransform normalization with variable regression set to mitochondrial reads using Seurat 4.0 (12-15) package followed by principal component analysis prior to clustering. We mathematically defined the optimal principal components (PC) to use by calculating the percent variation associated with each. Next, we calculated the cumulative percentage for each PC and determining which exhibits greater than 90% cumulative percent and less than 5% variation associated with

the PC. Next the difference between the variation of PC and subsequent PC was calculated where change of percentage of variation is more than 0.1% before finding the smaller of the two values as the optimal value for downstream analyses. Batch effects were removed using Harmony(16) and grouping variables by original sample identity. Resolution was initially set at 0.5-1 before adjusting until all unsupervised clusters represented transcriptionally unique groups as determined by post-hoc analysis of top 10 enriched transcripts per cluster on heatmap. Clustering was visualized by UMAP and clusters were annotated by expression of known lineage markers.

Western blotting

Protein lysates from whole duodenum, cortex, and colon were prepared in RIPA lysis buffer (Sigma, R0278) supplemented with cOmplete, EDTA-free Protease inhibitor (Sigma, 11836170001). Protein concentrations were measured using the BCA kit (Thermo Fisher, 23225) and then diluted in 4x Laemmli buffer (40% glycerol, 8% SDS, 240mM Tris-HCl pH 6.8, 5% b-mercaptoethanol, 12.5mM EDTA, 0.04% bromophenol blue). Lysate was resolved on 4-12% NuPAGE 1.5mm Bis-Tris gel (Thermo Fisher, NP0335BOX) and transferred to PVDF membrane (Thermo Fisher, LC2002). Membranes were blocked with 5% milk in Tris-buffered saline with 1% Tween 20 (TBST) for 1hr, followed by overnight incubation at 4°C with primary antibodies (PDE3A from ProSci 18-159 at 1:500, CDH10 from Novus NBP2-92600 at 1:1000, GHR from Affinity Biotech DF8425 at 1:1000, SORBS2 from Bioss BS-4905R at 1:500, B-ACTIN from Signa A3854 at 1:10,000). Membranes were washed three times with TBST before incubation for 1hr with either anti-rabbit IgG conjugated to horseradish peroxidase (HRP) or anti-mouse IgG-HRP.

Tissue sectioning and immunostaining

For sectioning, mouse duodenal tissue was dissected from the junction of the stomach to approximately 3cm distal at the turn of the intestine, flushed with PBS to remove luminal contents, and fixed overnight at 4 °C in 4% paraformaldehyde (PFA). Fixed tissues were washed 3 times in PBS before moving to 30% sucrose in PBS and stored at 4 °C overnight until tissue sank. Tissues were thoroughly wiped clean of sucrose using a Kim wipe, cut into smaller segments, and washed several times in OCT prior to embedding. Tissue blocks were sections on a Leica CM1950 Cryostat at 15-20 uM onto slides and stored at -80 °C.

For immunofluorescent staining, slides were blocked for 30 minutes room temperature with PBS+0.1% Triton X-100 (VWR) with 5% donkey serum (Jackson ImmunoResearch) then incubated overnight at 4°C in primary antibodies (RFP from Chromotek 5f8-100 at 1:200, GFAP from Agilent DAKO Z033429-2 at 1:500, GFP from Aves GFP-1020 at 1:100, NFIA from Signa HPA006111 at 1:100, VIM from Biolegend 919101 at 1:500, B-CATENIN from Thermo Fisher 71-2700 at 1:100, FIGN from MyBioSource MBS7048173 at 1:100, NPAS2 from GeneTex GTX105741 at 1:100, PIEZO2 from Novus NBP1-78624 at 1:100 validated with knockout and works best in whole mount LMMP tissue, PIEZO2 from NOVUS NBP1-78538 did not work in our hands, SOX10 from R&D AF2864 at 2ug/mL, GFAP from Thermo Fisher 13-0300 at 1:100, NRP1 from R&D AF3870 at 2ug/mL, GRIK3 from Thermo Fisher MA5-31743 at 1:100 with mouse on mouse pretreatment MKB-3313-1, SOX6 from Abcam ab30455 at 1:100, PPARd from Thermo Fisher PA1-823A at 1:100). Slides were washed, incubated with secondary antibody for 1 hour at room temperature, and washed. All secondary antibodies were purchased from Jackson ImmunoResearch. Slides were mounted in Fluoromount G (SouthernBiotech), covered with coverslips, and sealed with nail polish. Nuclei were stained with DAPI (Invitrogen). Human slides embedded in paraffin were deparaffinized prior to immunostaining by incubating in xylene two times, 1:1 xylene:100% ethanol once, 100% ethanol two times, followed by one time in 95% ethanol, 70% ethanol, and 50% ethanol before washing in running cold water to rehydrate.

Whole mount immunofluorescence

Longitudinal muscle and myenteric plexus tissue was isolated as described above and washed one time in PBS before fixing in 4% PFA for 20 minutes at room temperature. Tissue was washed 3 times in PBS before blocking for 30 minutes room temperature with PBS+0.1% Triton X-100 (VWR) with 5% donkey serum (Jackson ImmunoResearch) and incubating overnight at 4°C in primary antibodies (**Table S6**).

Tissues were washed, incubated with secondary antibody for 1 hour at room temperature, and washed. All secondary antibodies were purchased from Jackson ImmunoResearch. Nuclei were stained with DAPI (Invitrogen). Imaging was performed on two microscopes: (1) a Leica TCS SP8 gated super-resolution STED confocal laser scanner mounted on an inverted DMI6000 microscope and (2) an inverted Leica DMI8 microscope.

Longitudinal muscle myenteric plexus preparations

Mouse duodenal tissue was dissected from the junction of the stomach to approximately 3cm distal at the turn of the intestine, flushed with PBS to remove luminal contents, and placed in PBS on ice. Mesentery and pancreatic tissue were removed, then duodenal tissue was cut into 1.0-1.5cm segments. Tissue segments were individually slid onto a borosilicate glass Pasteur pipette and, using pressure from a finger for stability and under microscopy, forceps were used to create a thin nick along the serosa and muscle layers of the duodenum. A cotton swap was wetted with cold PBS and used to wipe along the nick while slowly turning the tissue using the stabilizing finger to peel away the longitudinal muscle and myenteric plexus layer. This region, when done properly, resembles the transparent membrane of an onion. Collected tissue was immediately used for subsequent assays including whole mount immunostaining, mouse primary cell culture, *ex vivo* contractility assays, and calcium imaging.

Mouse primary cell culture

Longitudinal muscle myenteric plexus layers were collected and placed in a digestive KREBS solution buffer with 1.3 mg/mL collagenase type II (Worthington, LS004176) and 0.3 mg/mL BSA (Sigma, A4161) through modification of established protocols (17, 18). KREBS solution was composed of the following: 121 mM NaCl, 5.9 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM NaH₂PO₄, 10 mM HEPES, 21.2 mM NaHCO₃, 1 mM pyruvic acid, 8 mM glucose. All tissues must be collected and placed into the solution on ice within 30 minutes of dissection. The digestive KREBS solution was moved to 37 °C and bubbled with carbogen for 1 hour. Following this hour, solution was quenched with base media and centrifuged at 356 g for 10 minutes at 4 °C. Enteric glial cell media was composed of 1:1 DMEM/F12 to Neurobasal, 1x B27 (ThermoFisher, 17504044), 1x N2max (Fisher, AR009), 10ng/mL GDNF (Stem Cell Technologies, 78058), 20ng/mL FGF2 (R&D Systems, 23-3FB-010M), and 1x penicillin/streptomycin (Thermo Fisher, 15070-063). The remaining pellet was gently resuspended in enteric glial cell media supplemented with 1x CloneR (Stem Cell Technologies, 5889) and 50ug/mL primocin (Invivogen, ant-pm-1), filtered through 100 uM filters (Fisher Scientific, 08-771-19), and plated onto 10cm 0.1mg/mL poly-ornithine primed, 10µg/mL laminin coated plates. Media was replaced every other day and cells were split into 96-well plates after reaching confluence or 2 weeks in culture.

For analysis, cells were washed one time in PBS, fixed for 20 minutes in 4% PFA at room temperature, and washed 3 times in PBS prior to blocking. Cells were blocked for 30 minutes room temperature with PBS+0.1% Triton X-100 (VWR) with 5% donkey serum (Jackson ImmunoResearch) then incubated overnight at 4°C in primary antibodies (**Table S6**). Plates were washed, incubated with secondary antibody for 1 hour at room temperature, and washed. All secondary antibodies were purchased from Jackson ImmunoResearch. Nuclei were stained with DAPI (Invitrogen). Brightfield imaging of primary enteric glial cells was performed on a Leica DMIL LED microscope.

Whole gut transit time assay

To measure the amount of time it took contents to fully pass through the gastrointestinal tract, we gavaged mice with 6% carmine red (Sigma, C1022) in methylcellulose (Sigma, 274429). To prepare this reagent, we dissolved 0.5% methylcellulose in hot distilled water prior to mixing 6% carmine red. The solution was used at room temperature but stored at 4°C until use.

Oral gavage of 300uL carmine red solution was performed using a 24-20 gauge feeding needle between 2.5-3.8 cm in length. Before the oral gavage procedure, we measure the distance from the oral cavity to the caudal point of the sternum with the feeding needle on the outside of the restrained animal to mark the distance needed to insert into the esophagus. This distance was marked using a marker. The

syringe was loaded with the volume to be administered (10 μ L/gram mouse weight). The outside of the needle was wiped to remove any compound on the outside.

Mice were gently scruffed and restrained in an upright position to immobilize the head and neck. The gavage needle was slid into the left side of the animals' mouth behind the teeth in front of the first molar along the roof of the animals' mouth. The gavage needle was used to gently tilt the mouse's head back towards the spine with gentle pressure to allow a straight line from the mouth to the esophagus and stomach, ensuring no resistance. The gavage was passed until the pre-marked line is reached. The solution was slowly injected over the course of 2-3 seconds to minimize fluid coming back up the esophagus.

Mice were housed in upside down empty p1000 pipette tip boxes on metal racks to suspend mice above a clean white paper mat. Mice were not disturbed during the assay other than during the gavage procedure to minimize effects due to handling (19). A timer was started after gavaging mice and fecal matter was observed immediately upon dropping to the mat for carmine red color. The time from gavage to the first carmine red defecation was documented.

Gastrointestinal transit time assay

Gastrointestinal motility was analyzed to determine the contribution of enteric glial subtypes to regulation of intestinal physiology. We performed pilot experiments to determine the optimal fasting time prior to starting the assay, in which mice were fasted for different lengths of time and autofluorescence of stomach contents were measured (**Fig. S9G**). Mice were fasted for 2-5 hours prior to oral gavage of a 70-kDa FITC labeled dextran solution (100 μ L, 25 mg/mL PBS; gavage procedure as described above). Initially, we collected timepoints between 20 and 50 minutes after gavage to determine when to assay experimental mice. All data in the manuscript were collected 30 minutes post gavage.

The gastrointestinal tract was removed from stomach to anus and placed into ice cold PBS to cold shock tissue and inhibit further peristalsis. We carefully cut the mesenteric and pancreatic tissue to straighten the gut and measured the small intestine length from the pyloric sphincter to the cecum. The small intestine was divided into 10 equal sections and each segment, in addition to the stomach and the cecum, was flushed with 2 mL of PBS. The colon tissue was measured and divided into 3 equal segments and flushed with 2mL of PBS. The flushed contents were centrifuged at 500 rpm for 10 minutes and 200 μ L of the supernatant from each section was placed in a 96 well plate. The quantification of the fluorescent signal in the supernatant from each segment was determined utilizing a Synergy neo 2 multi-well fluorescence plate reader from BioTek/Agilent (excitation 545 nm and emission 590 nm). The distribution of the fluorescent signal in the intestinal segments was mapped and used to calculate the rate of gastric emptying and the geometric center of fluorescence (GCF). GCF was determined by calculating the fraction of fluorescence per segment multiplied by the segment number (1-15) and adding all segments together before dividing by 10. GCF can range from 1 to 10 with a higher number indicating a faster motility and shorter intestinal transit time. Gastric emptying was calculated as: $[(\text{total fluorescence} - \text{fluorescence in stomach}) / (\text{total fluorescence})] \times 100$.

Fecal matter composition

Fecal water content was measured by weighing feces in a 1.5 mL tube with the cap on immediately after collection from the animal before opening the cap and heat drying the sample at 42 °C for 48 hours. After 48 hours, sample was capped and weighed before measuring the weight of the tube. Percent water content was calculated as $(\text{pre drying weight} - \text{tube weight}) - (\text{post drying weight} - \text{tube weight}) / (\text{pre drying weight} - \text{tube weight}) \times 100$.

Neurotransmitter analyses

Levels of acetylcholine and nitric oxide were measured from primary mouse tissues. The longitudinal muscle myenteric plexus was dissected from the proximal small intestine as described above and immediately homogenized in RIPA lysis buffer (Sigma, R0278) with cOmplete, EDTA-free Protease inhibitor (Sigma, 11836170001). The protein content was measured using a BCA assay (Thermo Fisher, 23225) to normalize neurotransmitter levels to protein content of the tissue samples. The level of

acetylcholine was determined using the Choline/Acetylcholine Quantification Kit (Sigma, MAK056) and measuring the difference between the level of total choline and free choline. Total nitrite/nitrate was measured as a proxy for nitric oxide concentration using the Nitric Oxide Assay Kit (Abcam, ab65327), with 5 hours of incubation with the enzyme cofactor to permit more than 99% conversion of nitric oxide to nitrite.

Epithelial barrier integrity assays

Mice were fasted for 2-5 hours prior to oral gavage of 4-kDa (Sigma-Aldrich, 600 mg/kg body weight, 80 mg/mL PBS) by gavage. The 4-kDa fluorescent dextran is a non-digestible dextran conjugated with dye that can transit through the gastrointestinal tract and passively cross the intestinal epithelium. The use of this dye allows for tracking of peristalsis and gut motility while also allowing for a readout of epithelial barrier integrity. All data in the manuscript were collected 30 minutes post gavage.

Mice were euthanized and blood was collected directly from the heart to ensure contents were not diluted by intraperitoneal fluids. Plasma was collected by centrifugation at 2000g for 5 minutes. Equal volumes of serum were loaded into a 96-well microplate in duplicate and read by spectrophotofluorometry with an excitation of 485nm and emission of 528nm using as standard serially diluted FITC-dextran (0, 125, 250, 500, 1000, 2000, 4000, 6000, 8000 ng/mL).

Biochip fabrication

Biochip microchannel geometry was formed via a laser micro-machined, double-sided adhesive film (3M, 8412KCL) and polymethyl methacrylate (McMaster-Carr, 8560K) components assembled in the following manner: a 1 mm polymethylmethacrylate spacer was sandwiched between two double-sided adhesive films and adhered to a glass slide for contraction imaging or to a cover slip for calcium imaging. At this point, microchannels were rinsed with ethanol and deionized water, then functionalized with N- γ -maleimidobutyryl-oxy succinimide ester (Fisher Scientific, PI22309) to facilitate Matrigel bonding. To chilled biochips, 50 μ L of Matrigel solution was added to each channel and dissected tissues were carefully laid flat in each channel to facilitate adhesion before incubation at 37 °C with 5% CO₂ for 10 minutes for curing. Culture media was then gently added to each channel and biochips were incubated at 37 °C with 5% CO₂ for at least 60 minutes for tissue recovery. Thereafter, a 3.175 mm polymethylmethacrylate block, with inlet and outlet holes was bonded on top of the open biochips to enclose each channel. Assembled microchannels were then perfused with fresh modified KREBS buffer (121 mM NaCl, 5.9 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM NaH₂PO₄, 10 mM HEPES, 21.2 mM NaHCO₃, 1 mM pyruvic acid, 8 mM glucose) to remove any residual air bubbles, then kept at 37 °C with 5% CO₂ until use.

Ex vivo motility assays

Longitudinal muscle myenteric plexus preparations from 6 week old mice were dissected fresh and washed one time in cold Matrigel before placing in 50 μ L of a 50% KREBS buffer: Matrigel solution in channels of chilled biochips and processed as described above. Biochips were incubated at 37 °C with 5% CO₂ for 1 hour prior to analysis to allow tissue to recover. Tissues were screened for quality and only tissues exhibiting spontaneous contractions were used for contractility assays under stimulation. Images were taken every 250 ms via a BX51WI Olympus microscope equipped with Retiga EXi CCD camera (QImaging) using microManager and ImageJ applications. Acquisition spanned for 30 seconds in brightfield at baseline and with 1 dyn/cm² flow (above threshold) or at baseline.

Calcium Imaging and Quantification

The longitudinal muscle myenteric plexus layer of Ai95(RCL-GCaMP6f)-D mice was prepared as described above. Tissues were carefully laid plated with 50 μ L of undiluted Matrigel on customized imaging biochips with channels mounted on coverglass. Tissues were carefully laid flat in each channel to facilitate adhesion before incubation at 37 °C with 5% CO₂ for 10 minutes for curing. KREBS buffer (121 mM NaCl, 5.9 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM NaH₂PO₄, 10 mM HEPES, 21.2 mM NaHCO₃, 1 mM pyruvic acid, 8 mM glucose) was then gently added to each channel and biochips were incubated at 37

°C with 5% CO₂ for at least 60 minutes for tissue recovery. Thereafter, a 3.175 mm polymethylmethacrylate block, with inlet and outlet holes was bonded on top of the open biochips to enclose each channel. Assembled microchannels were then perfused with fresh modified KREBS to remove any residual air bubbles. Fluorescent images of the mounted tissues were acquired via an inverted Olympus IX83 microscope at 40X objective. Acquisition was completed with the GFP shutter open acquiring 408 images over a 2 minute period of time.

Images were analyzed using Fiji and ImageJ plugin Time Series Analyzer ver3. Briefly, the GCaMP6f expressed cell regions were defined as regions of interest (ROI) and circled around then obtained the pixel intensity with Time Series Analyzer. Background nonspecific muscle staining/autofluorescence was subtracted from each picture by using the defined region without GCaMP6f expression. The sequence of the experiments was (1) background activity (2) with flow at 1 dyn/cm² (rates reported to activate PIEZO2 in other cell types (20)) (3) with Piezo2 inhibitor plus flow (4) washout. Same ROI were tracked and obtained the information of cell response to each experiment. XY drifts between each experiment were manually corrected so the ROI targeted the same cells throughout the sequence of experiments.

Statistical analysis

P values were calculated as indicated in figure legends using two-sided Welch's t-test, one way Welch's ANOVA, or two way Welch's ANOVA with multiple comparisons as indicated in the figure legends, calculated using either Prism9 or Microsoft Excel. Data are presented as mean ± SEM. N represents number of biological replicates, n represents number of independent experimental samples. Individual biological replicates are shown as dots on all plots.

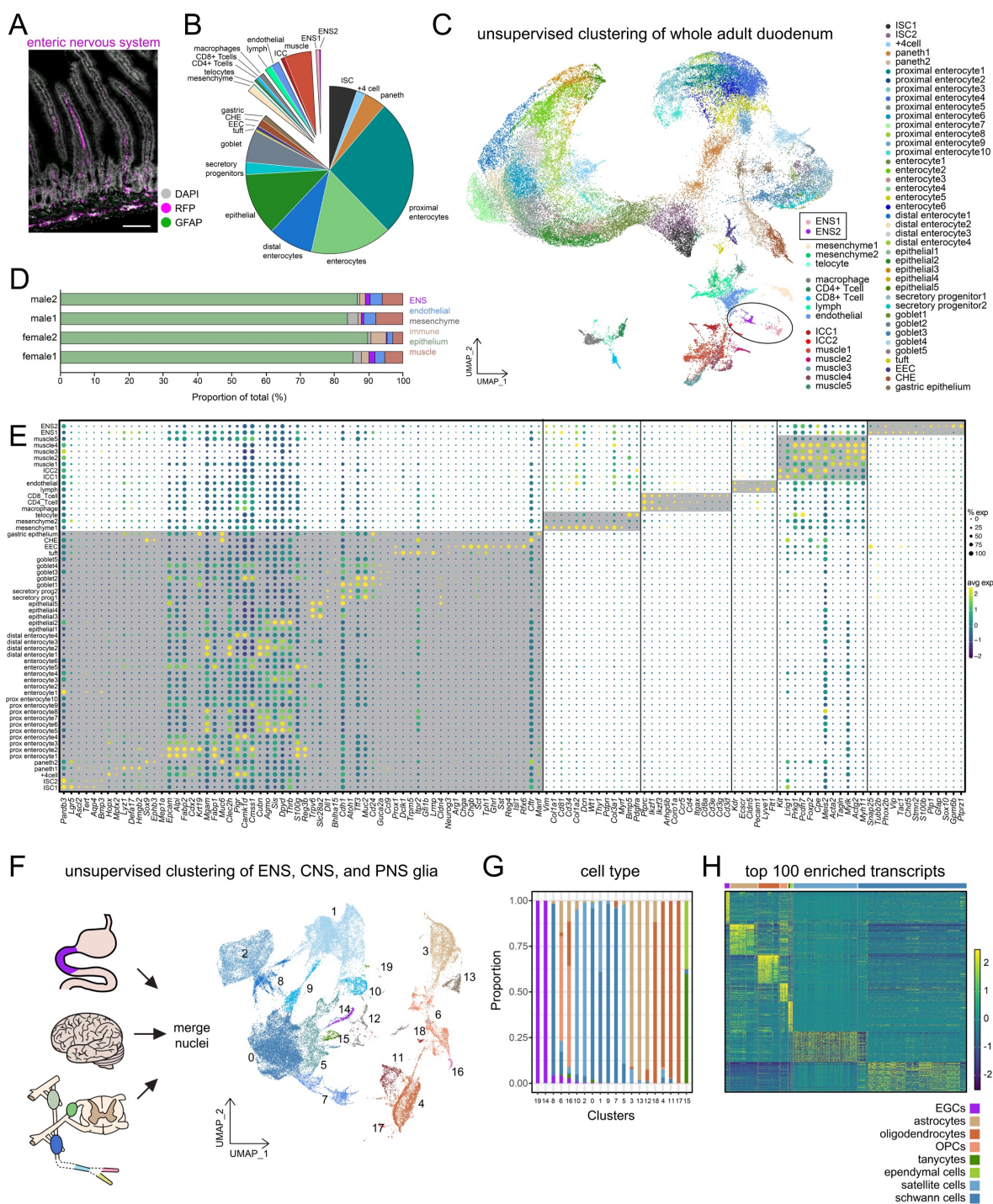
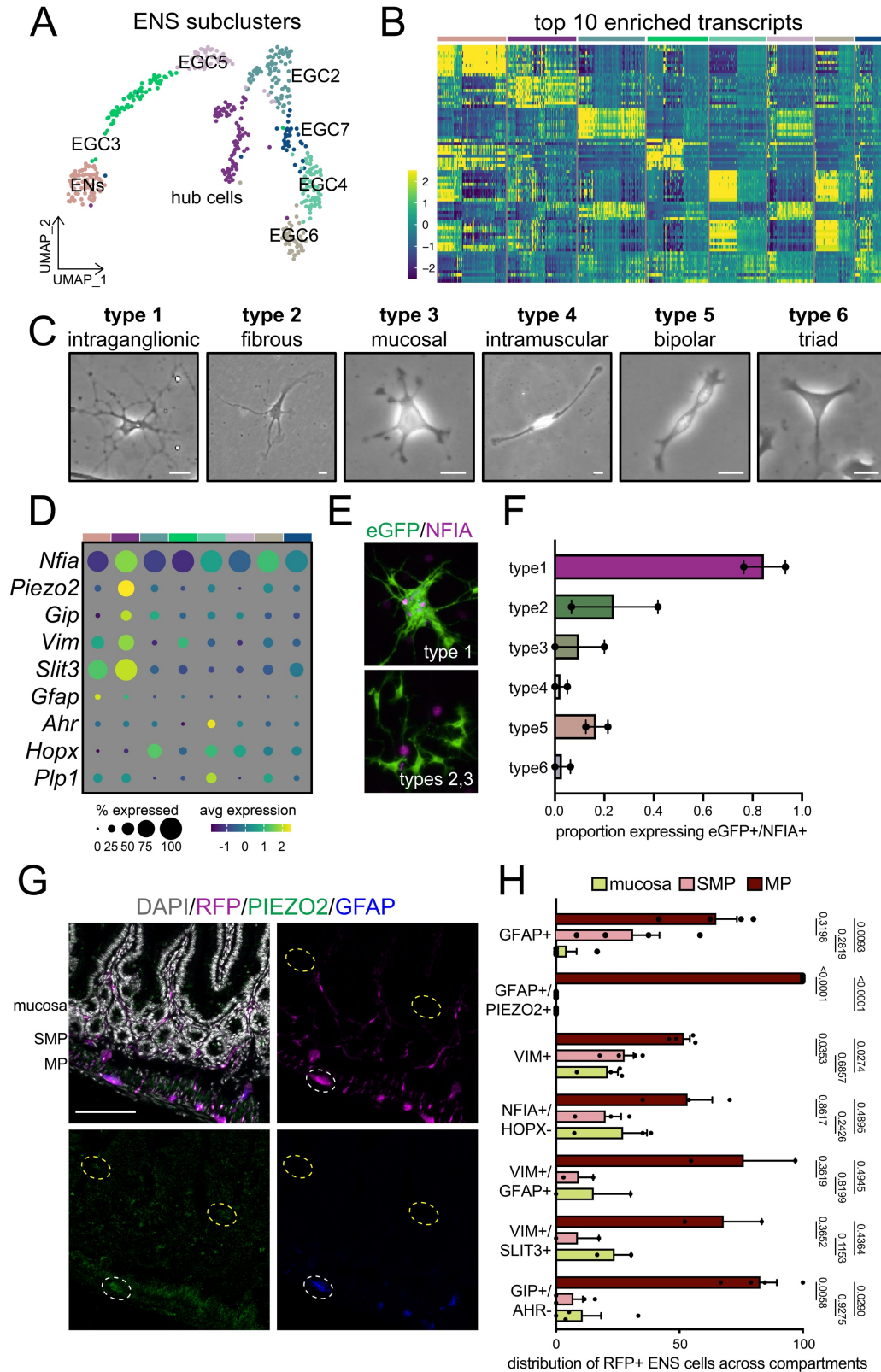


Figure 1. Enteric glia are a unique population of cells. (A) Labeling the enteric nervous system in the adult *Sox10-cre; Ai14(RCL-tdT)* mouse duodenum. RFP marks enteric nervous system cells, GFAP is shown in green, and nuclei are marked in white by DAPI. Scale bar = 100 um. (B) Proportion of cell types in the adult mouse duodenum from *CitraPrep* and snRNA-seq (n=4 mice, 57,033 nuclei). (C) snRNAseq of duodenal nuclei visualized by Uniform Manifold Approximation and Projection (UMAP), colored by cell type identity, and annotated post hoc. (D) Proportion of broad cell types captured in each of our biological replicates. (E) Cell type specific marker expression (columns) in clusters (rows) as shown in (C). Broad cell type classification is shown with dark gray boxes indicating from left to right epithelial cells,

mesenchymal cells, immune cells, vasculature, musculature, and enteric nervous system cells. Differentially expressed transcripts are detailed in Supplemental Table 1. The size of each circle indicates percentage of cells in the cluster that express the marker (≥ 1 UMI) while the color shows the average expression of transcript in cells. ISC, intestinal stem cell; EEC, enteroendocrine cell; CHE, *Cfr* high expressing epithelial cell; lymph, lymphocytes; ICC, interstitial cells of Cajal; ENS, enteric nervous system. Datasets are provided in Table S1. **(F)** On left, schematic of study design. Duodenal and cortical tissue were dissected and processed for snRNAseq. Datasets for comparison were obtained from the cortex (GSE132044), ventromedial hypothalamus (GSE172204), spinal cord (GSE103892), sciatic nerve (GSE182098), peroneal nerve (GSE182098), sural nerve (GSE182098), nodose ganglion and vagal nerve (GSE182098 and GSE138651), dorsal root ganglion (GSE175421), and superior cervical ganglion (GSE175421). Individual datasets shown in Fig. S4 and S5. On right, snRNAseq of glial cells spanning the central, peripheral, and enteric nervous systems colored by unsupervised clusters and visualized by UMAP (n=35 mice, 46,467 nuclei). **(G)** Proportion mapping of cell types to unsupervised clusters. Cell types are shown by color with key shown to the bottom right. Proportion of cells are shown on the y-axis while columns binned in the x-axis show unsupervised clusters. **(H)** Top 100 differentially expressed transcripts enriched in each glial cell type. Individual transcripts are shown in rows, cell types are clustered together in columns with color annotation above. Datasets are provided in Table S2.



provided in Table S4. **(C)** Representative images of enteric glial cells in culture capturing 6 different morphologies. Scale bars = 20 μ m. **(D)** Cell type specific marker expression (columns) in clusters (rows). The size of each circle indicates percentage of cells in the cluster that express the marker (≥ 1 UMI) while the color shows the average expression of transcript in cells. **(E)** Representative immunostaining of cultured enteric glial cells with different morphologies and marker expression. *Plp1*-eGFP is shown in green while NFIA is shown in magenta. **(F)** Quantification of NFIA/eGFP+ in each morphological group shown as percentage. P values show one-way ANOVA with unpaired t and Welch's correction. **(G)** Representative immunostaining of enteric glial cell subtype enriched markers in the adult *Sox10*-cre; RCL-tdT mouse duodenum. Intestinal compartments are labeled with boundaries marked by dashed lines. tdTomato is marked by RFP in magenta while nuclei are marked in white by DAPI. White circle shows co-expression of PIEZO2/GFAP in the ENS while yellow circles show PIEZO2 without GFAP outside of the ENS in the villi. Scale bars = 100 μ m. **(H)** Quantification of the spatial distribution of hub cell enriched markers in different intestinal compartments. The full spatial analysis of multiple enteric glial subtype enriched markers across compartments is shown in Fig. S6G. P values from two-way ANOVA with multiple comparisons. All error bars show SEM with biological replicates shown as individual dots.

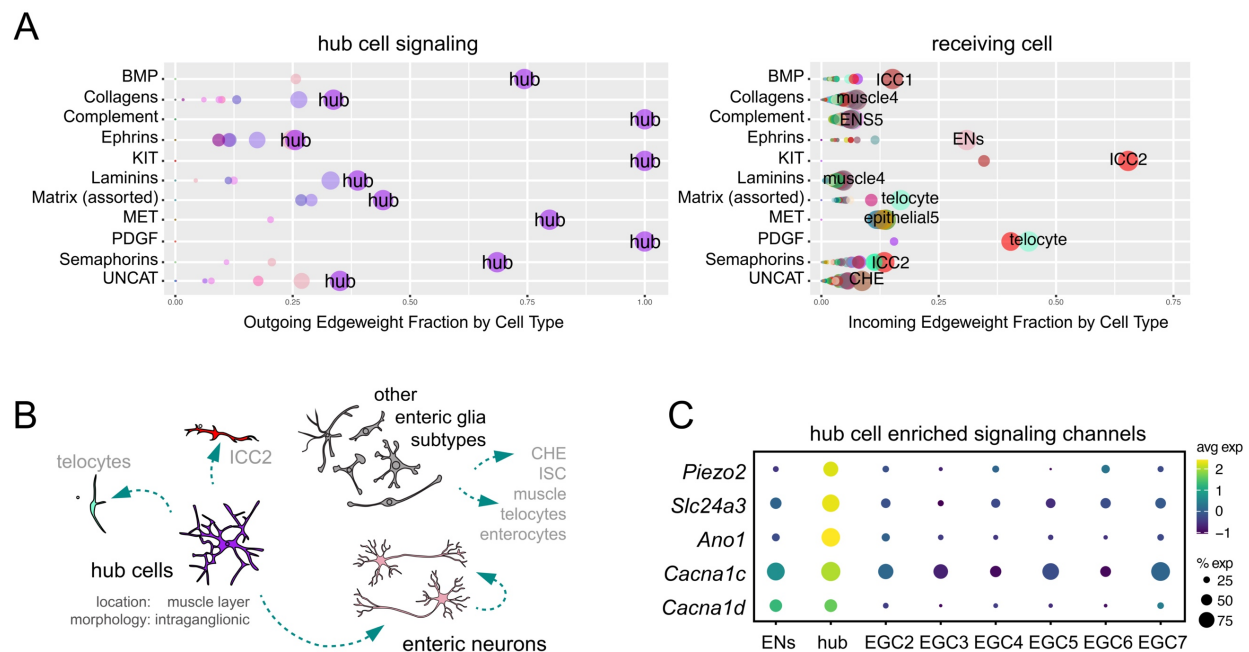


Figure 3. Subtype-specific enteric glia functional interaction network. (A) Interactome analyses of putative ligand-receptor pairs using the FANTOM5 database ($p < 0.00001$, percent > 0.2). Signaling classes are grouped in rows with enriched cell types shown across the x-axis and sending cells (ligands) on left and receiving cells (receptors) on right. Datasets are provided in Table S6. (B) Depiction of enteric nervous system signaling relationships (in teal). Hub cell transcripts are enriched in intraganglionic cells and reside in the muscle layer. (C) Dot plot showing expression of the top 5 significantly enriched transcripts ontologically associated with monoatomic ion channels. The size of each circle indicates percentage of cells in the cluster that express the marker (≥ 1 UMI) while the color shows the average expression of transcript in cells.

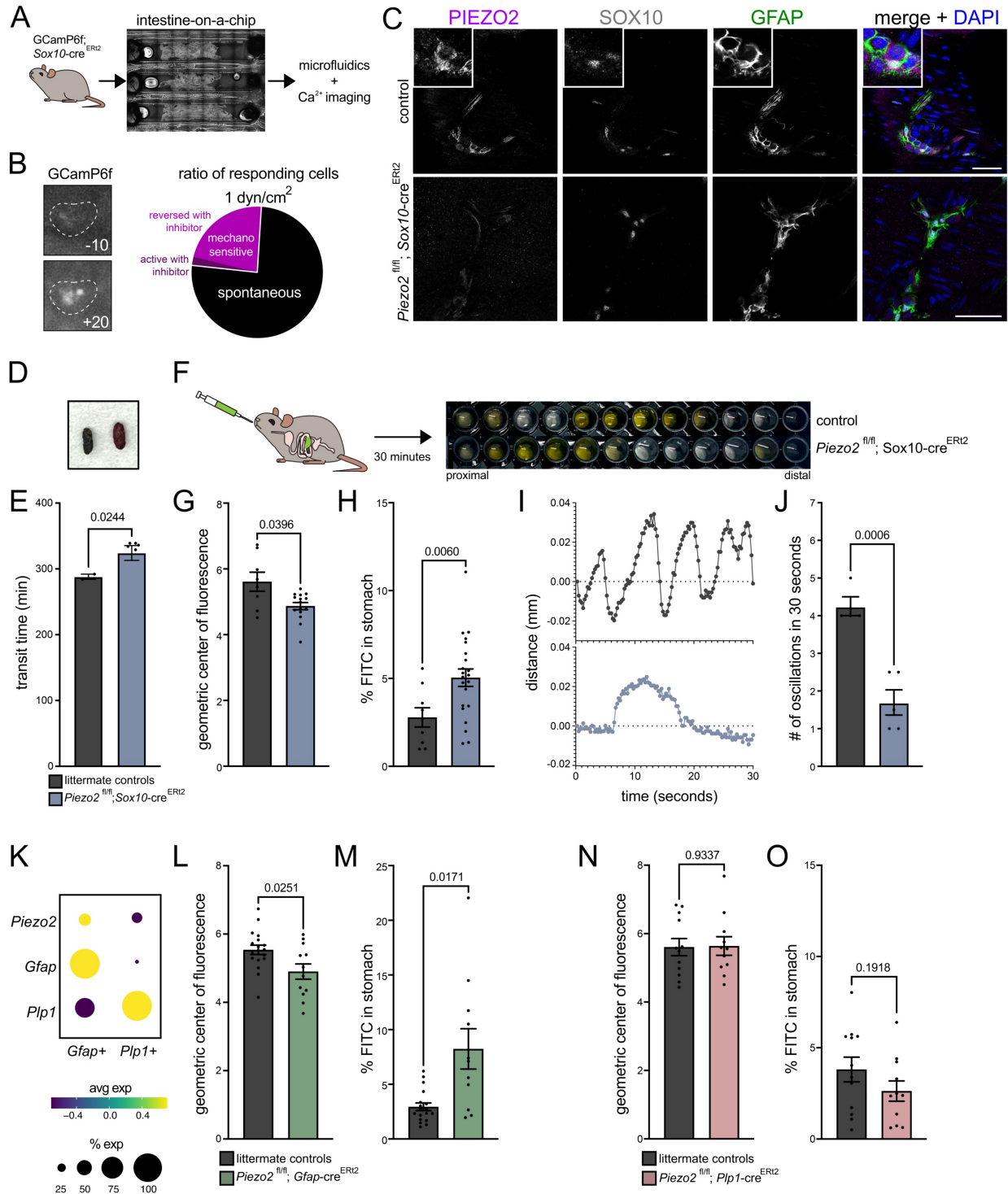


Figure 4. Hub cells regulate gut motility via mechanosensation. (A) Intestine-on-a-chip platform engineered on coverslips for high resolution, live Ca²⁺ imaging under shear stress with microfluidics. Longitudinal muscle with myenteric plexus was dissected from mice with the calcium indicator GCaMP6f expressed in enteric glia via tamoxifen driven expression of Cre in Sox10+ cells. Brightfield image shows an overview of the coverslip with tissues in chambers. (B) Representative image of mechanoresponsive enteric glia 10 seconds before and 20 seconds after 1dyn/cm² shear stress is applied. Pie chart shows

quantification of cells responding only at baseline (without shear stress), only after application of shear stress at 1 dyn/cm^2 and reversal of this response with D-GsMTx4, which inhibits PIEZO2. Cells that are responsive in both conditions are annotated, while cells active in the presence of inhibitor D-GsMTx4 at rest, after stimulation, or in both conditions are shown in a bracket. (C) Immunostaining of PIEZO2 (in magenta) in the muscle layer of the duodenum overlapping with pan-enteric glia marker SOX10 (in white) and subtype enteric glia marker GFAP (in green) with enteric glia specific adult PIEZO2 knockout shown on the bottom in *Piezo2^{fl/fl}*, *Sox10-cre^{ERT2}* mice. Separate channels are shown in grayscale. Scale bars = 50 μm . (D) Carmine red dye in fecal pellets marks transit time. (E) Total gastrointestinal transit time measuring time from gavage of carmine red dye to the appearance of red stool in littermate controls (n=2) and *Piezo2^{fl/fl}*, *Sox10-cre^{ERT2}* mice (n=5). (F) Gastrointestinal transit time assay with representative results from the stomach and small intestine. Each well represents contents from one equal sized segment of the small intestine, with the first well showing the stomach and the last well the cecum. (G) The geometric center of fluorescence shows the location of the bolus in the small intestine in littermate controls (n=8) and *Piezo2^{fl/fl}*, *Sox10-cre^{ERT2}* mice (n=15). (H) Gastric emptying in littermate controls (n=9) and *Piezo2^{fl/fl}*, *Sox10-cre^{ERT2}* mice (n=22). (I) Representative oscillations recorded from *ex vivo* longitudinal muscle with myenteric plexus under shear stress stimulation. (J) Quantification showing the number of oscillations over a 30 second time frame in littermate controls (n=4) and *Piezo2^{fl/fl}*, *Sox10-cre^{ERT2}* (n=5) mouse tissue stimulated with 1 dyn/cm^2 shear stress *ex vivo*. (K) Dot plot showing expression of *Piezo2*, *Gfap*, and *Plp1* in computationally selected and subsetted *Gfap*+ cells and *Plp1*+ cells. The size of each circle indicates percentage of cells in the cluster that express the marker (≥ 1 UMI) while the color shows the average expression of transcript in cells. (L) The geometric center of fluorescence shows the location of the bolus in the small intestine in littermate controls (n=18) and *Piezo2^{fl/fl}*, *Gfap-cre^{ERT2}* mice (n=12). (M) Gastric emptying in littermate controls (n=18) and *Piezo2^{fl/fl}*, *Gfap-cre^{ERT2}* mice (n=11). (N) The geometric center of fluorescence shows the location of the bolus in the small intestine in littermate controls (n=12) and *Piezo2^{fl/fl}*, *Plp1-cre^{ERT2}* mice (n=11). (O) Gastric emptying in littermate controls (n=12) and *Piezo2^{fl/fl}*, *Plp1-cre^{ERT2}* mice (n=11). For data in (E), (G), (H), (J), (L), (M), (N), (O), individual mice are shown as individual dots, error bars show SEM and *p* values are from Welch's *t* tests.

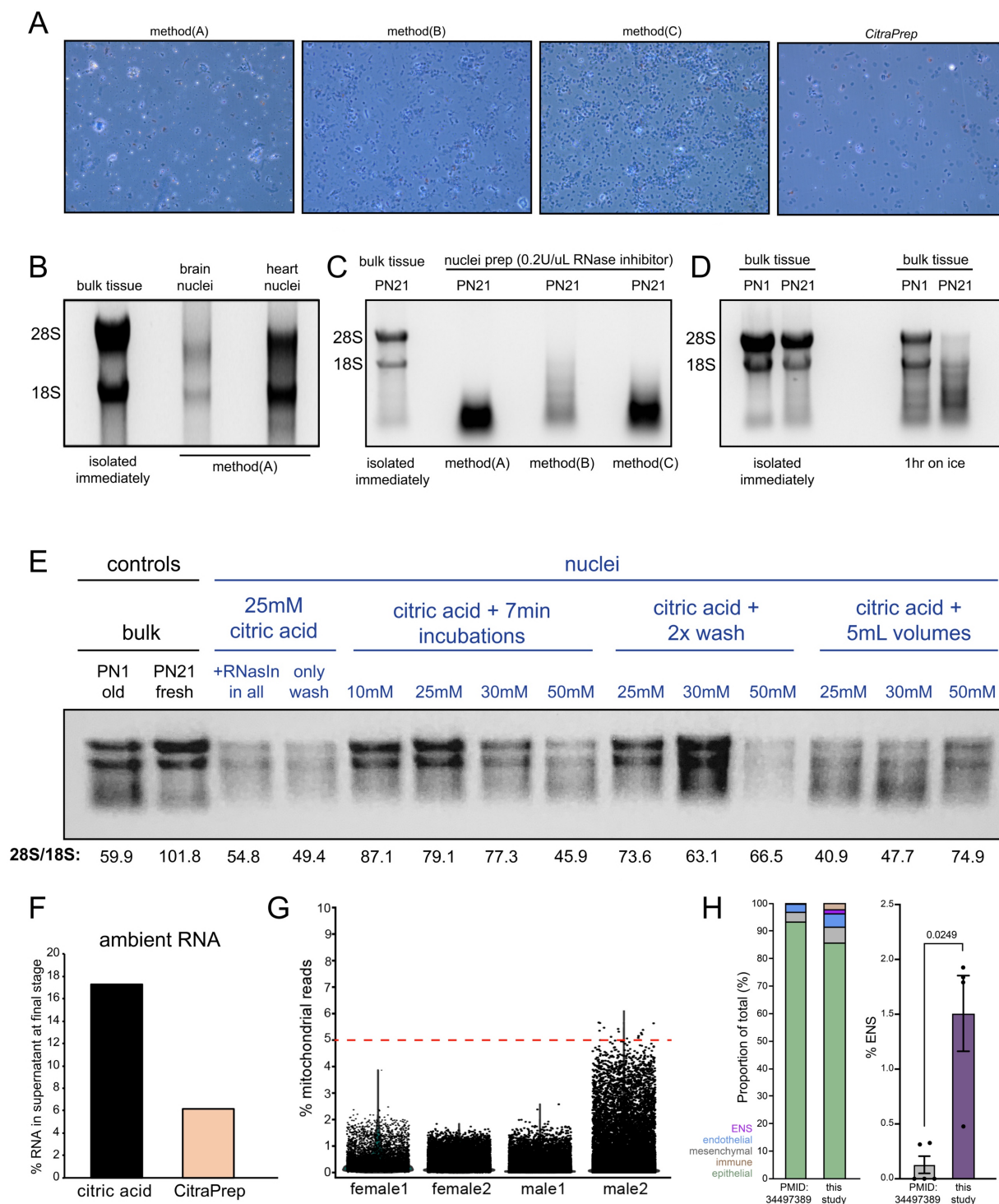


Fig. S1. Optimization of *CitraPrep* for single nuclei RNA-seq of high degradation tissue. (A) Brightfield images of isolated nuclei stained with 0.4% Trypan blue using three established methods (A)(21), (B)(22), and (C)(23), and *CitraPrep*. **(B)** RNA formaldehyde gel showing ribosomal RNA 28S and 18S bands in bulk postnatal day 21 duodenal tissue isolated immediately by acid-guanidinium-phenol based extraction compared to nuclei preparations from the cortex and the heart using an established method (21). **(C)** RNA formaldehyde gel showing ribosomal RNA 28S and 18S bands in bulk postnatal day 21

(PN21) duodenal tissue isolated immediately by acid-guanidinium-phenol based extraction compared to nuclei preparations from the duodenum using 3 different established methods (A)(21), (B)(22), and (C)(23). **(D)** RNA formaldehyde gel showing ribosomal RNA 28S and 18S bands in bulk PN1 and PN21 duodenal tissue isolated immediately by acid-guanidinium-phenol based extraction compared to snap frozen tissue that incubated undisturbed for 1 hour (1hr) on ice prior to isolation using acid-guanidinium-phenol extraction. **(E)** RNA formaldehyde gel showing ribosomal RNA 28S and 18S bands in bulk PN1 and PN21 duodenal tissue isolated immediately by acid-guanidinium-phenol based extraction compared to iterations of nuclei preparation chemistry. Ratio of 28S to 18S band is shown below to score each method. The 10mM citric acid, 7 minute incubation time resulted in the best results by 28S:18S ratio at 87.1. **(F)** Percent RNA concentration measured in supernatant after final centrifugation in nuclei preparation protocol in relation to nuclear RNA content compared to an established nuclear RNA protocol. **(G)** Percent mitochondrial reads sequenced in each individual animal after running CellBender but prior to any preprocessing or filtering showing low cytoplasmic contamination (23). Each dot is an individual nuclear transcriptome with percent mt- transcripts on the y-axis. Cutoffs for filtering for further analysis were set at 5% and shown by a dashed red line. **(H)** Comparison of our dataset with published whole proximal small intestine data from adult human (24) illustrating species- and preparation-based differences in cell proportions. Error bars show SEM with individual dots showing each biological replicate. *P* value shown determined by Welch's *t* test.

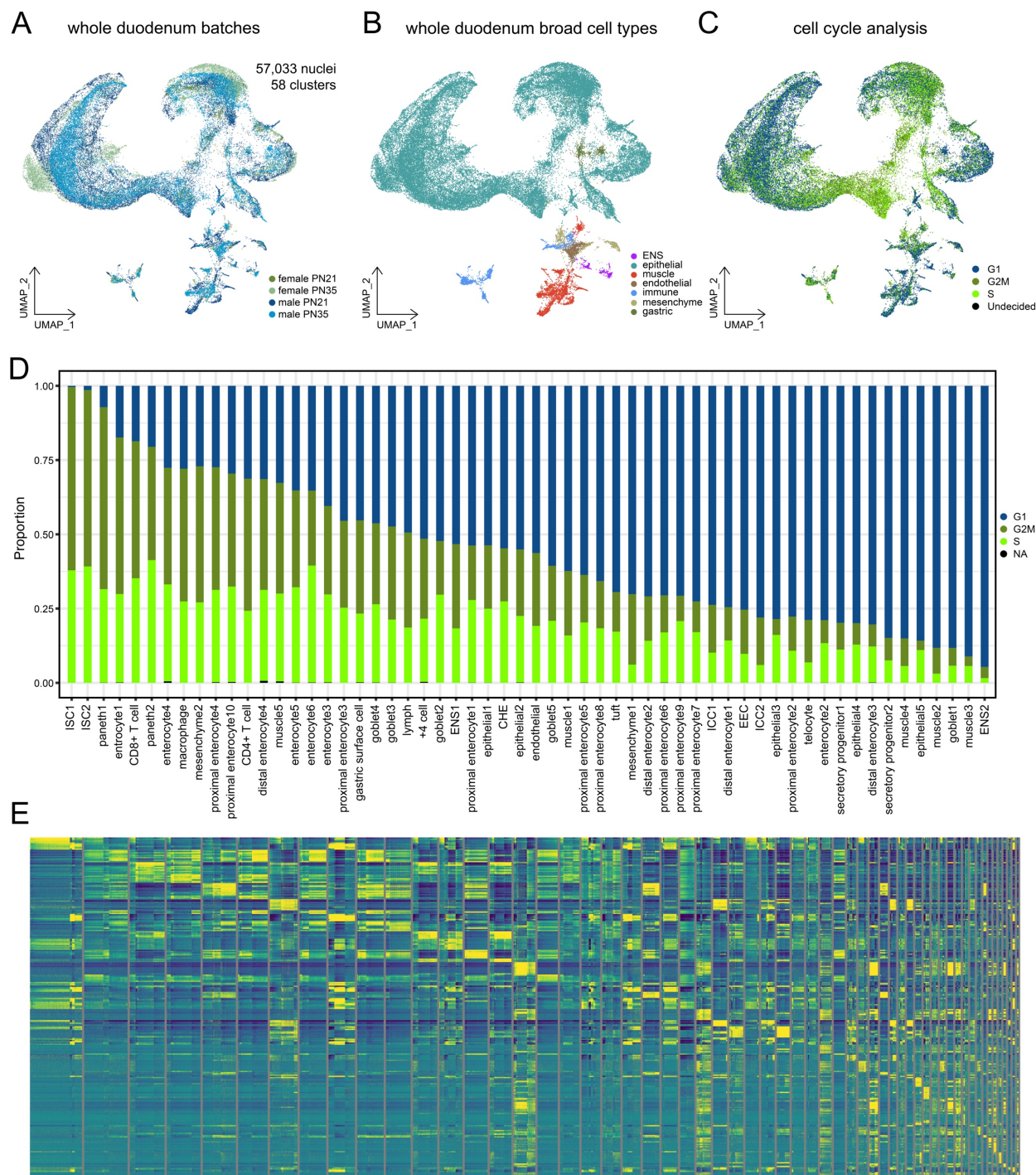


Fig. S2. Blueprints of the whole adult mouse duodenum. (A) snRNAseq of duodenal nuclei (n=4 mice, 57,033 nuclei) visualized by UMAP and colored by sample identity. PN=postnatal. (B) UMAP of duodenal nuclei visualized by colored by broad cell type identity. (C-D) Cell cycle scoring of duodenal nuclei based on expression of G1, G2/M, and S phase markers projected onto UMAP and shown as proportion of total per cell type in (D). (E) Top 10 differentially expressed transcripts enriched in each of the 58 unsupervised clusters in the duodenum. Individual transcripts are shown in rows, unsupervised clusters are shown together in columns. All data are in Table S1.

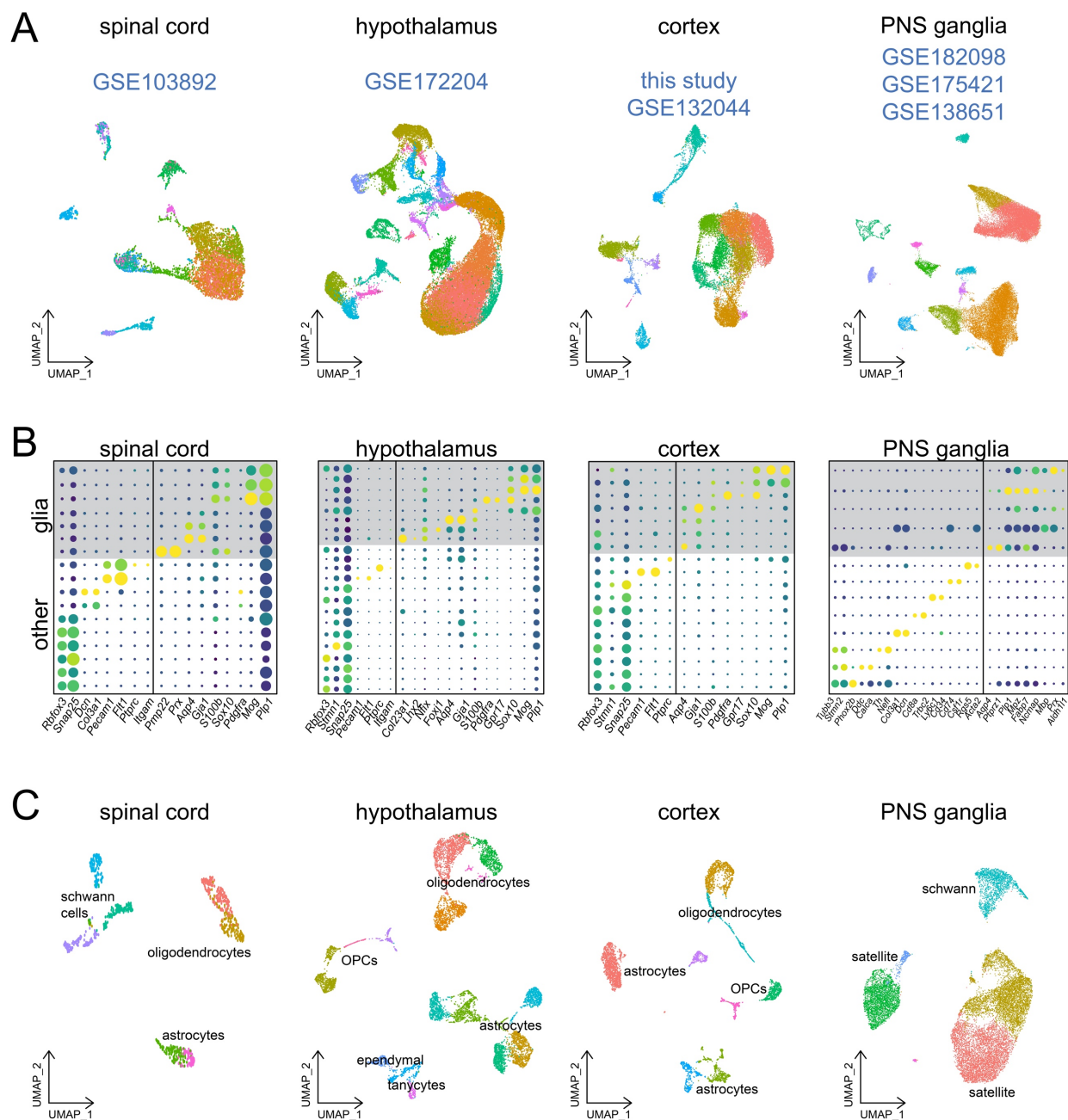


Fig. S3. Glial cell identification from central and peripheral nervous system datasets. (A) snRNAseq of tissues from studies visualized by UMAP and colored by unsupervised clustering. **(B)** Cell type markers shown by dot plot across unsupervised clusters. Transcripts are shown in each column and annotated below with unsupervised clusters shown in rows. Clusters with expression of glial markers and low expression of other markers (neurons, endothelial cells, microglia) are shown with gray boxes and selected for subclustering. The size of each circle indicates percentage of cells in the cluster that express the marker (≥ 1 UMI) while the color shows the average expression of transcript in cells. **(C)** Subclustered glial cell types from (C), with unsupervised clusters labeled with cell type identity. These nuclei were used in Fig. 1F-H.

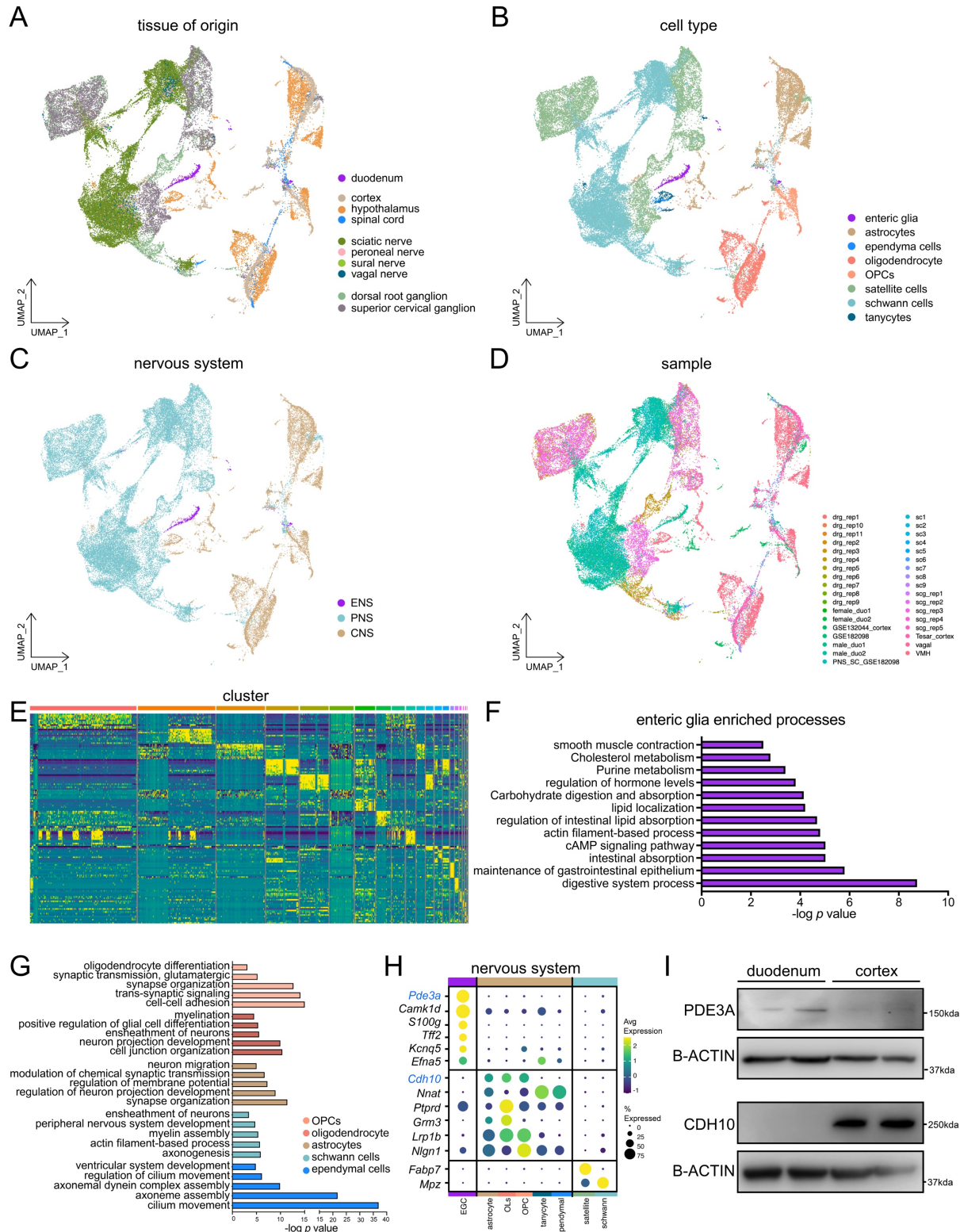


Fig. S4. Comparison of glial cells throughout the body. (A to D) Glial cells from Fig. 1F visualized by UMAP and colored by tissue of origin (A), cell type (B), nervous system (C), and individual sample (D). (E) Top 10 differentially expressed transcripts enriched in each of the unsupervised clusters in glia

throughout the body. Individual transcripts are shown in rows, unsupervised clusters are shown together in columns. All data are in Table S2. **(F-G)** Gene ontology analysis of top 100 enriched enteric glia (F) or other glial identity (G) transcripts when compared to other glia in the body using Metascape. **(H)** Dot plot showing expression of select differentially expressed genes across nervous systems. Cell types are shown in columns with nervous system depicted by colors on the top matching (C), with cell type annotated and colored at the bottom. Rows show transcripts with validated differential expression highlighted in blue. The size of each circle indicates percentage of cells in the cluster that express the marker (≥ 1 UMI) while the color shows the average expression of transcript in cells. **(I)** Western blot of select differentially expressed genes PDE3A and CDH10 in whole duodenum and whole cortex protein lysate showing differential protein expression. Each lane is a biological replicate.

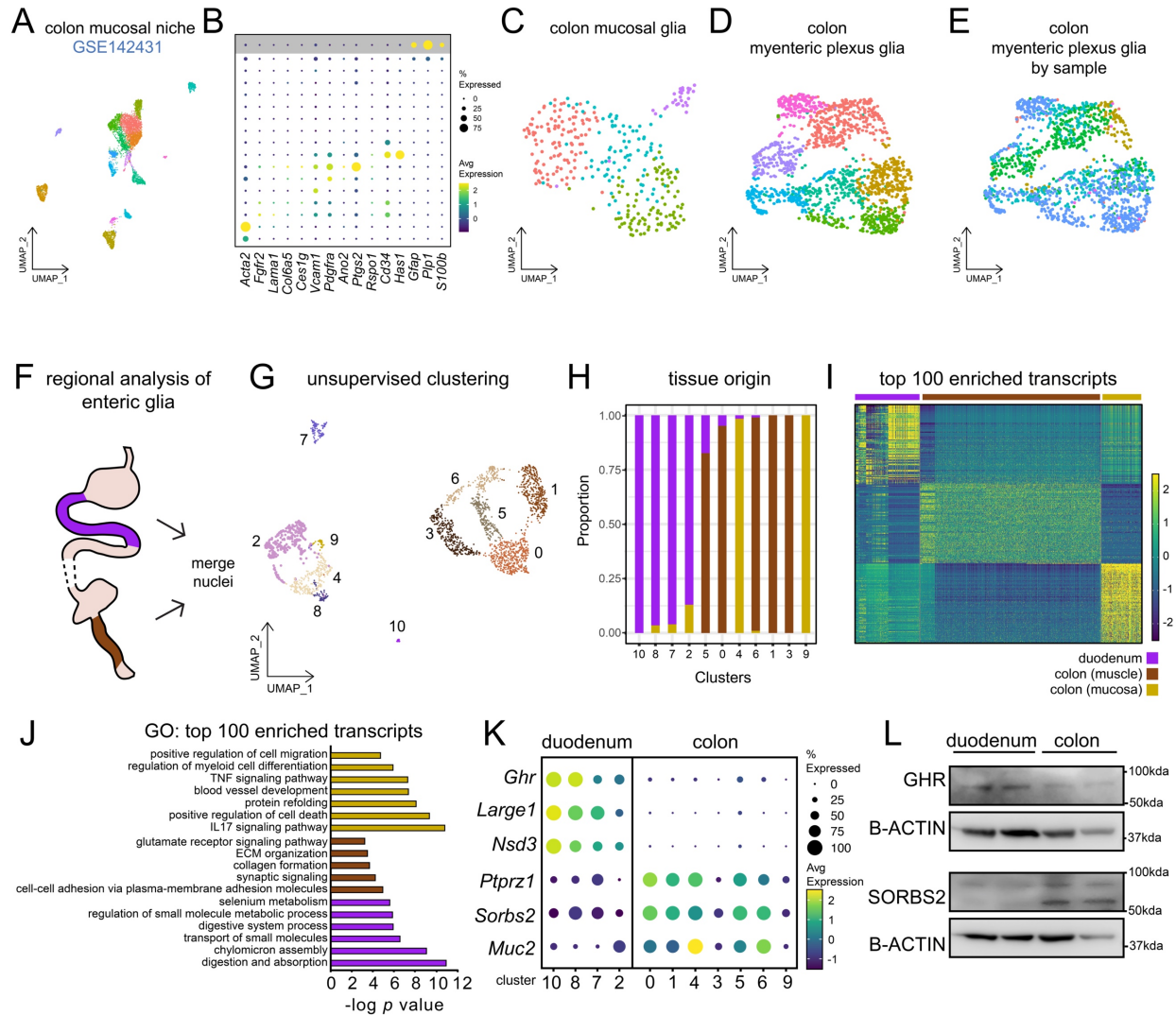


Fig. S5. Regional analysis of enteric glial cells. (A) snRNAseq of colon mucosal cells from GSE142431 colored by unsupervised clusters and visualized by UMAP. (B) Dot plot showing cell type markers shown in columns with unsupervised clusters in rows. One column containing glia are highlighted in gray. The size of each circle indicates percentage of cells in the cluster that express the marker (≥ 1 UMI) while the color shows the average expression of transcript in cells. (C) Subclustered enteric glia from the colon mucosa colored by unsupervised clusters. (D-E) Colon myenteric plexus glia from SCP1038(22) visualized by UMAP and colored by unsupervised clusters (D) or by biological replicate (E). (F) Schematic of study design. Duodenal tissue was dissected and processed for snRNAseq. Datasets were obtained from sorted *Sox10*-cre; INTACT nuclei from the myenteric plexus of the colon (SCP1038) and from mechanically isolated colonic mucosal nuclei (GSE142431). (G) snRNAseq of enteric glial cells in the duodenum and colon colored by unsupervised clusters and visualized by UMAP (n=15 mice, 2,303 nuclei). (H) Proportion mapping of cell types to unsupervised clusters. Cell types are shown by color with key shown to the bottom right. Proportion of cells are shown on the y-axis while columns binned in the x-axis show unsupervised clusters. (I) Top 100 differentially expressed transcripts enriched between tissues. Individual transcripts are shown in rows, tissue origins are clustered together in columns with color annotation above. All data are in Table S3. (J) Gene ontology analysis of top 100 enriched duodenal or mucosal and myenteric colonic transcripts using Metascape. (K) Dot plot showing expression of select differentially expressed genes across tissues. Unsupervised clusters from each tissue are shown in columns, rows show transcripts. The size of

each circle indicates percentage of cells in the cluster that express the marker (≥ 1 UMI) while the color shows the average expression of transcript in cells. (L) Western blot of select differentially expressed genes GHR and SORBS2 in whole duodenum and whole colon protein lysate showing differential protein expression. Each lane is a biological replicate.

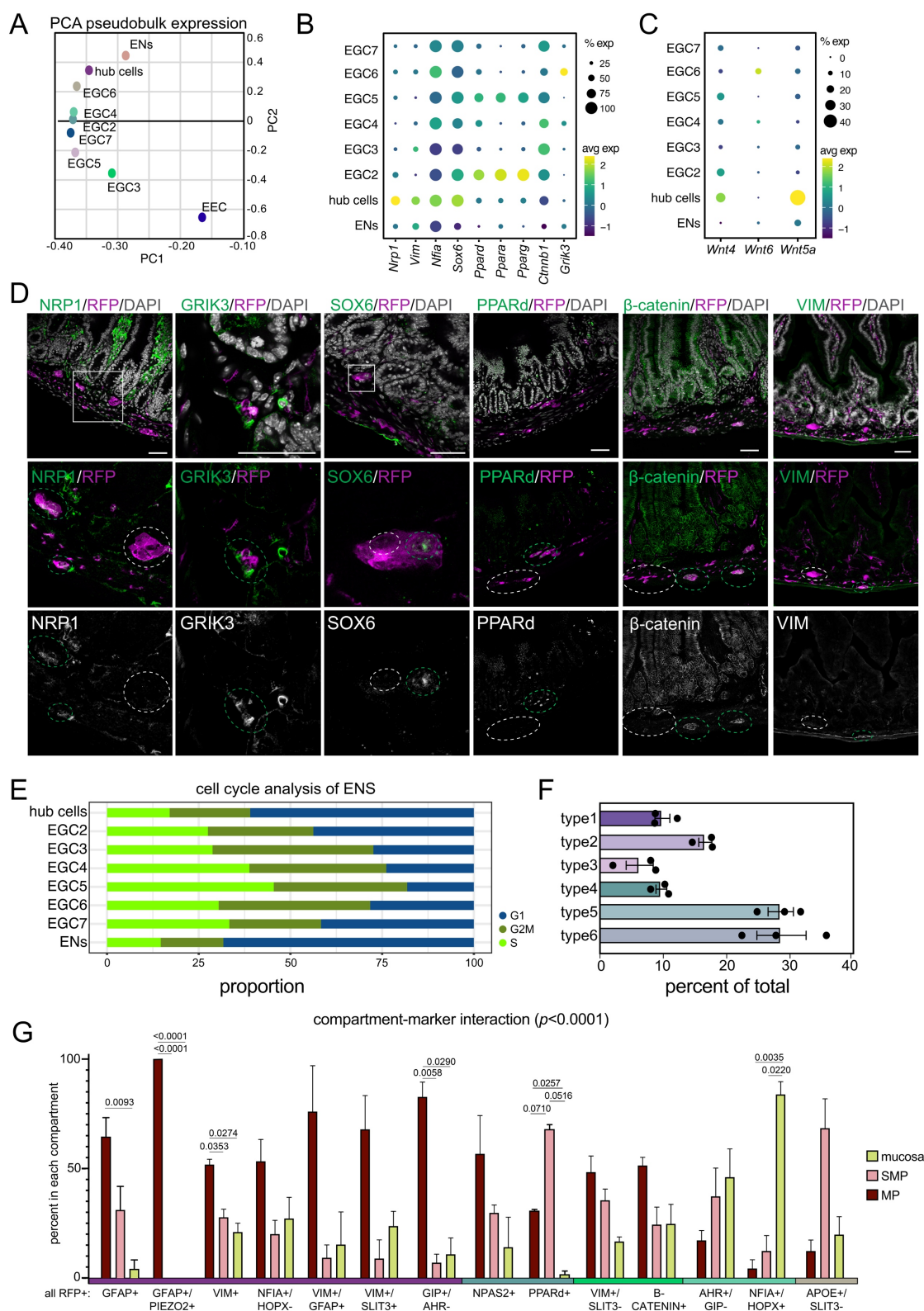


Fig. S6. Enteric glia are diverse. (A) Principal component analysis (PCA) of pseudobulk enteric nervous system clusters with enteroendocrine cells as a variable control. (B) Expression of select transcripts in enteric nervous system clusters. (C) Expression of *Wnt* ligands reported in a previous study (25) in enteric

nervous system clusters. **(D)** Immunostaining showing enteric glia subtype markers expressed only in subsets of cells. RFP marks tdTomato from *Sox10*-cre; RCL-tdT mice. DAPI marks nuclei in white. White boxes show area that is enlarged below in separate channels. Green dashed ovals show enteric nervous system cells co-expressing the marker of interest, while white dashed ovals show enteric nervous system cells not expressing the marker of interest. Scale bar = 50um. **(E)** Cell cycle scoring of enteric nervous system nuclei based on expression of G1, G2/M, and S phase markers shown as proportion of total per cell type. **(F)** Quantification of enteric glia morphologies in culture (n=3 mice). **(G)** Quantification of the proportion of enteric glial subtype enriched markers in each compartment of the gut, including mucosa, submucosa (SMP), and myenteric plexus/muscle layer (MP) using *Sox10*-cre; RCL-tdT mice. Color beneath bar plot indicates which enteric glia subtype the marker combination supports. P values are from two-way ANOVA with multiple comparisons. Error bars show SEM.

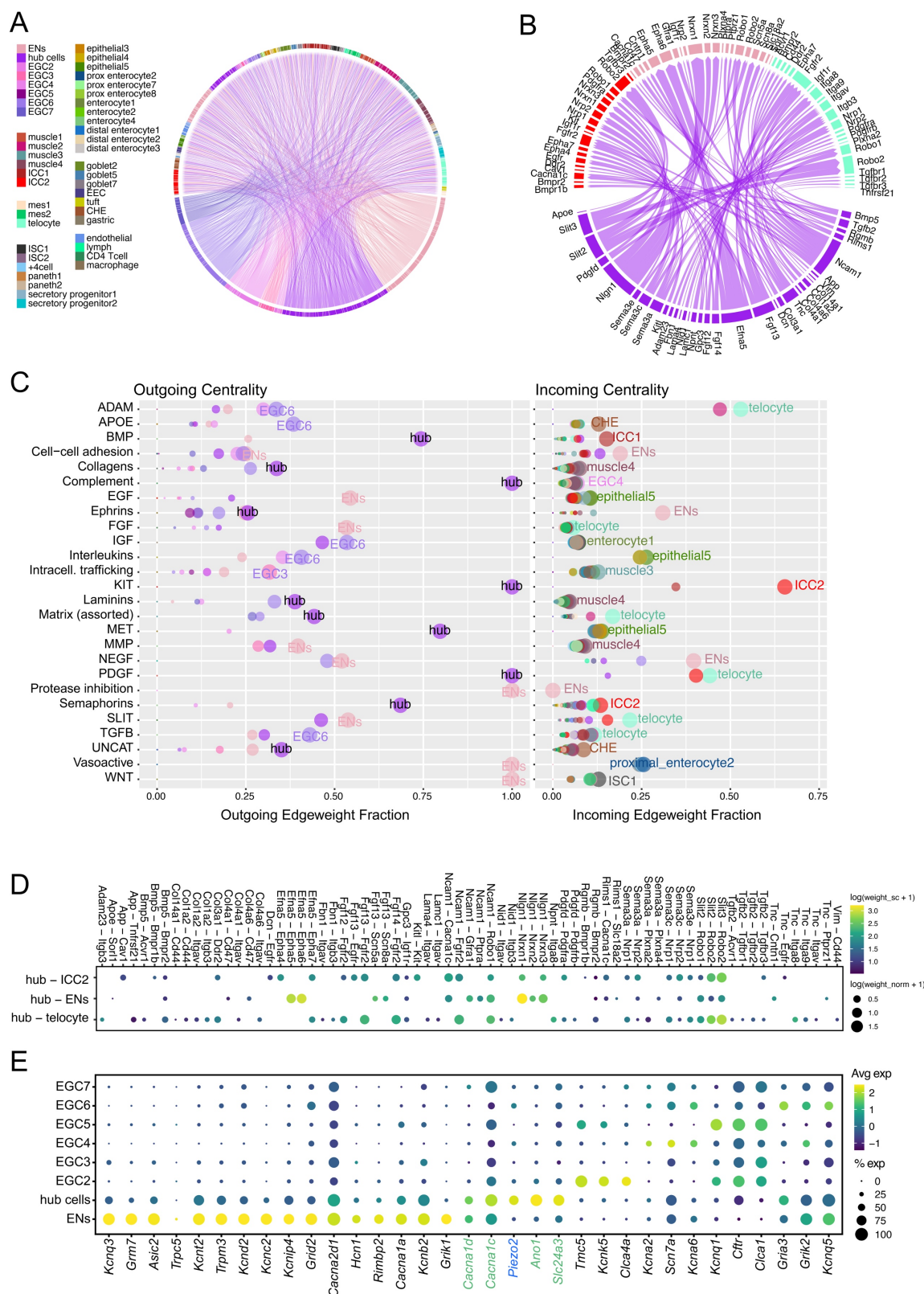


Fig. S7. Interactome analyses predicting functional links of enteric nervous system cells with other duodenal cell types. (A) Interactome analyses of putative ligand-receptor pairs using the FANTOM5 database ($p < 0.00001$, percent > 0.2) showing connections of enteric nervous system ligands expressed

(bottom) with receptor expression in all duodenal cell types (top). **(B)** Ligand-receptor pairs with ligands from hub cells matched to receptors in the top 3 predicted interaction partners, interstitial cells of Cajal or ICC2, enteric neurons or ENs, and telocytes. **(C)** Full centrality plot showing all predicted signaling between enteric nervous system cells and other duodenal cells as shown in Fig. 4A. **(D)** Dot plot showing signal strength and signal specificity together for hub cells and top predicted interaction partners, ICC2, ENs, and telocytes. **(E)** Full dot plot showing transcript levels of all monoatomic ion channels (GO: 0005216) significantly enriched in different enteric nervous system cell clusters as shown in Fig. 4C.

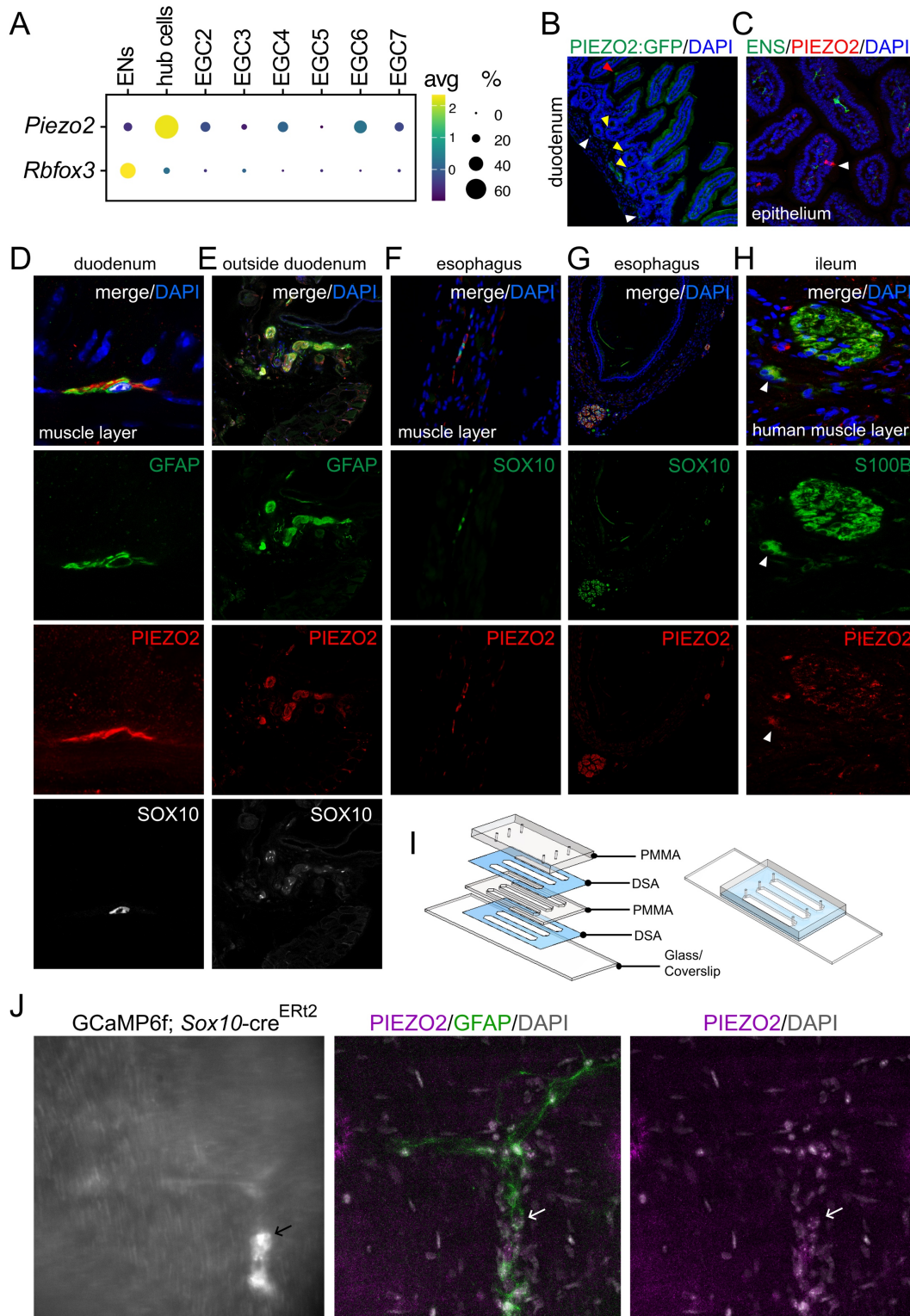


Fig. S8. PIEZO2 expression in the gut and surrounding tissue. (A) Quantification of PIEZO2+ cells and GFAP+ cells. All PIEZO2+ cells co-expressed GFAP. (B) Dot plot showing expression of *Piezo2* and neuron marker *Rbfox3* to illustrate expression of *Piezo2* outside of mechanosensory enteric neurons (MENs). The size of each circle indicates percentage of cells in the cluster that express the marker (≥ 1)

UMI) while the color shows the average expression of transcript in cells. **(C)** Expression of PIEZO2:GFP with DAPI shown in blue in the duodenum. Red arrow shows enterochromaffin cell in the epithelium. Yellow arrows show basement membrane cells beneath the epithelium with mesenchymal morphology. White arrows show ganglia like morphology in the muscle layer. **(D)** Expression of PIEZO2 using antibody staining in enterochromaffin cells in the epithelium in *Sox10*-cre; RCL-tdT mice. **(E)** Longitudinal muscle myenteric plexus preparation stained for SOX10, PIEZO2, and GFAP with DAPI shown in blue. **(F)** Immunostaining of ganglia on periphery of the intestine. **(G)** Immunostaining of PIEZO2 in enteric glia in the esophagus. **(H)** Immunostaining of ganglia on periphery of the esophagus. **(I)** Human enteric ganglia in the ileum muscle layer with enteric glia in green and PIEZO2 in red with DAPI showing nuclei in blue. **(J)** Assembled view of the intestine-on-a-chip. Channels are 4 mm wide, 24 mm long, and 1.1 mm in height. **(B)** Exploded view of the intestine-on-a-chip depicting the individual layers of polymethylmethacrylate (PMMA), double-sided adhesive (DSA) film, and glass. Top PMMA layer is 3.175 mm, DSA layers are 50 μ m, and middle PMMA layer is 1 mm. Two types of glass were used; for calcium imaging, cover slips were used for the glass layer while standard microscope slides were used for contraction imaging. **(K)** Left: still image from live imaging GCaMP6f upon stimulation with 1 dyn/cm² shear stress in GCaMP6f; *Sox10*-cre^{ERT2} mouse longitudinal muscle myenteric plexus. Middle and right: immunostaining of tissue shown on left for PIEZO2 with GFAP marking a subset of enteric glia and DAPI marking nuclei in white. Arrow points to mechanosensory cell co-expressing PIEZO2 with GFAP.

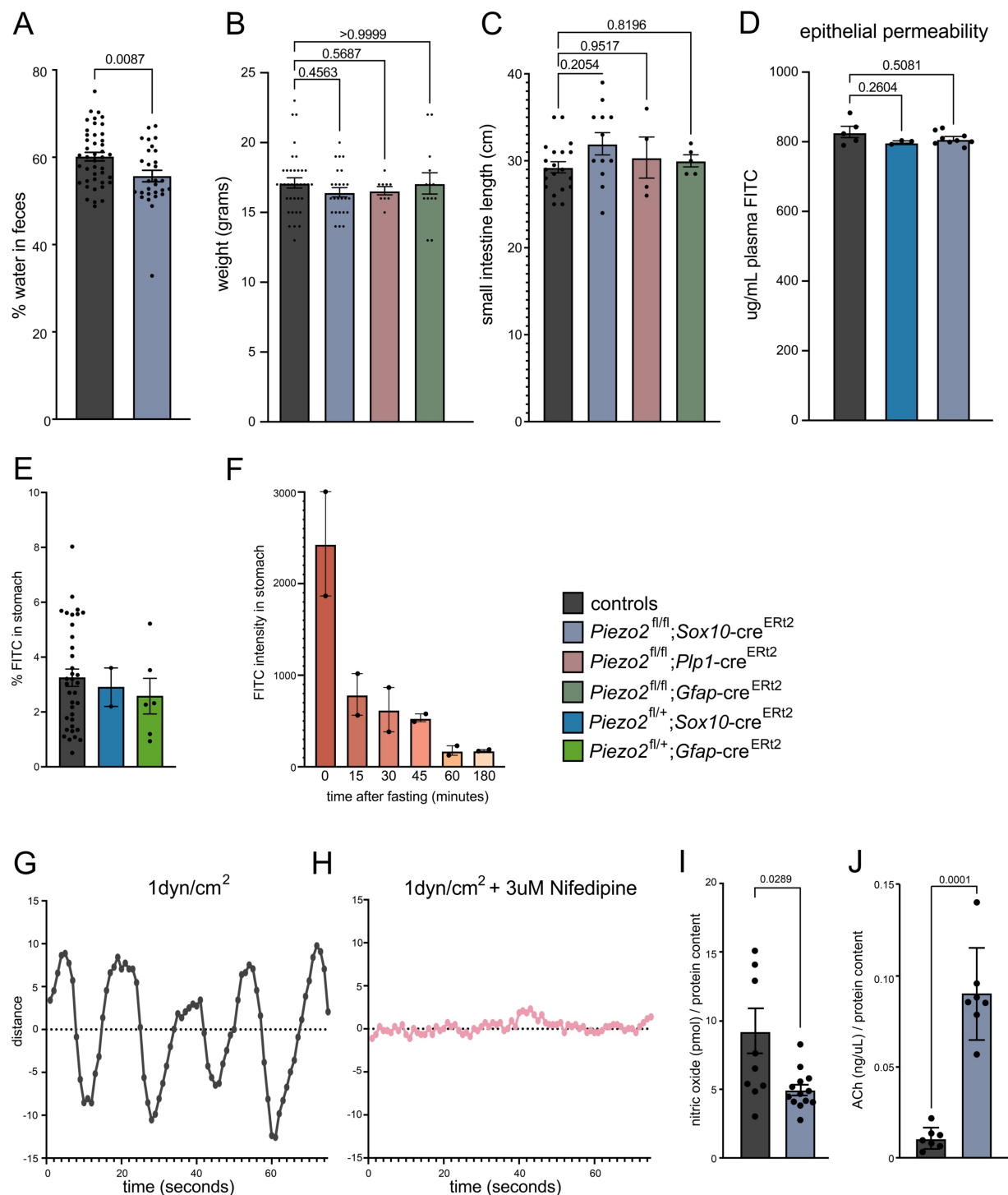


Fig. S9. Phenotyping mice without *Piezo2* in enteric glia. (A) Percent fecal water composition. (B-D) At six weeks of age, the weight (B), small intestinal length (C), and epithelial permeability (D) of mice. (E) Gastric emptying in mice with one functional copy of *Piezo2*. (F) Pilot study determining fasting length for emptying of chow from stomach. (G) Rhythmic oscillatory contractions in mice after stimulation with shear stress at 1dyn/cm². (H) Loss of rhythmic oscillations in mice treated with 3uM Nifedipine to block voltage gated calcium channels. (I) Levels of nitric oxide and (J) acetylcholine in the longitudinal muscle myenteric

plexus relative to total protein content. All error bars show SEM with individual mice shown as dots. P values by one-way Welch's ANOVA with multiple comparisons in (B-D) and Welch's t-test in (I-J).

Table S1.

Transcriptional blueprints of the whole adult mouse duodenum.

Table S2.

Differentially expressed genes across glia in the body.

Table S3.

Regionally enriched transcriptional profiles of glia in the duodenum and colon.

Table S4.

Molecular diversity of the enteric nervous system.

Table S5.

Functional interaction network of cells in the adult duodenum.

Movie S1.

Calcium imaging in GCaMP6f; *Sox10*-cre^{ERT2} mouse intestine-on-a-chip. Flash of light indicates the onset of flow as media washes over tissue. Two cells respond to mechanosensation after onset of flow.

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