

Age-associated changes in lineage composition of the enteric nervous system regulate gut health and disease

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Abstract

The enteric nervous system (ENS), a collection of neural cells contained in the wall of the gut, is of fundamental importance to gastrointestinal and systemic health. According to the prevailing paradigm, the ENS arises from progenitor cells migrating from the neural crest and remains largely unchanged thereafter. Here, we show that the lineage composition of maturing ENS changes with time, with a decline in the canonical lineage of neural-crest derived neurons and their replacement by a newly identified lineage of mesoderm-derived neurons. Single cell transcriptomics and immunochemical approaches establish a distinct expression profile of mesoderm-derived neurons. The dynamic balance between the proportions of neurons from these two different lineages in the post-natal gut is dependent on the availability of their respective trophic signals, GDNF-RET and HGF-MET. With increasing age, the mesoderm-derived neurons become the dominant form of neurons in the ENS, a change associated with significant functional effects on intestinal motility which can be reversed by GDNF supplementation. Transcriptomic analyses of human gut tissues show reduced GDNF-RET signaling in patients with intestinal dysmotility which is associated with reduction in neural crest-derived neuronal markers and concomitant increase in transcriptional patterns specific to mesoderm-derived neurons. Normal intestinal function in the adult gastrointestinal tract therefore appears to require an optimal balance between these two distinct lineages within the ENS.

eLife assessment

This paper identifies a subset of neurons within adult mouse myenteric ganglia that are not labeled via canonical neural-crest labeling, and argues, based on extensive lineage tracing, imaging and genomic data that these neurons are derived from mesoderm. There is **convincing** support for the existence of an unusual cell type in the gut, an intriguing and **important** observation that will be of interest to anyone studying the development of the nervous system. While the data are **incomplete** with respect to definitively establishing that these are mesodermally derived cells, there is sufficient evidence to support the provocative and paradigm-shifting hypothesis of a non-ectodermal origin for enteric neurons to warrant deeper investigation.

Introduction

The enteric nervous system (ENS) is a large collection of neurons, glial, and precursor cells that resides within the gastrointestinal wall and regulates gut motility and secretion along with modulating epithelial and immune cell function^{1, 2}. During fetal development, the mammalian ENS is populated by neurons and glia derived from neural crest (NC)-derived precursors^{3–9}. These precursors follow diverse migratory routes to colonize and innervate various parts of the gut before birth^{10–12}. It is not clear, however, that this lineage persists in its entirety in the adult gut, as indicated by the observed lack of expression of fluorescent reporter protein in a subpopulation of adult enteric neurons in NC-lineage-traced mice^{13, 14}. Alternative sources of enteric neurons that have been proposed in the literature include the ventral neural tube (VENT)¹⁵, or the Pdx1- expressing pancreatic endoderm¹⁴, but the interpretation of these studies has been limited by the lack of robust lineage markers for non-NC derived neurons¹⁶. In addition, while prior studies have documented cellular changes to the ageing ENS¹⁷, the developmental mechanisms behind these changes are unknown. Thus, confirmation of a second, distinct lineage of enteric neurons in adults is important for our understanding of the healthy post-natal development and aging of the ENS, as well as for the pathogenesis of acquired disorders of the ENS.

In this study, we found that while the early post-natal ENS is derived from the canonical NC-lineage, this pattern changes rapidly as the ENS matures, due to the arrival and continual expansion of a novel population of Mesoderm-derived Enteric Neurons (MENs) which represent an equal proportion of the ENS in young adulthood and with increasing age, eventually outnumber the NC-derived Enteric Neurons (NENs). We also found that, while the NEN population is regulated by glial derived neurotrophic factor (GDNF) signaling through its receptor RET, the MEN population is regulated by hepatocyte growth factor (HGF) signaling. Increasing HGF levels during maturation or by pharmacological dosing increase proportions of MENs. Similarly, decrease in GDNF with age decrease NENs; and increasing GDNF levels by pharmacological dosing increase NENs proportions in the adult ENS to impact intestinal motility.

These results indicate for the first time that the mesoderm is an important source of neurons in the second largest nervous system of the body. The increasing proportion of neurons of mesodermal lineage is a natural consequence of maturation and aging; further, this lineage can be expected to have vulnerabilities to disease that are distinct from those affecting the NEN population. These findings therefore provide a new paradigm for understanding the structure and function of the adult and aging ENS in health, age-related gut dysfunction and other acquired disorders of gastrointestinal motility.

Only half of all mid-age adult enteric neurons are derived from the neural crest

We analyzed small intestinal longitudinal muscle-myenteric plexus (LM-MP) from adult (post-natal day 60; P60) Wnt1-cre:*Rosa26*-tdTomato mice, in which tdTomato is expressed by all derivatives of Wnt1⁺ NC-cells.¹⁸ In these tissues, while GFAP, a glial marker, was always co-expressed with tdTomato (Fig 1a), tdTomato-expression was absent in many myenteric neurons (Fig 1b, c). By careful enumeration, almost half of all myenteric neurons expressing the pan-neuronal marker Hu were found to not express the reporter (percent tdTomato- neurons: 44.27 ± 2.404 SEM; enumerated from 2216 neurons from 6 mice, Fig 1d). In these lineage-traced mice, myenteric ganglia were found to contain tdTomato^{high} and tdTomato^{low} neurons and due care was taken to image subsets (Suppl. Fig 1a). Both tdTomato^{high} and tdTomato^{low} neurons were classified as tdTomato+ and only neurons that did not show any tdTomato-expression were classified as tdTomato- neurons. Immunofluorescence staining of small intestinal LM-MP tissue from adult Wnt1-cre:*Rosa26*-tdTomato mice using antibodies against tdTomato protein showed a consistent lack of reporter expression in a population of myenteric neurons (Suppl. Fig 1b). This lack of reporter expression is not a function of tissue peeling or fixation, as the tdTomato- cells within the myenteric ganglia were also observed in freshly harvested unpeeled gut tissue, when imaged using a live tissue fluorescence microscope (Suppl. Fig 1c). In addition, we noticed that myenteric ganglia of the Wnt1-cre:*Rosa26*-tdTomato transgenic mouse show similar tdTomato aggregation in cells of the myenteric plexus from both freshly harvested and immediately fixed tissue (Suppl. Fig 1d, e). Since we previously showed ongoing neuronal loss in healthy ENS,¹⁹ we reasoned that cells that show hyper-aggregation of tdTomato are in advanced stages of cell death. Cells with hyper-aggregation of tdTomato showed a lack of staining for the nuclear marker DAPI (Suppl. Fig 1e), suggesting that these are indeed cells in the advanced stages of death, and given that they are not labeled with antibodies against Hu, their presence does not alter our estimation of tdTomato-expressing neurons (Suppl. Fig 1f). Thus, the absence of reporter expression in myenteric ganglia of freshly harvested tissue and the presence of tdTomato aggregation in both freshly harvested and fixed tissues shows that our observations are not caused due to any technical issues in tissue isolation and preservation.

We next sought additional confirmation that the lack of tdTomato expression in this often-used lineage fate mapping mouse model was not due to incorrect activity at the *Rosa26* locus where the floxed reporter transgene is located, due to aberrant cre activity under the Wnt1 promoter, or due to issues with the antibodies used to detect the pan-neuronal protein Hu. This was provided by an analogous Wnt1-cre:*Hprt*-tdTomato lineage-traced mouse line, in which tdTomato was expressed from the *Hprt* locus in a Wnt1-cre-dependent manner (*Hprt* locus is X-inactivated in females, hence only adult male mice were used)²⁰; and by the Pax3-cre:*Rosa26*-tdTomato lineage-traced mouse line, where *Rosa26*-tdTomato driven by the Pax3-cre transgene labels the derivatives of the neural tube and pre-migratory NC (Suppl. Fig 1g, h).²¹ Similar lack of reporter expression was previously observed by the Pachnis Lab in adult myenteric neurons from Sox10- cre NC-lineage-traced mice, and more recently by the Heuckeroth Lab in significant numbers of myenteric neurons from Wnt1-cre:*Rosa26*-H2B-mCherry mice, which expresses nuclear localized reporter mCherry in NC-lineage cells, further confirming our observations of the presence of non- NC-derived neurons in the adult ENS.^{13, 22} In addition to the ANNA-1 antisera, which is known to detect all neuronally significant Hu proteins (HuB, HuC, and HuD),²³ we found that another often used antibody thought to be specific to HuC and HuD also detects HuB protein (Suppl. Fig 1i) – showing that these antibodies do not detect the expression of a specific neuronal Hu antigen.

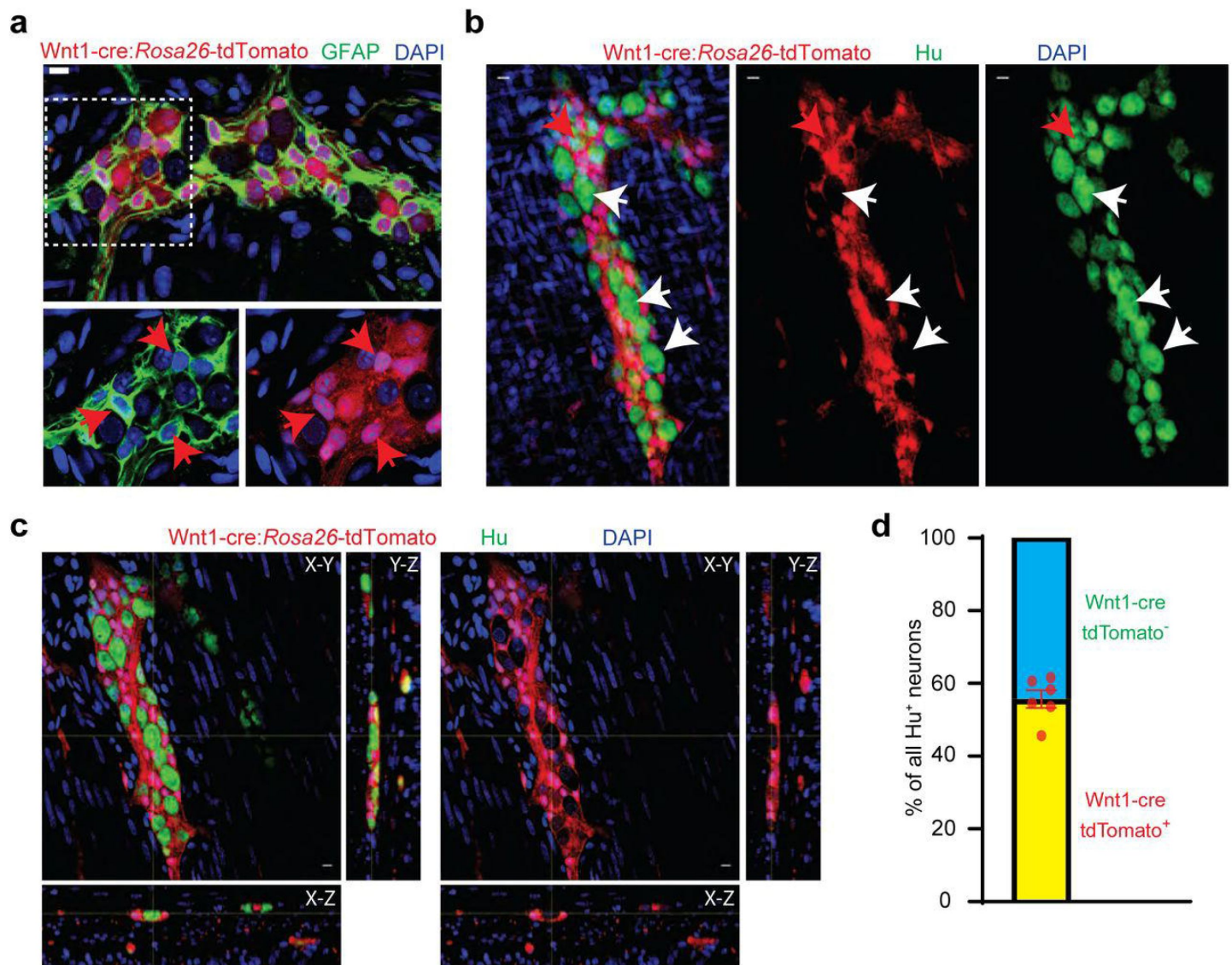


Fig 1

Half of all adult small intestinal myenteric neurons are derived from a non-neural crest lineage.

- (a) Enteric glia, labeled with GFAP (green) in the myenteric plexus from an adult Wnt1- cre:Rosa26-tdTomato mouse. Inset is magnified in color segregated panels, showing GFAP⁺ glia co-expressing tdTomato (red, red arrows). Nuclei are labeled with DAPI (blue). Scale bar = 10 μ m.
- (b) 2 dimensional representation of a 3 dimensional stack of images shows enteric neurons, labeled with Hu (green), in the myenteric plexus from an adult Wnt1-cre:Rosa26-tdTomato mouse. Enteric neurons either express tdTomato (red arrow) or not (white arrows). Nuclei are labeled with DAPI (blue). Scale bar = 10 μ m.
- (c) Orthogonal views of the image (b) shows the lack of tdTomato expression in a particular Hu-immunolabeled enteric neuron.
- (d) Quantification of tdTomato⁺ and tdTomato⁻ neurons in the myenteric ganglia of 6 P60 Wnt1- cre:Rosa26-tdTomato mice. Data represents Mean $\pm$ SEM.

Lineage-tracing confirms a mesodermal derivation for half of all adult myenteric neurons

Alternative sources of enteric neurons proposed previously include the ventral neural tube¹⁵, and the pancreatic endoderm¹⁴, but the interpretation of these studies was limited by the lack of robust lineage markers¹⁶. Brokhman et al.¹⁴ found evidence of labeled neurons in inducible Pdx1-cre, Foxa2-cre, and Sox17-cre lineage-traced mouse lines and inferred their derivation from pancreatic endoderm. However, in a Pdx1-cre lineage-traced mouse line, many neuroectoderm-derived neurons of the central nervous system have also been previously shown to be derived from Pdx1-expressing cells²⁴, suggesting that Pdx1 is not an exclusive endodermal marker. Foxa2 is expressed by mesoderm-derived cells in the heart²⁵ and Sox17 also labels mesoderm-derived cells, especially cells of the intestinal vasculature^{26–29}. We therefore hypothesized that the embryonic mesoderm may be the true developmental source of the non-NC enteric neurons.

Mesoderm-derived cells during embryogenesis express Tek²⁵ and analysis of LM-MP tissues from adult male Tek-cre:*Hprt*-tdTomato lineage-traced mice revealed the presence of a population of tdTomato⁺ neurons (**Fig 2a**). Since Tek is not expressed in the adult ENS (Suppl. Fig 2a), the presence of Tek-derived adult myenteric neurons suggested their mesodermal origin. We then used *mesoderm posterior 1* or Mesp1 as a definitive developmental marker for the embryonic mesoderm^{30–34} to confirm the mesodermal derivation of the non-NC derived adult enteric neurons. Reporter expression in this mouse line has been previously studied in cardiac development, which we confirmed by observing its expression in the adult heart tissue (**Fig 2b**). Expression of tdTomato in vascular cells of both Tek-cre and Mesp1-cre mice was more pronounced than in myenteric neurons (**Fig 2a, c, d**) and in cardiac myocytes of Mesp1-cre mice (**Fig 2b**, Suppl. Fig 2b), which reflects the variable expression of the reporter gene in both the neural crest-lineage and mesoderm-lineage specific transgenic mouse lines (**Fig 2b, c**; Suppl. Figs 1a, 2c). Using an analogous Mesp1-cre:*Rosa26*-EGFP lineage-traced mouse line, in which EGFP was expressed from the *Rosa26* locus in a Mesp1-cre-dependent manner, we next confirmed the derivation of a population of myenteric neurons from Mesp1-expressing mesoderm (**Fig 2e**, Suppl. Fig 2d, e). Similar to our observations with the tdTomato reporter (**Fig 2a–d**), we observed lower expression of the reporter EGFP in myenteric neurons as compared to vascular cells (**Fig 2e**; Suppl. Fig 2d, e). Variable expression of CAG and other pan-cellular promoters in various cell-types and during processes of maturation have previously been reported^{35–38}. In addition, an earlier study by Agah et al. showed differing degrees of CAG-driven LacZ reporter activation in a cardiac-specific transgenic cre mouse line, which were unrelated to copy number, suggesting insertional and positional effects or, potentially, differential methylation³⁹. These reports are consistent with our observation and could potentially help explain the observed variable expression of CAG and CMV promoter-driven reporter genes in our study.

Analysis of small intestinal LM-MP from P60 Mesp1-cre:*Rosa26*-tdTomato lineage-traced mice showed that tdTomato-expression was observed in about half of all myenteric neurons (**Fig 2f**; percent tdTomato⁺ neurons: 50.28 ± 2.89 SEM; enumerated from 484 neurons from 3 mice). Because of the variable expression of the reporter gene in the transgenic mice described above, we also labeled Mesp1-cre:*Rosa26*-tdTomato⁺ neurons with the S46 antibody, which was raised against the slow tonic myosin heavy chain protein MHCst derived from avian embryonic upper leg muscle, and is expressed exclusively by mesoderm-derived cell populations (**Fig 2g**)^{40–43}. MHCst immunostaining was exclusively observed in all Tek-cre:*Rosa26*-tdTomato⁺ MENs (Suppl. Fig 2f). None of the 107 MHCst⁺ intra-ganglionic cells observed (35.67 ± 6.17 SEM MHCst⁺ cells per mouse) across 3 Wnt1-cre:*Rosa26*-tdTomato mice expressed tdTomato, suggesting that MHCst immunostaining was exclusive to non-NC lineage neurons (**Fig 2h**, Suppl. Fig 3a shows the same image where the MHCst immunostained ganglia can be observed with and without the tdTomato channel for better visualization). MHCst antibody was also found to label extra-ganglionic smooth muscle cells in the LM-MP tissue (Suppl. Fig 3a). Thus, both lineage tracing and protein biomarker

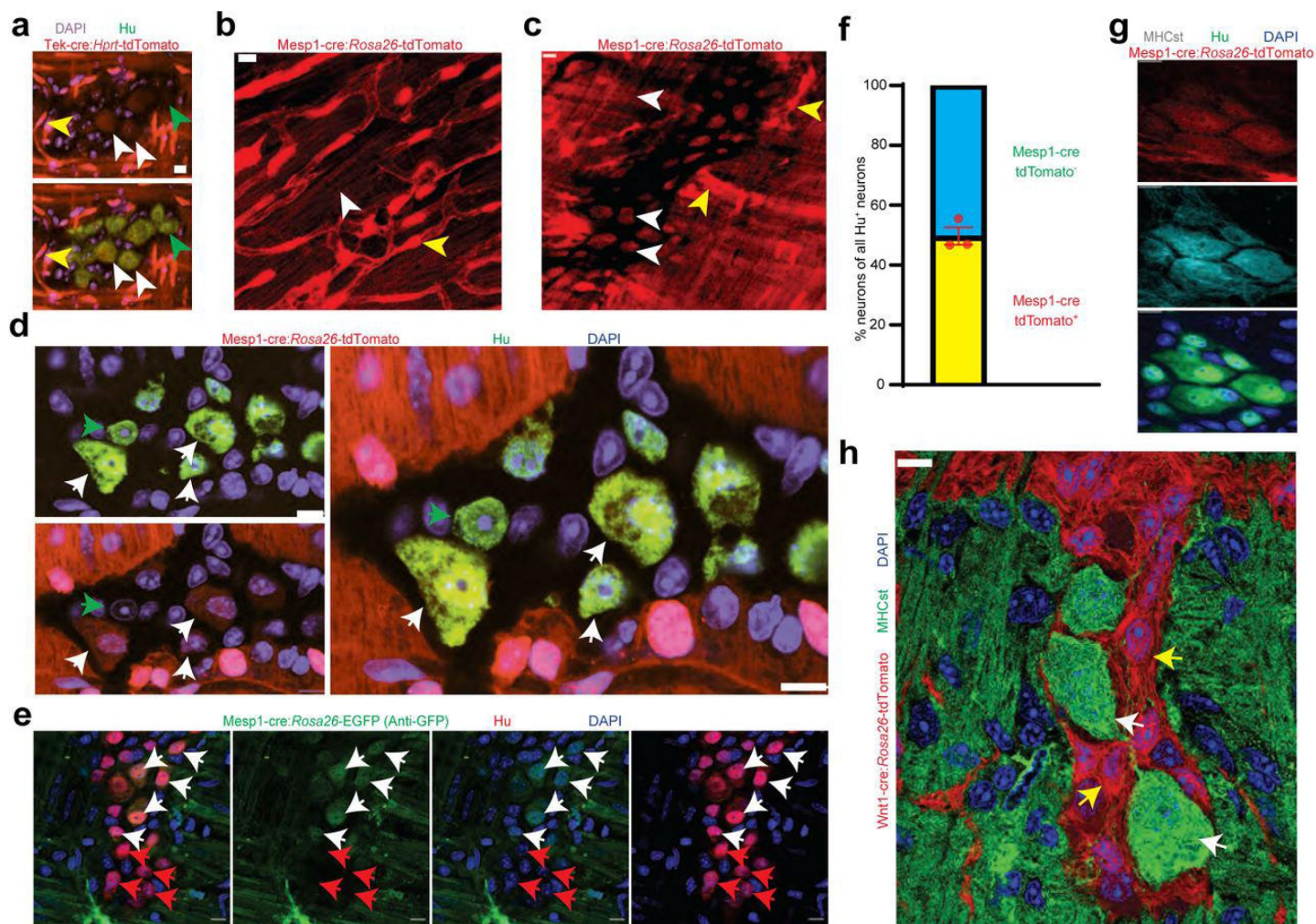


Fig 2

Mesoderm-lineage-tracing and marker expression provide evidence of the mesodermal-derivation of half of all adult small intestinal myenteric neurons.

(a) Small intestinal LM-MP from adult male Tek-cre:Hprt-tdTomato shows the presence of Tek- derived and hence mesoderm-derived tdTomato⁺ (red) Hu⁺ (green) neurons (white arrows) and non-mesoderm-derived tdTomato⁻ neurons (green arrows). Vascular cells depict higher fluorescence intensity (yellow arrow) than cells in the ganglia. Nuclei are labeled with DAPI. Scale bar = 10 μ m.

Cardiac and small intestinal tissues from **(b)** heart and **(c)** small intestinal LM-MP layer of adult male Mesp1-cre:Rosa26-tdTomato mice shows the presence of variable tdTomato expression in cells of these tissues. While cardiomyocytes in heart and cells within the myenteric ganglia (white arrows) exhibit lower tdTomato fluorescence, similar to the tissues from Tek-cre:Hprt-tdTomato mice in **(a)**, the vascular cells exhibit higher fluorescence intensity (yellow arrows). Scale bar = 10 μ m.

(d) Small intestinal LM-MP from adult male Mesp1-cre:Rosa26-tdTomato mouse shows the presence of mesoderm-derived tdTomato⁺ (red) Hu⁺ (green) neurons (white arrows) and non-mesoderm-derived tdTomato⁻ neurons (green arrows). Nuclei are labeled with DAPI. Scale bar = 10 μ m.

(e) Small intestinal LM-MP from adult male Mesp1-cre:Rosa26-EGFP mouse, when immunostained with antibodies against GFP and against Hu, shows the presence of mesoderm- derived EGFP⁺ (green) Hu⁺ (red) neurons (white arrows) and non-mesoderm-derived EGFP⁻ neurons (red arrows). Nuclei are labeled with DAPI. Scale bar = 10 μ m.

(f) Quantification of tdTomato-expressing and non-expressing neurons in the myenteric ganglia of 3 P60 Mesp1-cre:Rosa26-tdTomato mice. Data represents Mean $\pm$ SEM.

MHCst immunolabeling exclusively marks all the **(g)** mesoderm-derived adult Hu⁺ (green) neurons in the small intestinal LM-MP of Mesp1-cre:Rosa26-tdTomato mice; and **(h)** all the non- NC-derived neurons in the small intestinal LM-MP of Wnt1-cre:Rosa26-tdTomato mice (white arrows). MHCst does not label tdTomato⁺ NC-derived cells (yellow arrows). Nuclei are stained with DAPI (blue). Scale bar = 10 μ m.

expression provides strong support for their mesodermal origin (**Fig 2g**, Suppl. Fig 3a). Along with our observations on *Wnt1-cre:Rosa26-tdTomato* mice (**Fig 1c**), our results indicate that these two distinct lineages together account for all the adult small intestinal myenteric neurons.

The proteins RET, a receptor tyrosine kinase that transduces GDNF signaling in NC-derived enteric neuronal precursors, and MET, a receptor for hepatocyte growth factor (HGF), are expressed by different subsets of adult myenteric neurons⁴⁴. MET is classically expressed by mesoderm-derived cells⁴⁵, and by using immunostaining of small intestinal LM-MP tissues from *Wnt1-cre:Rosa26-tdTomato* and *Mesp1-cre:Rosa26-tdTomato* mice, we found that the expression of MET was restricted to a sub-population of adult MENs (**Fig 3a, b**). By contrast, RET expression was confined to NENs (**Fig 3c**).

We then studied whether MENs and NENs differed phenotypically. In the current paradigm regarding the neurochemical basis for the functional classification of adult enteric neurons, inhibitory enteric motor neurons express the nitric oxide-producing enzyme nitric oxide synthase 1 (NOS1), excitatory motor neurons express the acetylcholine-producing enzyme choline acetyl transferase (ChAT), and enteric sensory neurons, called intrinsic primary afferent neurons (IPANs) express the neuropeptide calcitonin gene related peptide (CGRP)^{9, 44, 46}. Immunostaining using antibodies against NOS1, ChAT, and CGRP in the LM-MP from P60 *Wnt1-cre:Rosa26-tdTomato* mice showed that both *tdTomato*⁺ NENs and *tdTomato*⁻ MENs express these neuronal markers (**Fig 3d, f, h**). Quantification of proportions in these neuronal lineages shows that the majority of NOS1⁺ inhibitory neurons and ChAT⁺ excitatory neurons are NENs (**Fig 3e**; of the 616 NOS1⁺ neurons observed across 3 mice, NOS1⁺ *tdTomato*⁻: 27.06% ± 4.46 SEM; *p* < 0.0001; Fisher's exact test); (**Fig 3g**; of the 912 ChAT⁺ neurons observed across 3 mice, ChAT⁺ *tdTomato*⁻: 26.34% ± 2.54 SEM; *p* < 0.0001; Fisher's exact test). By contrast, the majority (~75%) of CGRP⁺ neurons were found to be MENs (**Fig 3i**; of the 146 CGRP⁺ neurons observed across 3 mice, CGRP⁺ *tdTomato*⁻: 75.76% ± 0.63 SEM; *p* < 0.0001; Fisher's exact test). These results are in keeping with a previous report by Avetisyan et al. that shows low expression of NOS1 (0% of MET⁺ neurons were NOS⁺) and ChAT (<8% of MET⁺ neurons were ChAT⁺) and abundant expression of CGRP by MET⁺ neurons in the adult ENS⁴⁴, previously not known to be derived from a different lineage. In addition, MENs also express Cadherin-3 (CDH3, Suppl. Fig 3b), which is known to also mark a sub-population of mechanosensory spinal neurons⁴⁷. Apart from their derivation from a distinct developmental lineage, the mean cell size of MENs was significantly larger than that of NENs (Suppl. Fig 4a; *n* = 143 neurons/group observed across 3 mice; Feret diameter (μm) MENs: 17.47 ± 0.50 SEM, NENs: 13.03 ± 0.36 SEM, *p* = 0.002; Student's *t*-test).

An expanded molecular characterization of MENs using unbiased single cell RNA sequencing (scRNAseq)-based analyses

Recent studies have used single cell or single nuclei-based transcriptomic analyses to query molecular diversity of enteric neurons. These studies have used either used enrichment methods based on FACS sorting neural crest-derived cells (using reporter-expression from neural crest-specific lineage fate mapping mice)^{22, 48, 49}, Phox2b- expressing cells⁵⁰, Baf53-derived cells⁵¹ or used classical NENs markers such as Snap25 to drive identification and/or enrichment of enteric neurons⁴⁹. Instead of using these strategies, we relied upon another accepted use of scRNAseq, which allows for sequencing of diverse un-enriched cell populations from a tissue, to discover any novel cell types in that tissue⁵². We performed truly unbiased and agnostic clustering of the scRNAseq data from tissues of two 6-month-old adult male C57BL/6 wildtype mice (**Fig 4a**; Suppl. Fig 4b, c). We identified clusters of NC-derived cells by exclusive expression of canonical NC markers *Ret* and *Sox10* (which include the clusters of *Ret*-expressing NENs and *Sox10*-expressing Neuroglia); and the MENs cluster by its co-expression of the genes *Calcb* (CGRP), *Met*, and *Cdh3* (**Fig 4a, b**). With cells from both samples pooled together, we compared 1,737 NC-derived cells across the NENs (77 cells) and Neuroglia (1660 cells) clusters with 2,223 cells in the MENs cluster. A list of top marker genes for every cluster are provided in Supplementary Table 2.

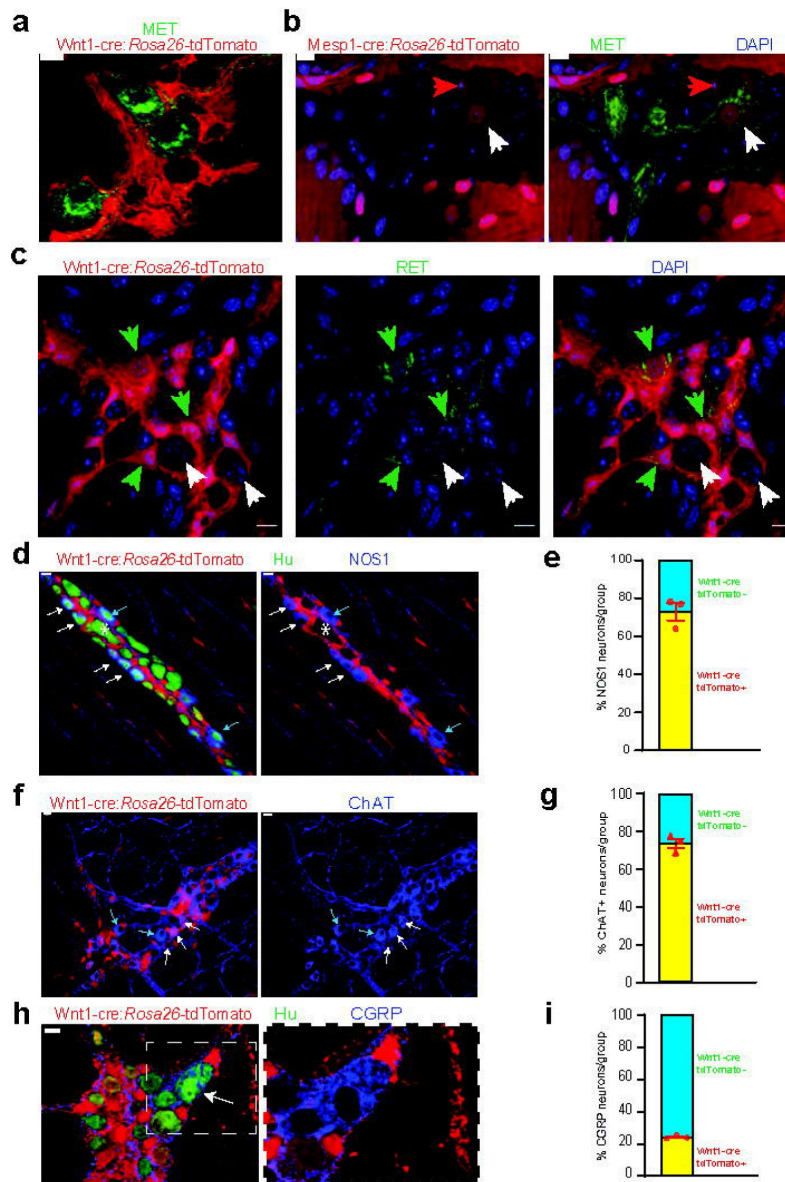


Fig 3

Cellular and molecular phenotyping of MENs and NENs.

MET immunostaining (green) labels (a) *Wnt1-cre:Rosa26-tdTomato*⁻ MENs and (b) a population of *Mesp1-cre:Rosa26-tdTomato*⁺ MENs (white arrow) while not labeling all MENs (red arrow).

(c) RET immunostaining (green) only labels *Wnt1-cre:Rosa26-tdTomato*⁺ NENs (green arrow) and not *tdTomato*⁻ MENs (white arrows).

(d) NOS1 is expressed by both *tdTomato*⁺ NENs (red, white arrows) and *tdTomato*⁻ MENs (blue arrows) in an adult *Wnt1-cre:Rosa26-tdTomato* mouse, but most MENs do not express NOS1 (marked by *).

(e) NEN lineage contains significantly higher proportions of NOS1⁺ neurons compared to MEN lineage in *Wnt1-cre:Rosa26-tdTomato* mice. Data represent mean $\pm$ S.E.M. $p < 0.0001$; Fisher's exact test.

(f) ChAT is expressed by both *tdTomato*⁺ NENs (red, white arrows) and *tdTomato*⁻ MENs (blue arrows) in an adult *Wnt1-cre:Rosa26-tdTomato* mouse.

(g) NEN lineage contains significantly higher proportions of ChAT⁺ neurons compared to MEN lineage in *Wnt1-cre:Rosa26-tdTomato* mice. Data represent mean $\pm$ S.E.M. $p < 0.0001$; Fisher's exact test.

(h) Both *tdTomato*⁺ (red) and *tdTomato*⁻ neurons in the myenteric plexus of an adult *Wnt1-cre:Rosa26-tdTomato* mouse (Hu, green) express CGRP (blue) Inset showing a *tdTomato*⁻ CGRP⁺ neuron (white arrow) is magnified on the right.

(i) MEN lineage contains significantly higher proportions of CGRP⁺ neurons compared to NEN lineage in *Wnt1-cre:Rosa26-tdTomato* male mice. Data represent mean $\pm$ S.E.M. $p < 0.0001$; Fisher's exact test.

Nuclei in (b) and (c) are labeled with DAPI (blue). Scale bar for all images denotes 10 μ m.

It is important to note here that it is well known that enzyme-based dissociation methods are unequally tolerated by diverse cell types, which is known to cause over- or under-representation of several cell types in scRNAseq^{53, 54}. The same is true for neurons, where certain neuronal subtypes may be significantly over- or under-represented in scRNAseq databases, compared to their true representation in tissue⁵⁵. Thus, the relative sizes of the NENs and MENs clusters in the scRNAseq data should not be taken as a reliable indicator of their actual proportions in tissue.

The neuronal nature of MENs was first established by testing our scRNAseq dataset for expression of genes known to be expressed by enteric or other neurons. We found detectable expression of genes *Elavl2* (HuB), *Hand2*, *Pde10a*, *Vsnl1*, *Tubb2b*, *Stmn2*, *Stx3*, and *Gpr88* in the MENs as well as in the NC-derived cell clusters (**Fig 4b**). While expression of *Elavl2*, *Stmn2* and *Hand2* has been previously observed in enteric neurons^{56, 57}, the expression of neuronal marker genes *Pde10a* (expressed by medium spiny neurons in the striatum⁵⁸), *Vsnl1* (expressed by hippocampal neurons⁵⁹), *Gpr88* (expressed by striatal neurons⁶⁰), *Stx3* (expressed by hippocampal neurons⁶¹), and *Tubb2b* (expressed by adult retinal neurons⁶²) has not been studied. Using antibodies against these markers along with co-staining with anti-Hu ANNA1 antisera to label neurons, we visualized the expression of these neuronal markers in both tdTomato⁺ NENs and tdTomato⁻ MENs of the small intestinal myenteric plexus tissue from adult Wnt1-cre:*Rosa26*-tdTomato mice (**Fig 4c**, Suppl. Fig 3l-q). In addition, the MENs cluster was also enriched in previously characterized ENS markers, such as *Ntf3* and *Il18*⁶³ (**Fig 4b**). We validated the MEN-specificity of these markers using immunochemical analyses (**Fig 4c**; Suppl. Fig 3b - k). In addition, we also detected >40 neuronally significant genes whose expression was unique to or enriched in MENs scRNAseq cluster, when compared with other larger cell clusters of macrophages, smooth muscle cells, neuroglia, and vascular endothelium (Suppl. Fig 5). These genes include neurotransmitter receptor genes (such as *Gabra1*, *Gabra3*, *Gria3*, *Grik5*, *Grind2d*, *Npy1r*); ion channels genes (such as *Cacna1a*, *Cacna1g*, *Cacnb3*, *Clcn3*); gap junction genes (*Gjc1*, *Gjb5*); transient receptor potential channel genes (*Trpv4*, *Trpc1*, *Trpc6*); potassium channel genes (such as *Kcnn1*, *Kcnj8*, *Kcnd3*, *Kcnh3*, *Kcns3*); hormone encoding genes (*Ghrh*, *Gnrh1*, *Nucb2*) along with other neuronal genes such as, *Prss12* (encoding for Neurotrypsin)⁶⁴, *Uchl1* (encoding for pan-neuronal marker PGP9.5)⁶⁵, *Cplx2* (encoding for Complexin 2)⁶⁶, *Gpm6a* (encoding for neuronal membrane glycoprotein M6-A)⁶⁷, and *Vamp2* (encoding for Synaptobrevin 2)⁶⁸ (Suppl. Fig 5). Owing to the small size of the NENs cluster, a comparative analysis between these two neuronal lineages for these neuronally significant genes was not possible. However, the discovery of these neuronally significant genes in the MENs cluster, and the subsequent validation of a set of them provides evidence for the neuronal nature of MENs. Further examination of the MENs cluster yielded additional MENs-specific marker genes *Slpi*, *Aebp1*, *Clic3*, *Fmo2*, *Smo*, *Myl7*, and *Slc17a9*, whose expression by adult enteric neurons has not been previously described and which we also validated (**Fig 4b**).

Next, we examined the MENs cluster for expression of a mesenchymal gene that would underscore the mesodermal nature of MENs. Decorin (*Dcn*) is a gene coding for a chondroitin-dermatan sulphate proteoglycan, which is expressed by mesoderm-derived cells, including fibroblasts and smooth muscle cells⁶⁹. Decorin was found to be highly expressed in the MENs cluster in our data (**Fig 5a**), and not by the neurons in the Zeisel et al. database (**Fig 5b**)⁴⁸. We used a validated antibody against DCN⁷⁰ and found that this protein, known to be specifically expressed by mesoderm-derived cells, is expressed specifically by tdTomato-negative small intestinal myenteric neurons of the Wnt1-cre:*Rosa26*-tdTomato lineage fate mapping mouse (**Fig 5c**).

These data show that the MENs scRNAseq cluster, which can be identified by its co-expression of neuronal markers (*Elavl2*, *Pde10a*, *Hand2*, etc), validated cell surface marker (*Cdh3*), and the mesodermal marker *Dcn* is the same as the population of Hu-expressing neurons that do not express tdTomato in the Wnt1-cre:*Rosa26*-tdTomato neural crest lineage fate mapping mouse. We thus establish the distinct nature of MENs based on the co-expression of many neuronally

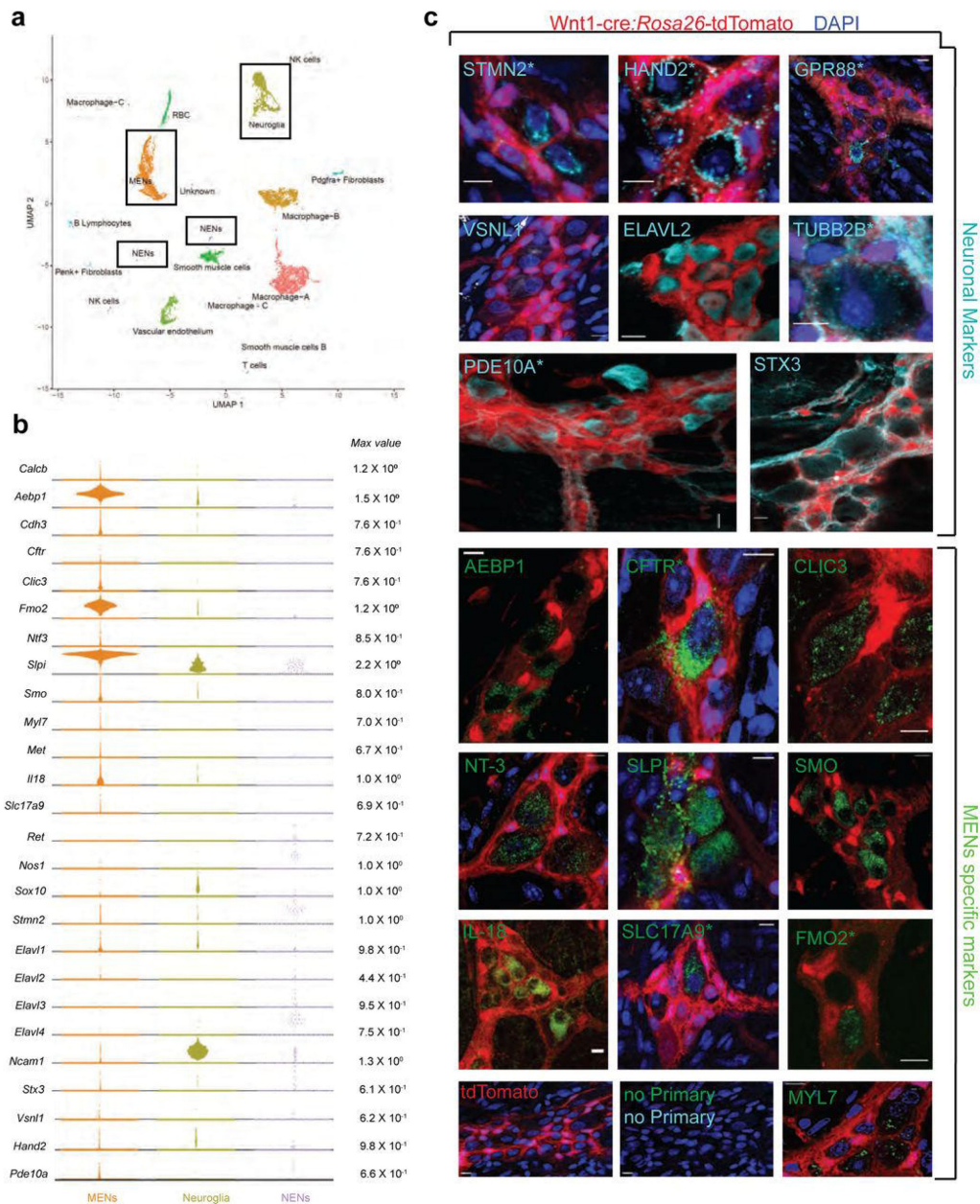


Fig 4

scRNAseq-analyses identifies the distinct transcriptomic profile of the MENs.

(a) UMAP representation of 11,123 sequenced high-quality cells that were identified as meeting a 200 UMI minimum threshold with a mitochondrial read ratio of less than 20%. Clusters were annotated by markers that were found to be cell-type specific by searching UniProt, Allen Cell Atlas and Pubmed databases. Cells of the neural crest lineage were then identified as NENs by their expression of neural crest marker gene *Ret* and Neuroglia by their expression of *Ncam1* and *Sox10*, or as MENs by co-expression of *Calcb* (CGRP), *Cdh3*, and *Met* genes.

(b) Visualization of expression of select MEN-specific and neuronal markers using quasi-violin plots. "Max value" represents the scale for the log-normalized expression of each gene.

(c) Validation of the MENs-specific marker genes discovered in the scRNAseq analyses by immunohistochemistry and confocal microscopy of small intestinal LM-MP from adult male *Wnt1-cre:Rosa26-tdTomato* mice. Expression of neuronal markers STX3, VSNL1, STMN2, HAND2, GPR88, PDE10A, ELAVL2, and TUBB2B (Gray, green arrows) was found in tdTomato- MENs. Immunostaining of the proteins AEBP1, CFTR, CLIC3, NT-3, SLPI, SMO, IL-18, SLC17A9, FMO2 and MYL7 (green; green arrows) was found to be localized to tdTomato- MENs. tdTomato⁺ (red) NC-cells did not immunostain for these markers (red arrows). Panel also shows *Wnt1-cre:Rosa26-tdTomato* tissue with no primary controls (stained with AlexaFluor 488 and AlexaFluor 647 antibodies). Figures with * annotations are 2D representation of 3D stacks of images. Nuclei in some panels were labeled with DAPI (blue). Scale bar denotes 10 μ m.

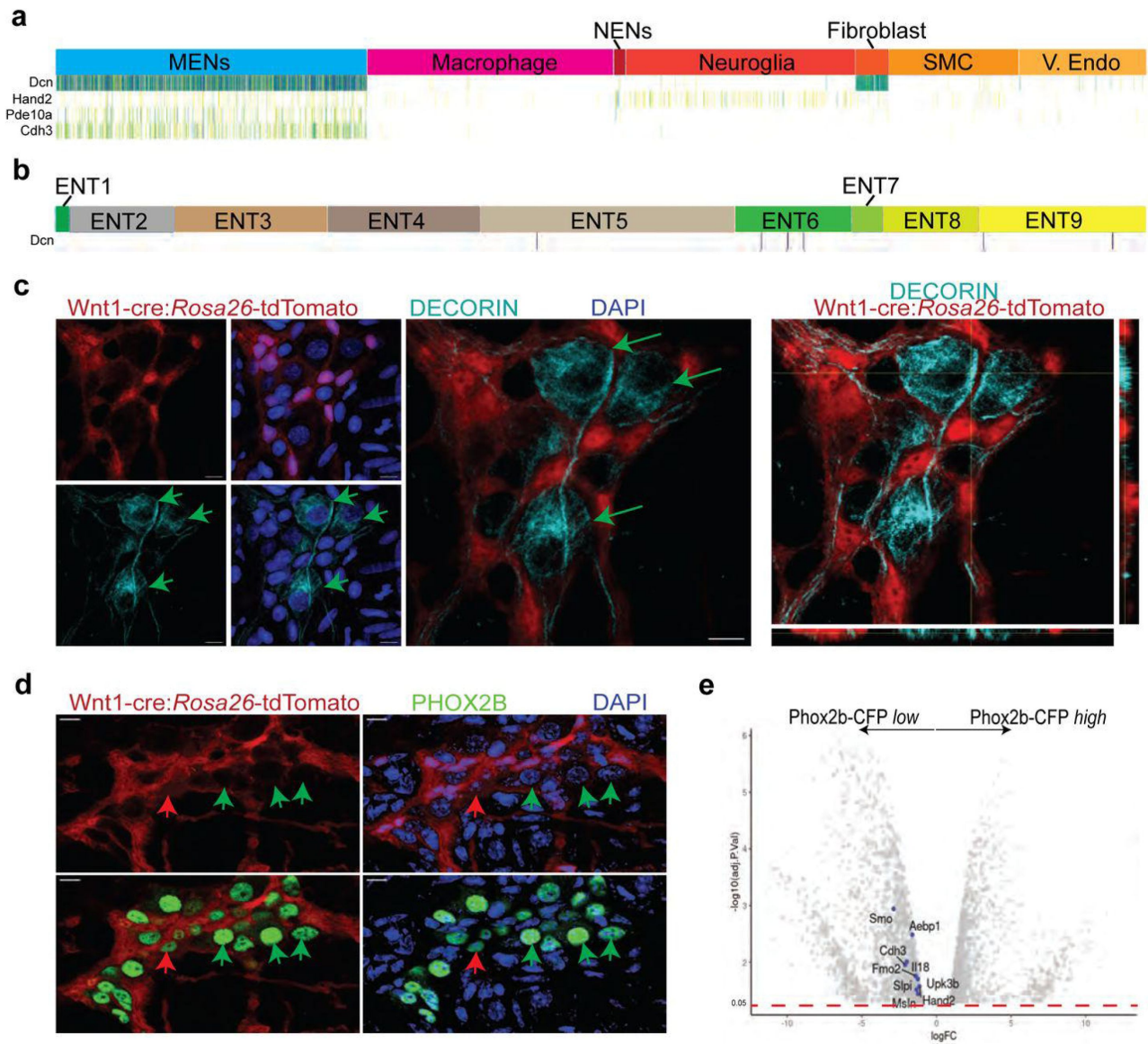


Fig 5

MENs express the mesenchymal gene Decorin and the ENS-specific gene Phox2b.

(a) Sparkline representation plot of the top 90 percentile of expressed genes in the various scRNAseq cell clusters from the adult murine LM-MP tissue of a 6-month-old mouse shows that the MENs cluster, which expresses the genes *Cdh3*, *Pde10a*, and *Hand2*, also expresses significant amounts of the mesenchymal gene *Decorin* (*Dcn*). Darker colors of the sparkline plot represent higher expression.

(b) Sparkline representation plot of the neural crest-derived enteric neurons from the Zeisel et al. dataset shows that the *Decorin* gene is not found expressed by most neural crest-derived enteric neurons.

(c) 2 dimensional representation views and orthogonal view of a myenteric ganglion from the small intestinal LM-MP of adult male *Wnt1-cre:Rosa26-EGFP* mouse, where tdTomato (red) is expressed by neural crest-derived cells, when immunostained with antibodies against DECORIN (green) shows that the DECORIN-expressing myenteric cells (green arrows) do not express tdTomato and hence are non-neural crest-derived MENs. Nuclei are labeled with DAPI. Scale bar = 10 μ m.

(d) Representative image of a myenteric ganglion from the small intestinal LM-MP of adult male *Wnt1-cre:Rosa26-EGFP* mouse, where tdTomato (red) is expressed by neural crest-derived cells, when immunostained with antibodies against PHOX2b (green) shows that the PHOX2b- expression is found in myenteric cells that do not express tdTomato (green arrows, and hence are not neural crest-derived cells, or MENs) as well as in tdTomato-expressing neural crest- derived NENs (red arrow). Nuclei are labeled with DAPI. Scale bar = 10 μ m.

(e) Volcano plot of gene expression profiles of Phox2b-expressing cells, which were sorted based on their CFP expression level, shows that the expression of MENs marker genes *Smo*, *Aebp1*, *Cdh3*, *Fmo2*, *Il18*, *Slpi*, *Upk3b*, *Msln*, and *Hand2* is significantly enriched in the Phox2b-CFP^{low} fraction. Red dotted line shows the $p_{adjusted}$ value of 0.05.

significant genes and of mesodermal genes by this cell population of Wnt1-cre:*Rosa26*-tdTomato-negative neuron. Our experimental strategy of performing scRNAseq on LM-MP cells without any pre-enrichment helps detect this newly characterized cell type. However, it does not allow us to compare and contrast the transcriptomic profiles of MENs and NENs – especially given that we do not know the true size of NENs represented in our dataset. This is exemplified by the fact that cells within the Neuroglia cluster showed expression of glial genes such as *Sox10* as well as neuronal genes such as *Ncam1*, *Hand2* and *Stmn2*, suggesting that NC-derived neurons may be present within both the smaller NENs cluster as well as the larger Neuroglia cluster (**Fig 4b**).

Detection of MENs in other murine and human datasets

Since *Phox2b*, the transcription factor expressed by all ENS cells, shows significant higher expression in some neurons than in all ENS glial cells⁷¹, May-Zhang et al.⁵⁰ reasoned that flow sorted nuclei of *Phox2b*-CFP^{high} cells from a *Phox2b*-CFP reporter mouse would provide for an enriched population of ENS neurons that can be used for single nuclei RNA sequencing (snRNAseq). They first performed bulk-RNAseq on *Phox2b*-CFP⁺ nuclei that were flow sorted based on CFP-expression level into CFP^{low} and CFP^{high} fractions, of which the CFP^{low} fraction was found enriched with glial-specific gene expression profile. May-Zhang et al.⁵⁰ used this to provide a rationale for performing snRNAseq on *Phox2b*-CFP^{high} nuclei, which they presumed to consist of all enteric neurons. Since, MENs make up roughly half of all adult ENS neurons, we hypothesized that they should also express *Phox2b*. By using a *PHOX2b*-specific antibody, we validated the expression of this important transcription factor in MENs (**Fig 5d**). Next, we tested whether we could detect some of the genes that show enriched expression in MENs (namely, *Smo*, *Aebp1*, *Cdh3*, *Fmo2*, *Il18*, *Slpi*, *Upk3b*, *Msln*, *Hand2*) in the *Phox2b*⁺ bulkRNAseq data from May-Zhang et al.⁵⁰, and found that not only were these genes expressed by their *Phox2b*⁺ cells, but expression of these genes was also significantly enriched in the *Phox2b*-CFP^{low} fraction (**Fig 5e**) that was not used for subsequent snRNAseq experiments. These data provide evidence that MENs express *Phox2b*, both transcriptomically, as well as at the protein level. We next queried whether any MENs were represented in the May-Zhang et al.'s snRNAseq data generated on *Phox2b*-CFP^{high} nuclei, given that they reported on the presence of a cluster of adult enteric neurons that expressed mesenchymal markers. We again tested whether the MENs-expressed genes, such as *Decorin*, etc were also expressed by the 'mesenchymal' neurons in the May-Zhang et al.⁵⁰ dataset. We found a high degree of similarity between the top genes expressed by the MENs cluster in our scRNAseq dataset and the 'mesenchymal' neurons in the May-Zhang et al.'s snRNAseq dataset (**Table 1**, Suppl. Fig 4d). Furthermore, examination of the top genes shows that this *Decorin*-expressing neuronal sub-population is significantly different from the other *Calcb*-expressing neuronal populations, which we presume to be the CGRP-expressing NENs (**Table 1**).

To confirm that the 'mesenchymal' neurons identified by May-Zhang et al.⁵⁰ are MENs, we used an agnostic bioinformatics projection-based approach which we have previously published⁷². This approach allows us to learn latent space representations of gene expression patterns using single cell transcriptomic data, generating patterns that correspond to cell-type-specific gene expression signatures⁷². We can then project other single cell and bulk RNA transcriptomic datasets into these learned patterns to accurately quantify the differential use of these transcriptional signatures in target data across platforms, tissues, and species⁷². In this instance, gene expression patterns were learned on our murine single cell transcriptomic data using non-negative matrix factorization (NMF) dimensionality reduction and the data was decomposed into 50 distinct NMF-patterns^{72, 73} (**Fig 6a**). Four NMF patterns were identified that were specific to MENs (**Fig 6a, b**; Suppl. Fig 4e). We next projected the snRNAseq dataset from May-Zhang et al.⁵⁰ into the four MEN-specific patterns and found that all these patterns specifically labeled the cluster of 'mesenchymal' neurons in the May-Zhang et al. dataset of the adult ileal ENS (**Fig 6b**). Further, upon clustering the nuclei based on their projection weights from the four MEN-specific patterns, we identified a cluster of nuclei that showed higher usage of all four patterns, when compared to all the other nuclei sequenced in the dataset (Suppl. **Fig 4f**). The genes that define this cluster of cells are similar to the top-expressed genes that define our MENs

Calcb+ cluster corresponding to Ileum cluster in May-Zhang et al. which expresses Calcb, Calb2, Nmu, Pcdh10, Cyslr2			Calcb+ cluster corresponding to Ileum cluster in May-Zhang et al. which expresses Calcb, Calb2, Sst			MENs-like cluster corresponding to Ileum cluster in May-Zhang et al. which expresses Utpk3b and Igfbp6			MENs Cluster (Our Data)			Cluster annotated as Mesothelial (Drokhlyansky et al data)		
Gene	FDR	summary.logFC	Gene	FDR	summary.logFC	Gene	FDR	summary.logFC	Gene	FDR	summary.logFC	Gene	FDR	summary.logFC
Zfp804a	0	5.314372906	Nlgn1	1.56E-227	4.503778932	Abi1	4.98E-36	3.316005072	Bcam	0	1.310889756	Lrrn4	5.4657E-206	16.886354
Pcdh10	0	2.727131014	Pknox4	5.40E-170	3.513261638	Dcn	2.08E-52	5.091738535	Mgst1	0	1.466554292	Rspo1	3.6499E-210	9.4753895
Gpr149	0	2.928565459	Pcdh15	6.75E-269	4.552606891	Col3a1	7.95E-31	3.039859427	Krt18	1.02E-252	0.722676212	Wt1	1.5809E-227	10.114356
Necab1	0	3.126115371	Nrg3	9.23E-246	4.051010208	C3	1.11E-38	4.827731701	Igfbp6	0	4.277079428	Utpk3b	5.8625E-282	8.9449835
Gria3	0	2.477871618	Enk1b	1.02E-168	3.739255875	Sipa	2.75E-31	4.589255236	Rspo1	0	1.358288212	Bnc1	4.04195E-85	15.045354
Fam19a1	0	4.658451729	Ryr2	4.15E-122	2.724692337	Lrrn4	7.45E-21	2.797813295	Gpmf6a	0	2.007236002	Wt1os	2.3697E-23	9.9661905
Ano2	0	3.443068822	Nrxn3	1.20E-156	4.845162261	S100a6	9.36E-29	3.230324987	Utpk3b	0	2.136663175	Utpk1b	1.277E-215	8.0787953
Ntrk3	0	4.380099903	Rbfox1	0.00E+00	4.833341666	Utpk3b	1.90E-26	3.101085936	Aebp1	0	2.502295294	Aldh1a2	1.5275E-143	6.3791433
Cntn5	0	6.308342507	Sst	6.45E-135	5.862426929	Gpmf6a	1.13E-24	3.730157809	Dcn	0	4.082052904	Main	8.3474E-159	7.3877455
Ccbe1	0	3.076522552	Chsy3	4.53E-122	3.602794203	Sparc	1.37E-28	3.162147448	Slpi	0	4.928306229	Igfbp6	1.7674E-307	7.7565747
Zbtb7c	0	2.658109043	Gria2	3.32E-126	3.083755369	Meg3	3.89E-23	2.952544748	Rarres2	0	4.202866323	Lvrn	4.18762E-92	7.8098721
Astr2	0	3.368494926	Ptprn	3.17E-139	2.903737928	Igfbp6	4.99E-28	2.811931349	Gabap1	0	1.672848073	C3	0	8.057149
Dapk2	0	1.703870376	Pde4b	3.69E-210	3.947198917	Agap1	4.03E-21	2.507795342	Aldh1a2	4.60E-277	0.796236425	Muc16	7.8925E-269	7.3116341
Nrxn3	0	5.160806272	Fam19a2	8.41E-100	3.227261386	Pbx1	4.47E-25	2.503783608	Fmo2	0	1.582315372	Gpmf6a	0	6.5940179
Dgkq	0	4.15397884	Mdga2	2.01E-132	3.667573403	Etfemp1	2.67E-22	2.915648257	Utpk1b	0	1.081912196	Cybrd1	4.39858E-60	9.0017763
Gair1	0	1.673088209	Snhg11	6.24E-112	4.344115315	Gas6	1.69E-22	2.742900314	Gas1	0	1.464482623	Fam180a	1.90191E-30	13.526168
Nmu	0	3.521779106	Raly1	1.59E-128	3.809356707	Muc16	2.43E-21	3.168081514	Krt7	0	1.315380677	Myrf	1.71228E-56	8.6635402
Hspb8	1.42E-236	1.244433448	Bnc2	1.59E-137	3.131344621	Gas1	2.13E-23	2.708510446	Cnp1	0	4.58883961	Aebp1	4.5764E-247	6.6271763
Cpna4	0	1.818980809	Rbpb1	2.40E-134	2.418320757	Dpp4	4.40E-21	2.764472346	Cnp2	0	1.502041377	Cr2	2.91628E-72	7.3719161
Tmemf2	0	3.495305459	Nrxn1	1.51E-150	4.652680188	Colb1	1.10E-20	2.321556143	C3	0	3.494711523	Zdbf2	3.25895E-75	7.0872463
My1	0	3.653772641	Pcsk2	5.79E-169	3.258681935	Rarres2	1.64E-20	3.226437143	Dpp4	1.12E-210	0.556370013	Sulf5a1	2.85305E-24	13.31773
Calcb	0	3.200679451	Syn2	1.17E-132	3.619085064	Myo1d	6.05E-21	2.158327438	Lrrn4	1.58E-271	0.736486954	Cth	4.6319E-250	5.7592104
Cyslr2	0	2.487957211	Ntrk3	1.05E-108	2.657539356	Tbce	1.69E-12	3.139158995	Nkain4	0	1.298360281	Wdr17	3.1501E-282	6.6320991
Cln2	0	2.655215539	Scube1	1.03E-95	2.265338351	Ilfst	6.05E-21	2.338914462	S100a6	0	4.128650316	Ildr2	3.4601E-136	6.7198792
Robo1	1.98E-297	1.9960805	Man2a1	9.20E-139	3.375536511	Nfb	7.62E-23	2.57202827	Krt19	0	1.900927424	Slpi	1.7684E-135	7.6326894
Hunk	0	1.818980809	Kcnr1	7.39E-85	2.418320757	Nfbp1	1.70E-19	2.764472346	Ctb	8.74E-203	0.808707666	Fmod	2.29777E-40	7.8768629
Snhg11	0	2.986542626	Fam19a1	1.70E-147	3.629692675	Sema5a	1.97E-19	2.852546212	Gabaprap1	0	1.709728292	Cpxm1	7.29311E-19	12.558464
Aff2	0	2.087576143	Nk1	3.21E-100	2.793128177	Fmo2	1.80E-18	2.353371192	Lgals3	0	1.26072921	If205	2.62948E-18	12.864742
Otd7a	3.38E-263	2.545208984	Gaintf6	1.72E-109	3.815091788	Ezr	2.84E-20	2.166036039	Krt8	1.39E-245	0.689105423	A730046J19	8.01459E-17	12.793582
Pcdh9	0	4.216229123	Scgcl	5.24E-120	2.88253651	Ahnak	6.05E-21	2.714251351	Tm4sf1	0	1.367716307	Eya4	4.5788E-136	6.8974953
Htr3b	0	1.553388667	Pde4d	1.18E-97	2.285404689	Bnc2	6.54E-20	3.061860372	Slc39a8	7.17E-197	0.49898021	Nkain4	1.08326E-93	5.7592624
Scn11a	0	1.908978823	Slc8a1	1.08E-180	3.289543426	Krt19	6.15E-120	2.380492921	Sema3c	2.63E-255	0.71554962	Col2a2	5.81471E-44	5.2884497
Igkap2	0	1.755993309	Tcf4	1.52E-159	2.582805317	Tim1	3.00E-20	2.434254443	Poolee	0	1.883134360	Ptp1b5	1.12186E-43	7.3412329
Rbfox1	0	4.702418413	Pknox1	2.80E-97	3.629692675	Col2a2	2.00E-19	2.707560817	Cst4	1.09E-222	0.627337521	Dcn	6.4597E-292	5.6187432
Tcf12	0	2.841306652	Kcnma1	1.27E-136	2.945051283	Sox6	3.93E-19	2.803585619	Ezr	0	1.019712533	Gm20400	7.4045E-135	7.0357895
Tbx2	0	1.871954414	Adgrb3	5.63E-143	4.396153392	Aebp1	1.57E-19	2.548178022	Cldn15	4.76E-291	0.798661223	Gas1	2.6758E-182	5.7763744
Robo2	0	4.088780154	Unc5c	2.09E-114	3.291001111	Cth	9.08E-18	2.422456138	Serpinb6b	2.66E-212	0.562587863	Cdn3	2.1841E-97	6.6406251
Kcnk2	0	3.9267211	Cpna4	4.42E-81	2.730862875	Col1a1	2.72E-13	2.033502612	Nbl1	0	1.239754358	Efnh5	1.6397E-264	4.7533032
Srrm4	2.23E-261	2.303709088	Meg3	7.04E-124	4.308953414	Wt1	2.03E-17	2.196826568	Slc16a1	2.28E-244	0.65901005	Tmem151a	1.08326E-93	5.7592624
Pknox4	0	3.31892195	Snap25	4.22E-135	3.917101813	Ccdc171	1.09E-14	2.022034827	Smm1	2.76E-245	0.859125894	Bnc2	5.1982E-206	5.1670469
Socx	0	4.739595341	Nk1	3.21E-100	2.793128177	Pknox4	2.00E-19	2.707560817	Cav13	6.15E-293	0.85731531	Gm12381	7.0467E-102	7.693331
95300590t1	0	3.800146025	Grik4	8.53E-115	2.35016852	Arhgap29	3.72E-18	2.409211927	Gpc3	0	1.393181642	Serpinb6b	1.16024E-44	5.3575251
Sox4	7.89E-259	1.211156367	Ptprn2	1.75E-115	3.282060046	Ano1	2.38E-18	3.039709984	Serpinb1a	3.54E-178	0.472277374	Pknox4	1.3438E-213	5.1141234
Hcn1	8.40016528	1.861283774	Arhgap26	1.46E-133	2.381300601	Col1a2	5.02E-13	2.132928671	Chst4	3.42E-232	0.598828207	Etfemp1	6.3046E-195	5.3926198
Scube1	0	2.077912292	Bcl2	1.53E-91	2.274340682	Cav1	5.10E-18	2.23545211	Esam	1.50E-188	0.475123823	Gm765	4.94209E-43	6.7967853
Boc	5.82E-199	0.854637195	Ppm1h	6.46E-139	3.466729894	Bicc1	2.27E-19	2.486459966	Ptgis	5.99E-231	0.598750529	Fmco3	1.17735E-20	8.4299989
Cbln2	0	2.229769856	Fgf14	3.30E-126	4.330212767	Main	2.28E-14	1.829701181	Elec3r1	7.03E-265	0.887889897	Fmco2	2.9871E-243	5.3187177
Kcnk3	0	1.869022238	Kcnk21b	9.54E-78	2.107925878	Utpk1b	1.96E-17	1.970013911	Ecm1	1.88E-172	0.514390319	Prss12	3.67853E-61	5.4000171
Rph3a	0	1.741082515	Fgf13	5.32E-123	3.932808506	Sulf1	3.39E-17	2.542517734	Igfbp7	0	2.347561986	Vwa3a	1.02294E-65	5.8157216
Cpna8	0	2.360533782	Ppp3ca	1.96E-101	2.03501536	Vim	1.68E-16	2.452909991	Main	0	2.266588157	Abcb1b	1.7423E-115	6.470465
Lingo2	1.12695240	4.714175039	Adams9	1.24E-60	1.993937158	Sema3c	1.17E-17	2.547300887	Gpx3	0	2.209730215	Rarres2	6.6617E-142	5.7715962
Slc4a4	0	2.575716085	Calcb	4.08E-76	2.288867891	Ilfm3	6.57E-15	2.071790767	Col14a1	1.17E-163	0.438755664	Lrp2	2.01232E-42	6.2276692
Cux2	0	2.614397251	Pcbp3	1.17E-136	3.893280923	Rspo1	1.99E-17	1.964750591	Anxa1	4.24E-246	0.891007825	Cldn10	6.74497E-17	5.6397899
Cbat	1.62E-252	1.470549345	Nlgn2	1.21E-92	2.470976738	Alb1a2	1.21E-36	2.494302786	Utpk2	7.94E-272	0.803204775	Spock2	1.9494E-138	5.1124963
Htr3a	0	1.824344275	Nkain2	1.01E-107	1.826125797	Serpinp1	4.91E-14	2.014097588	Cth	0	1.512747523	Chrd	1.6918E-22	7.4139259
Hs3st2	1.18369186	1.564650584	Chat	2.41E-66	1.619335139	Tim2	8.53E-10	1.596556888	Tmem176a	1.85E-170	0.915766916	Ltp3	1.94368E-88	5.07873
Cdh8	0.00E+00	2.544193879	Gfra2	4.04E-106	2.379843878	Sdc4	9.47E-13	1.640281507	Tmsb10	0	2.299200297	Pkhd11	4.889E-120	4.4235079
Gm45881	0	2.054509731	Pde10a	7.37E-115	2.924056301	Cd34	2.04E-10	1.324668076	Tim2	0	1.918063461	Svopl	4.52096E-42	6.0169611
Cdh6	0	2.996825969	Ptpr	1.01E-130	3.346248491	Serpinh1	2.72E-11	1.617681505	Pdpn	2.36E-252	0.846386263	Ptgis	1.487E-109	5.035511
Gm12847	9.83E-301	2.668713923	Pfod2	7.61E-111	2.338515945	Heg1	1.56E-17	1.795303412	Prss23	0	0.980677037	Ilf6	1.51074E-97	4.7890361
Frmf4b	0	3.613502554	Srm4	1.17E-82	2.302208949	Igfbp7	3.27E-15	2.014097588	Spin2	2.85E-277	0.57273732	Wnt5a	1.97493E-27	4.860321
Ndr4	5.28E-172	1.033574628	Rbm3	2.24E-117	1.826125797	Utpk1b	3.27E-15	2.014097588	P3M4	8.94E-212	0.512747523	Gabra3	2.92934E-37	4.659901
Efr3a	0	2.922824682	Ccbe1	2.81E-69	1.972703173	Wdr17	9.82E-15	3.265554301	Cd200	0	0.975177086	Medag	2.3697E-99	4.7556656
Syt2	0	1.996197662	Trpm3	3.63E-112	3.250658539	Anxa2	1.55E-16	1.783316817	Ltpb4	1.80E-268	0.859534481	Atp5f0v4	1.46514E-75	5.0974539
Pak7	2.65E-280	1.50563649	Frmf4a	4.22E-106	3.509014084	Cell2	8.01E-17	2.016354207	Gstt1	2.26E-142	0.347320974	Rpp25	1.288E-19	5.45417515
Lhfp12	0	1.681014338	Gaintf7	1.01E-130	3.346248491	Slo4a4	2.64E-14	2.058956296	Ssb					

cluster (Suppl. Fig 4g, **Table 1**). This approach, together with the high similarities in the top-expressed genes, establishes that cluster of neurons annotated as ‘mesenchymal’ neurons by May-Zhang et al. are indeed MENs. Further, one of the markers used by May-Zhang et al. to describe their mesenchymal neuronal cluster was the gene *Myh11*⁵⁰, which is known to be expressed by mesoderm-derived smooth muscle cells in the adult gut⁷⁴. To test whether *Myh11* is expressed by MENs, we immunolabeled the small intestinal LM-MP tissue from an adult *Wnt1-cre:Rosa26-tdTomato* mouse with a monoclonal antibody against MYH11 (Invitrogen) and found that *tdTomato*-negative and *Hu*⁺ neurons exclusively immunolabeled for MYH11 (Suppl. Fig. 6a). We confirmed that a subpopulation of adult enteric neurons expressed *Myh11* by immunolabeling small intestinal LM-MP tissues from a tamoxifen treated *Myh11-cre^{ERT2}:Rosa26-YFP* transgenic mouse line with anti-YFP/GFP and anti-Hu antibodies (Suppl. Fig. 6b). Anti-MYH11 antibodies labeled circular smooth muscle in the human duodenum, where it did not immunolabel nerve fibers and some submucosal neurons immunostained with antibodies against the pan-neuronal marker PGP9.5 but showed immunolabeling against PGP9.5-expressing myenteric neurons in the adult healthy small intestinal tissue (Suppl. Fig 6c, d). Thus, we confirm that the MENs identified by us correspond to the cluster of ‘mesenchymal neurons’ observed by May-Zhang et al. As compared to our scRNAseq dataset, where MENs comprise of a significantly large numbers of cells, the MENs cluster in the May-Zhang et al. dataset is significantly smaller, possibly due to the fact that a large number of MENs were left unsequenced in their *Phox2b*-CFP^{low} fraction.

Similarly, Drokhlyansky et al.⁴⁹ using MIRACL-seq found a small cluster of cells that express the MENs-signature genes (*Cdh3*, *Dcn*, *Slpi*, *Aebp1*, *Wt1*, *Msln*, *Fmo2*). However, the neuronal nature of these cells was not correctly identified as the study used a gene signature profile specific for neural crest-derived neurons (derived from their experiment with enriched *Wnt1-cre2:Rosa26-tdTomato*-expressing neural crest-derived cells). As a result, this cluster of cells – which shows similarity to the MENs transcriptomic profile, was instead annotated as *Mesothelial*. This ‘mesothelial’ cluster in the Drokhlyansky et al.⁴⁹ dataset shows similar gene expression profile as that of the cluster of ‘mesenchymal neurons’ from the May-Zhang et al.⁵⁰ dataset (**Table 1**), especially given that many of the marker genes for both clusters are similar (*Lrrn4*, *Rspo1*, *Msln*, *Upk3b*, *Upk1b*, *Gpm6a*, *Wnt5a*, *Gpc3*, *Sulf1*, *Muc16*). The ‘mesothelial’ cluster also expresses neuronally significant genes such as *Gabra3* (GABA receptor A subunit 3⁷⁵), *Prss12* (Neurotrypsin^{64, 76}), *Synpr* (Synaptoporin⁷⁷), *Trpv4* (Transient receptor potential cation channel subfamily V member 4⁷⁸), and *Nxph1* (Neurexophilin-1⁷⁹). Unfortunately, a deeper interrogation of this dataset was not possible due to the size and the manner in which the original data was processed⁴⁹. In addition, MENs were not represented in the snRNAseq carried out in Wright et al.²², which was based solely on neural crest-derived cells.

In contrast to these snRNAseq based studies, the Marklund Lab in two studies performed single cell RNA-sequencing (scRNAseq) on juvenile ENS cells, first on *tdTomato*⁺ cells from P21 *Wnt1-cre:Rosa26:tdTomato* lineage fate mapping mice in Zeisel et al.⁴⁸ and then from the newly characterized *Baf53b-cre:Rosa26-tdTomato* BAC-transgenic mouse line⁸⁰ in Morarach et al.⁵¹. Barring 2 small clusters (Clusters 5 and 11), the other neuronal clusters identified in Morarach et al.⁵¹ mapped to the neural crest-derived clusters identified earlier by them in Zeisel et al.⁴⁸. The gene expression signature of these two clusters (Cluster 5: *Sst*, *Calb2*; Cluster 11: *Npy*, *Th*, *Dbh*) did not match the signature of our MENs cluster or that of the cluster of mesenchymal neurons in the May-Zhang et al.⁵⁰ study (**Table 1**). We reasoned that the lack of MENs in the Morarach et al.⁵¹ data may be driven by the non-comprehensive nature of *Baf53b-cre* in this BAC transgenic mouse line labeling all neurons. On immunolabeling LM-MP tissues from two P21 *Baf53b-cre:Rosa26-tdTomato* mice with antibodies against Hu, we enumerated 1312 *Hu*⁺ cells in the ileum (759 from mouse 1 and 553 from mouse 2), 5758 *Hu*⁺ cells in the proximal colon (3411 from mouse 1 and 2347 from mouse 2), and 3352 *Hu*⁺ cells in the distal colon (1894 from mouse 1 and 1458 from mouse 2), and found that a significant population of myenteric neurons did not express *tdTomato* (Suppl. Fig. 6e, f). Given this non-comprehensive nature of the *Baf53b-cre* BAC transgenic line in labeling all myenteric neurons, and the congruence between the transcriptomic

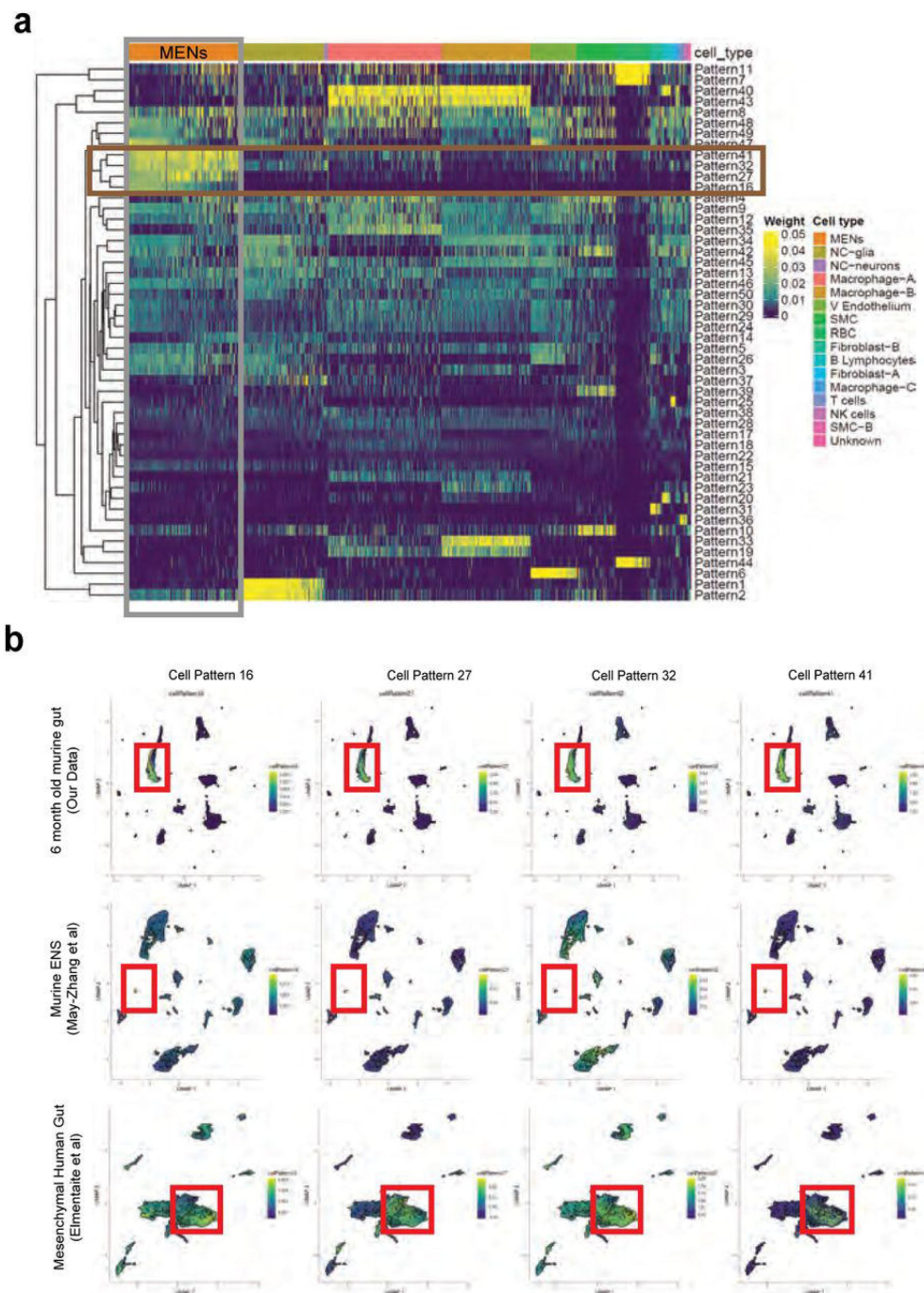


Fig 6

Computational analyses using *projectR* identifies MENs in publicly available murine and human transcriptomic datasets.

(a) Heatmap of cell weights for the patterns learned by NMF ($k=50$). Hierarchical clustering was calculated using Euclidean distance. Multiple clusters annotated as the same cell type are merged. The four most specific MENs patterns (16, 27, 32 and 41) are selected.

(b) In addition to using the four MEN-specific NMF patterns to label the MENs cluster in our scRNAseq dataset (plots in top row), two other transcriptomic datasets: May-Zhang et al.'s murine ileal ENS snRNAseq dataset⁵⁰ (plots in second row), and Elmentaite et al.'s gut mesenchymal scRNAseq dataset⁸¹ (plots in third row), were projected into the defined four MEN-specific pattern space using *ProjectR*. Raw projected cell weights were visualized using previously learned UMAP embedding. The cell clusters that show high pattern usage are shown bounded by the red square. Plots in the final row shows projection of healthy post-natal mesenchymal cells from Elmentaite et al.'s data⁸¹ into the four MEN-specific NMF patterns, which again show a population of cells showing high MEN-specific pattern utilization.

profiles of cells analyzed using the Baf53b-cre and the neural crest-specific Wnt1-cre lines, we infer that molecular taxonomy performed on enteric neurons in the Morarach et al.⁵¹ dataset is restricted to NENs.

In a recent report, Elmentaite et al.⁸¹ performed scRNAseq on dissociated single cells from the human gut at fetal, juvenile, and adult ages and catalogued multiple intestinal cell-types to generate a comprehensive gut cell atlas. Canonical enteric neurons, while adequately represented in the fetal ages, were not found in the juvenile and adult ages which were dominated by mesenchymal and other cell types.⁸¹ Given that our scRNAseq approach of sequencing diverse cell types from the murine gut wall without any marker-based pre-selection was similar to this approach in human tissue, we reasoned that MENs would also be represented in their description of mesenchymal cell clusters, especially in the juvenile and adult ages. To detect putative MENs in this human gut cell atlas data, we projected the entire human mesenchymal cell scRNAseq dataset into the four murine MEN-specific NMF patterns and detected putative human MENs clusters (Suppl. Fig 4h). Since our identification of MENs and the generation of MEN-specific NMF patterns was based on post-natal healthy murine tissue, we next projected data from only post-natal healthy mesenchymal clusters into the four MEN-specific NMF patterns (**Fig 6b**). The four patterns identified a population of cells within the post-natal intestinal mesenchymal cell clusters suggesting that these cells are putative MENs in the human gut (**Fig 6b**). These putative MENs, which map to Cluster 1 in our representation of the intestinal mesenchymal cells from the post-natal gut cell atlas data (**Fig 7a**) were annotated previously by Elmentaite et al. as transitional stromal cells (**Fig 7b**). We next tested and found that the MENs marker *Dcn* (**Fig 7c**) along with pan-neuronal *Uchl1* among other neuronal markers *Hand2*, *Stx3*, *Slc17a9*, *Pde10a* and *Tubb2b* (**Fig 7d**) that we earlier showed to be expressed by adult murine MENs, were expressed by the putative MENs in the adult human gut.

We hypothesized that the reason these cells were not annotated as enteric neurons was due to the use of a NENs-restricted gene list. Apart from the NENs marker *Ret*, such gene lists often contain the pre-synaptic gene *Snap25*, a component of the SNARE complex, which is widely assumed to be a pan-neuronal marker in the adult ENS.^{48, 49, 82, 83} While we have previously observed the NENs-specific nature of *RET*, our transcriptomic data suggests that MENs either do not express *Snap25* or do so in very small amounts (Suppl. Fig 4d). We tested the expression of *Snap25* by NENs and MENs in the myenteric ganglia of an adult *Wnt1-cre:Rosa26-tdTomato* mouse and found that *Snap25* expressing neurons expressed *tdTomato*, but not the MENs- marker *MHCst*, suggesting the NEN-specific expression of this canonical marker for synaptic neurons (**Fig 8a**). Next, we used a validated *Snap25-Gcamp* knock-in mouse line^{84–86}, where *GCamp/GFP* is knocked in at the *Snap25* locus and hence is expressed by *Snap25*-expressing cells, to confirm that the expression of *Snap25* is indeed restricted to a subset of myenteric neurons in the adult LM-MP layer. By using antibodies against Hu and against GFP, we found that the expression of *Snap25-GFP* is indeed restricted to a subset of adult myenteric neurons (**Fig 8b**). Finally, by immunolabeling adult small intestinal LM-MP of *Snap25-Gcamp* mice with antibodies against *MHCst* and against GFP, we confirmed the lack of *Snap25* expression by a population of MENs (**Fig 8c**). The low or lack of expression of *Ret*, *Snap-25*, *Elavl3* and *Elavl4* in MENs as observed both by us and May-Zhang et al.⁵⁰ may help explain why these canonical gene lists missed the correct identification and annotation of MENs' neuronal nature in human datasets.⁸¹ Next, we examined the expression of MENs markers *MHCst*, *MET*, *SLC17A9*, and *DECORIN* in LM-MP tissues from adult humans with no known gut motility disorder and found them to be expressed by a population of myenteric neurons in normal adult human ENS (**Fig 9**; Suppl. Fig 7a, b, c). Thus, our bioinformatic approaches and immunofluorescence analyses of MEN-specific markers in adult human gut together shows that the human gut similarly contains a population of MENs.

We next tried sub-clustering the MENs cell cluster in our scRNAseq data to study whether individual subclusters could be discriminated on the basis of MENs markers or other genes. However, at the current sequencing depth, these clusters did not yield meaningful information on

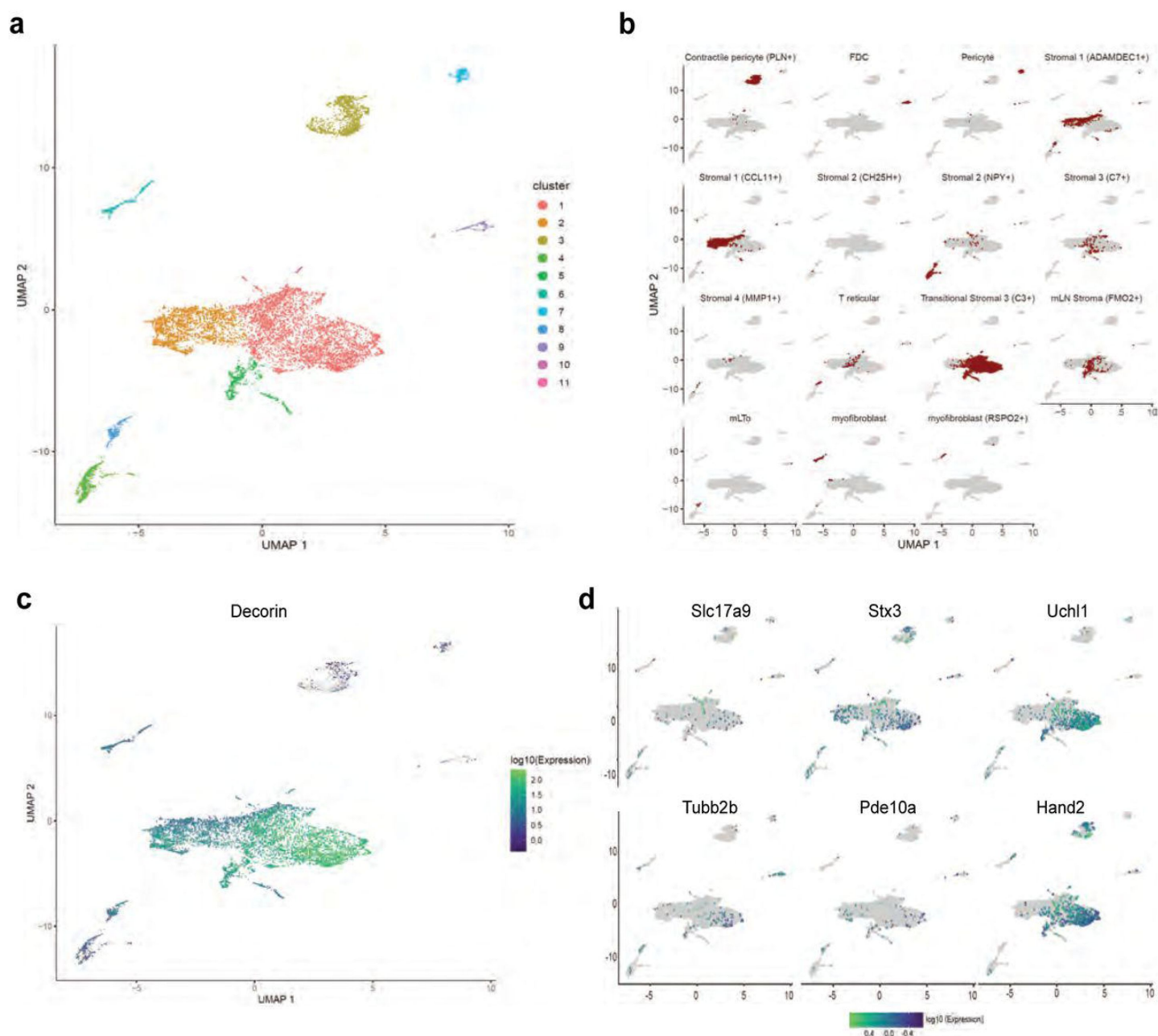


Fig 7.

Detection of MENs in human single cell RNA sequencing data.

- (a) UMAP representation of scRNAseq data from Elmentaite et al.'s healthy post-natal mesenchymal cells from the human gut⁸¹.
- (b) Breakdown for previously published annotated features of the post-natal health subset of the gut cell atlas as presented in Elmentaite et al.⁸¹.
- (c) Expression of the MENs marker gene *Dcn* in the UMAP representation of healthy post-natal data from Elmentaite et al.⁸¹, which was annotated as clusters of mesenchymal cells.
- (d) Expression of neuronal marker genes *Slc17a9*, *Stx3*, *Uchl1*, *Tubb2b*, *Pde10a*, and *Hand2* in the UMAP representation of healthy post-natal data from Elmentaite et al.⁸¹, which was annotated as clusters of mesenchymal cells.

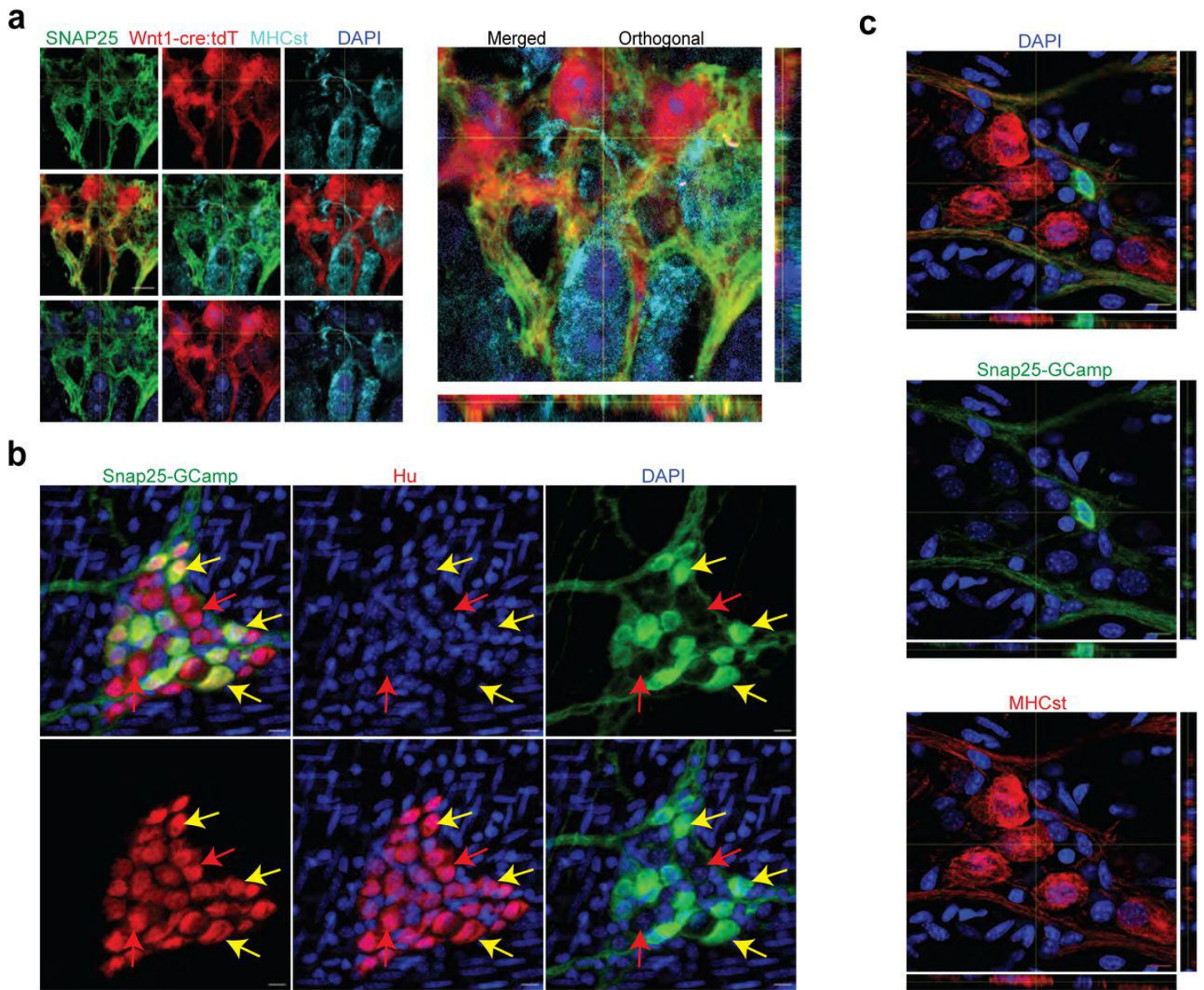


Fig 8

Validation of SNAP-25 as a NENs marker

- (a)** SNAP-25 expression is restricted to the neural crest lineage in the adult myenteric ganglia. SNAP-25 expression (green) co-localizes with tdTomato (red) but not with the MENs marker MHCst (cyan) as observed in 2D and orthogonal views of a myenteric ganglia from a *Wnt1-cre: Rosa26-tdTomato* mouse that was immunolabeled with antibodies against MHCst and SNAP-25. Nuclei were labeled with DAPI (blue). Scale bar denotes 10 μ m.
- (b)** In the adult male Snap25-Gcamp knock-in mice, the expression of Snap25-Gcamp/GFP (green) is restricted to a subset of Hu-expressing (red) myenteric neurons in the adult murine small intestinal myenteric ganglia (yellow arrows), while many neurons (red arrows) do not show any detectable expression of Snap25. Nuclei were labeled with DAPI (blue). Scale bar denotes 10 μ m.
- (c)** Orthogonal views of z-stack of an image of the myenteric ganglion from the small intestinal LM-MP tissue from an adult male Snap25-Gcamp knock-in mice shows that the expression of Snap25-Gcamp/GFP (green) is exclusive of the expression of the MENs marker MHCst (red). Nuclei were labeled with DAPI (blue). Scale bar denotes 10 μ m.

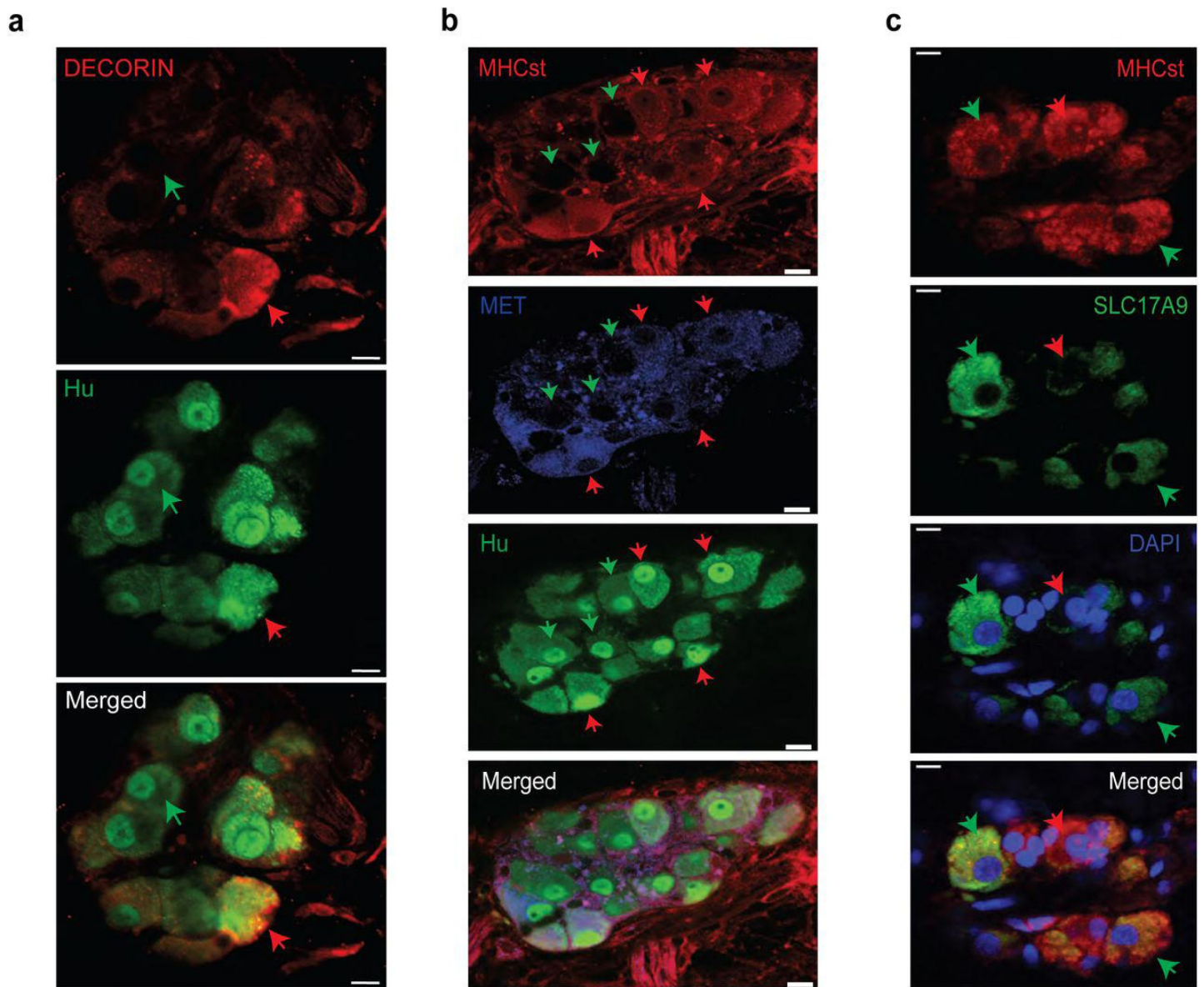


Fig 9

Observation of MENs marker expression in the adult human myenteric ganglia

(a) Hu-expressing small intestinal myenteric neurons (green) from the normal human duodenal tissue, when immunolabeled with antibodies against the MENs marker DECORIN (red) identifies putative human MENs (red arrows) and NENs (green arrows) by presence or absence of DECORIN immunolabeling. Scale bar denotes 10 μ m.

(b) Hu-expressing small intestinal myenteric neurons (green) from the normal human duodenal tissue, when immunolabeled with antibodies against the MENs marker MHCst (red) and MET (blue) identifies putative human MENs (red arrows) and NENs (green arrows) by presence or absence of these MENs markers. Scale bar denotes 10 μ m.

(c) Immunolabeling myenteric tissue with antibodies against the MENs markers SLC17A9 (green, green arrows) and MHCst (red) shows SLC17A9 expression in a subset of MHCst-expressing (red) neurons. Subset of MHCst-expressing cells do not express SLC17A9 (red arrow). Nuclei are labeled with DAPI (blue) in the normal human duodenal tissue. Scale bar denotes 10 μ m.

The proportion of mesoderm-derived neurons expands with age to become the dominant population in the aging ENS

Since the ENS at birth consists solely of NENs¹³, we next studied the birth-date and eventual expansion of the MEN lineage in the post-natal gut. Using Wnt1- cre:*Rosa26*-tdTomato mouse line, we enumerated the tdTomato- MENs in LM-MP at different ages and found a significant age-associated increase in MENs proportions (**Fig 10a, b, c**; One-way ANOVA, $F = 117.6$, $DFn, DFd = 5, 12$; $p < 0.0001$). At P11, MENs were found only in few isolated myenteric ganglia (**Fig 10a**) and together represented only ~5% of all myenteric neurons (tdTomato- neurons: $4.12\% \pm 2.98$ SEM; $n = 1327$ Hu⁺ neurons counted from 3 mice), suggesting that they originate shortly before this age. The proportion of MENs in myenteric ganglia rises sharply thereafter: by P22, they account for ~30% of all neurons (tdTomato- neurons: $29.63\% \pm 1.229$ SEM; $n = 742$ neurons counted from 3 mice); and at P60 they represent ~45% of all neurons (tdTomato- neurons: $46.38\% \pm 4.62$ SEM; $n = 700$ neurons counted from 3 mice). During the adult ages, the proportions of MENs in the adult ENS remains relatively stable as at P180, MENs continue to represent roughly half of all myenteric neurons (tdTomato- neurons: $57.29\% \pm 3.62$ SEM; $n = 586$ neurons counted from 3 mice). However, MENs dominate the proportions of the aging ENS as at the very old age of 17 months (P510), the ENS is populated almost exclusively by MENs (**Fig 10b**; tdTomato- neurons: $95.99\% \pm 1.62$ SEM; $n = 996$ neurons counted from 3 mice). Thus, the proportions of NENs and MENs in the myenteric ganglia can be used as a biomarker for deducing ENS age, as a healthy ENS dominant in NENs would be juvenile, one with roughly equal proportions would be adult, and an aging ENS is dominated by MENs.

We next tested whether the representation of MEN-specific transcriptomic signatures show a similar increase during the maturation and aging of the human gut. We tested whether the representation of the four MEN-specific NMF patterns (**Fig 5a**) increased with age in the human transcriptomic data from Elmentaite et al.⁸¹. We grouped the transcriptomic data from the wide-range of healthy post-natal specimens of the gut cell atlas into three groups: Juvenile (containing data on 2910 cells represented by 10 samples within the age range of 4 – 12 years), Adult (containing data on 3696 cells represented by 36 samples within the age range of 20 – 50 years), and Aging (containing data on 2848 cells represented by 37 samples within age range of 60 – 75 years) and tested whether the representation of the four MEN-specific patterns changed significantly between these age groups. Barring pattern 16 which showed a non-significant increase in projection weights between the Juvenile and Aging samples (One-way ANOVA, $F = 2.10$, $DFn, DFd = 2, 8$, $p = 0.18$), projection weights of MEN-specific pattern 27 (One-way ANOVA, $F = 16.08$, $DFn, DFd = 2, 8$, $p = 0.0016$), pattern 32 (One-way ANOVA, $F = 8.13$, $DFn, DFd = 2, 8$, $p = 0.01$) and pattern 41 (One-way ANOVA, $F = 9.818$, $DFn, DFd = 2, 8$, $p = 0.007$) all show significant age-associated increase in the human gut tissue (**Fig 10d**, every datapoint refers to mean projected pattern weight for cells within a defined age or age range). This suggests that analogous to the age-associated increase in MENs we observed in murine gut (**Fig 10b**), the proportions of MENs in the human gut also might increase with age. We also found that the representation of MENs cluster (Cluster 1) in the post-natal human gut cell atlas data expanded with maturation and age (Suppl. Fig 7d)

GDNF and HGF levels regulate the populations of the neural crest-derived and the mesoderm-derived neurons, respectively, in the maturing ENS

GDNF-RET signaling is responsible for proliferation and neurogenesis from NC-derived ENS precursor cells during development as well as for the survival of Ret-expressing enteric neurons^{87–90}. Similarly, HGF signaling has been shown to be essential for the proliferation of mesoderm-derived cells⁹¹. Since the expression of the HGF receptor MET and the GDNF receptor

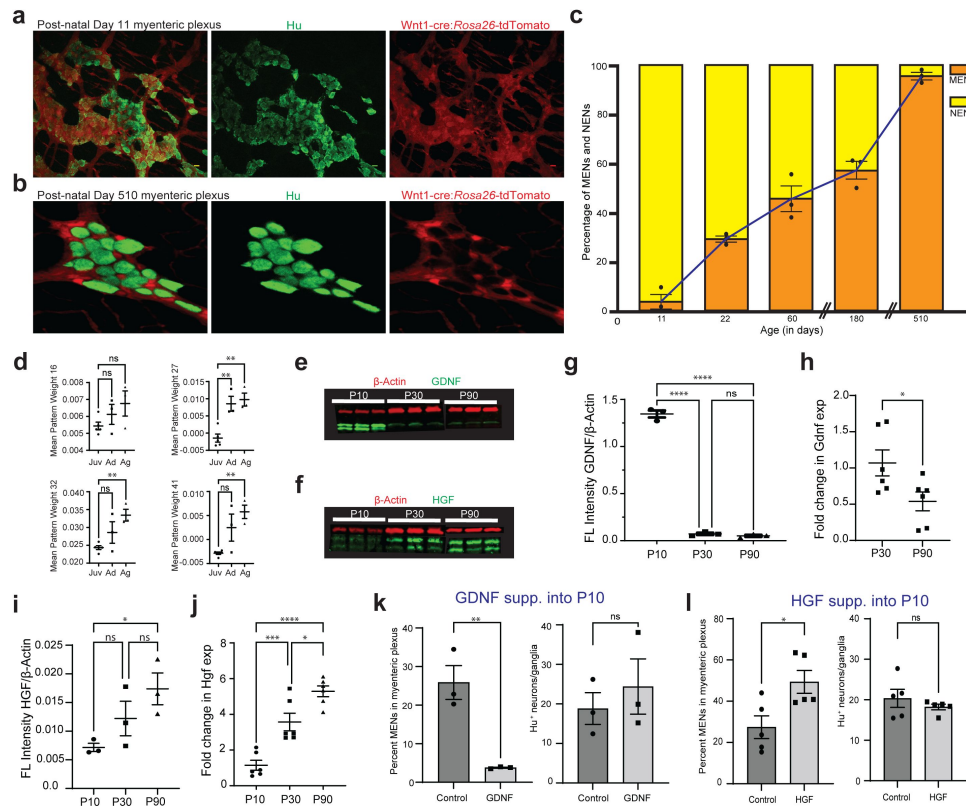


Fig 10

GDNF and HGF signaling regulate age-dependent changes in NENs and MENs proportions.

- (a, b) Immunostaining myenteric plexus tissue from juvenile and mature *Wnt1-cre:Rosa26-tdTomato* mice with antibodies against the pan-neuronal marker Hu (green).
- (c) Age-associated loss of NENs and gain of MENs in the small intestinal LM-MP of maturing and aging *Wnt1-cre:Rosa26-tdTomato* mice. Data represent mean $\pm$ S.E.M.
- (d) Mean $\pm$ SEM of the four MEN-specific pattern weights in the human mesenchymal cell populations from Elmentaite et al.⁸¹, wherein data from age ranges of 4 – 12 years was clubbed together as Juvenile (Juv), data from age ranges of 20 – 50 years was clubbed together as Adult (Ad), and data from age ranges of 60 – 75 years was clubbed together as Aged (Ag). Every datapoint refers to mean projected pattern weight for cells within a defined age or age range, where Juv data comprise of ages 4, 6, 9, 10, 12 years; Ad data comprise of age ranges 20 – 25, 25 – 30, and 45–50 years; and finally Ag data comprise of age ranges 60 – 65, 65 – 70, and 70 – 75 years. One-way ANOVA ** = $p < 0.01$.
- (e) Western blot analyses of GDNF (green) and the house-keeping protein β -actin (red) expression in LM-MP tissues from mice of ages P10, P30, and P90. ($n = 3$ mice per group; each sample is a biological replicate). Fluorescent intensities of the two bands of GDNF (that correspond to ~25 kD bands of protein marker) were quantified together. The lower band of GDNF is present only in the P10 tissues and disappears in P30 and P90 adult murine tissues.
- (f) Western blot analyses of HGF (green) and the house-keeping protein β -Actin (red) expression in LM-MP tissues from mice of ages P10, P30, and P90. ($n = 3$ mice per group; each sample is a biological replicate). Fluorescent intensities of the two bands of HGF (that are between 50 kD and 37 kD bands of the protein marker) were quantified together.
- (g) The normalized fluorescent intensity of GDNF protein to house-keeping protein β -Actin compared between the three age groups. GDNF presence was highest in P10 group and was significantly reduced in P30 and P90 groups. Data represent mean $\pm$ S.E.M. One-way ANOVA **** $p < 0.001$.
- (h) Age-dependent decrease in *Gdnf* mRNA transcript expression (normalized to the house-keeping gene *Hprt*) in the myenteric plexuses of P30 and P90 old mice. Data represent mean $\pm$ S.E.M. Student's t-test * $p < 0.05$.
- (i) The normalized fluorescent intensity of HGF protein to house-keeping protein β -Actin was compared between the three age groups. HGF expression significantly increased from P10 through P90. Data represent mean $\pm$ S.E.M. One-way ANOVA * $p < 0.05$.
- (j) Age-dependent increase in *Hgf* mRNA transcript expression (normalized to the house-keeping gene *Hprt*) in the myenteric plexuses of P10, P30, and P90 old mice. Data represent mean $\pm$ S.E.M. One-way ANOVA * $p < 0.05$, *** $p < 0.01$, **** $p < 0.001$.
- (k) Percent proportions of tdTomato- MENs and mean Hu+ neurons/ganglia in LM-MP of cohorts of *Wnt1-cre:Rosa26-tdTomato* mice that were dosed with GDNF or Saline from P10 to P20 age. Data represent mean $\pm$ S.E.M. Student's t-test ** $p < 0.01$.
- (l) Percent proportions of tdTomato- MENs and mean Hu+ neurons/ganglia in LM-MP of cohorts of *Wnt1-cre:Rosa26-tdTomato* mice that were dosed with HGF or Saline from P10 to P20 age. Data represent mean $\pm$ S.E.M. Student's t-test * $p < 0.05$.

RET is exclusive to MENs and NENs respectively, we studied the correlation between age and levels of HGF and GDNF in LM- MP (**Fig 10e, f**). Given that both GDNF, as well as HGF expression is found in the LM-MP layer^{44, 92, 93}, we used LM-MP tissue to study how the levels of these two trophic factors change in and around the ENS tissue during maturation. We found that in agreement with a previous report⁹¹, GDNF protein levels are markedly reduced between the P10 and the P30 ages and remains reduced thereafter up to the P90 age (**Fig 10g**; $n = 3$ mice/age-group, $F = 6.821$, $DFn, DFd = 1, 7$, $p = 0.0348$, One way ANOVA). In addition, expression of *Gdnf* transcripts show significant reduction between the ages of P30 and P90 (**Fig 10h**; $n = 6$ mice/age-group, P30: 1.070 ± 0.179 SEM, P90: 0.539 ± 0.129 SEM, $p = 0.037$, Student's t-test). On the other hand, HGF expression increases progressively between P10 and P90 ages (**Fig 10i, j**; $n = 3$ mice/age- group, Protein levels: $F = 8.820$, $DFn, DFd = 1, 7$, $p = 0.02$; mRNA levels: $F = 36.98$, $DFn, DFd = 1, 16$, $p < 0.0001$, One way ANOVA). The ratio of HGF to GDNF expression in ileal LM-MP tissue shows significant increase from the age of P10 to P90 (Suppl. Fig 9a, $F = 48.60$, $DFn, DFd = 1, 7$, $p = 0.0002$, One way ANOVA), and HGF expression is consistently higher than GDNF expression in the full thickness small intestinal tissue from adult and aging mice in the *Tabula muris* data⁹⁴ (Suppl. Fig 9b). We then queried the plasma proteome levels from the LonGenity cohort of 1,025 aging individuals and found that HGF levels correlated positively, while GDNF and RET levels correlated negatively with age (Suppl. Fig 9c)⁹⁵, suggesting parallels between our data from murine intestine and human plasma proteome data.

Since GDNF tissue levels are correlated with NENs proportions, we hypothesized that GDNF signaling regulates NENs proportions in maturing and adult ENS. On administration of GDNF or saline to cohorts of P10 *Wnt1-cre:Rosa26-tdTomato* mice over 10 days^{87, 96}, we found that GDNF treatment promoted the juvenile phenotype by enhancing the proportions of *tdTomato*+ NENs and correspondingly reduced the proportion of *tdTomato*- MENs in P20 mice to a level similar to that seen at the P10 age, while retaining the MENs proportions in saline-treated control mice remained at a level expected of its age (**Fig 10k**; Controls: %MENs: 25.87 ± 4.37 SEM of 1350 neurons from 3 mice; GDNF: %MENs: 3.86 ± 0.07 SEM of 1301 neurons from 3 mice; $p = 0.0072$; Student's t-test). Consistent with a previous report⁸⁷, the GDNF-driven expansion of NENs and associated contraction of MENs conserved the total neuronal numbers (**Fig 10k**; Controls: neurons/ganglia = 20.60 ± 4.00 SEM; GDNF: neurons/ganglia = 24.39 ± 6.96 SEM; $p = 0.52$; Student's t-test).

Since HGF tissue levels increase with age, we hypothesized that increasing HGF signaling drives the expansion of MENs in the maturing gut. HGF administration to cohorts of P10 *Wnt1-cre:Rosa26-tdTomato* mice over 10 days promoted an increase in the *tdTomato*- MENs population in P20 mice to levels previously observed in P60 mice, while tissues from saline - treated control mice exhibited a MENs:NENs ratio that is expected at P20 (**Fig 10l**; Controls: %MENs: 27.40 ± 5.49 SEM of 1970 neurons from 5 mice; HGF: %MENs: 49.37 ± 5.52 SEM of 1704 neurons from 5 mice; $p = 0.02$, Student's t-test). Similar to GDNF treatment, HGF treatment also did not cause any significant change in total neuronal numbers (**Fig 10l**; Controls: neurons/ganglia = 20.35 ± 2.22 SEM; HGF: neurons/ganglia = 18.21 ± 0.69 SEM; $p = 0.38$, Student's t-test).

Transcriptomic evidence of MENs genesis

As the proportions of MENs in the myenteric plexus rise significantly between the ages of P10 and P30, we reasoned that this phase was accompanied by significant neurogenesis of MENs. To provide transcriptomic evidence that MENs populations indeed expand during this phase, using 10X Genomics v3.1, we again performed unbiased scRNAseq and agnostic clustering, this time on unsorted cells from the myenteric plexus layer of two male mice of P21 age, when the MENs population is still expanding (Suppl. Fig 10a). Using NMF-generated MENs patterns to run projectR-based analyses (Suppl. Fig 10b) and by using the NENs and Neuroglia-specific markers, we again annotated the neuroglia, the NENs, and the MENs as before and found that at this age, we were able to sequence a similar number of MENs and NENs (~500 cells) along with a large number (~800) of neuroglia cells (**Fig 11a**). The expression of *Met* was again detected in the MENs

cluster, as well as in a small subset of Ret-expressing NENs in the P21 cluster (Suppl. Fig 10c). MENs showed significantly higher UMI per cell, when compared to neural crest-derived cells (NENs and neuroglial cells) (Suppl. Table 4, One-way ANOVA; $F = 187.4$, DF_n , $DF_d = 2$, 1877 , $p < 0.0001$), which we expected given the larger size of MENs⁹⁷ (Suppl. Fig 4a). By immunolabeling LM-MP tissue from 3 P21 *Wnt1-cre:Rosa26-tdTomato* male mice with antibodies against MET, we found that proportions of MET-expressing neurons at this age were significantly higher in the population of tdTomato-negative MENs than in the population of tdTomato⁺ NENs (of 152 MET-immunolabeled neurons across 3 mice, 81.51 ± 1.55 S.E.M. were tdTomato-negative MENs). The Met and Ret co-expressing NENs cluster in our data were described as populations of neural crest-derived Chat⁺ Calcb⁺ neurons by Zeisel et al.⁴⁸ at the P21 age (Suppl. Fig 10d), where they were annotated as ENT6, 8, and 9 clusters (and which correspond to clusters 1, 3, 4, 7, 6, and 7 of the Morarach et al.⁵¹ database). These Met- and Ret-co-expressing neurons were also described by Avetisyan et al.⁴⁴ as fetal-born neural crest-derived neurons that respond to both GDNF and HGF. By contrast, MENs do not express Ret and hence are the only cell population that can respond to solely to HGF. This data provides further evidence on the HGF-induced expansion of MENs during the juvenile phase.

We next performed *in silico* analyses on the MENs cluster using our recently published Tricycle software⁹⁸, which is capable of inferring continuous cell-cycle position and can be applied to datasets with multiple cell types, across species and experimental conditions, including sparse data and shallow sequenced droplet-based dataset - thus allowing us to find evidence of cell cycling in the MENs subset. P21 MENs scRNAseq data was projected into universal cell cycle principal components (PCs) defined by the expression of 500 cell-cycle correlated genes and the continuous cell cycle position (theta) was measured as the angle from the origin (Fig 11b). Based on expression of 500 cell-cycle correlated genes, cells between 0.5π and 1.5π show hallmarks of being cycling cells (Fig 11b). Our analyses showed that a significant number of cells in the MENs cluster (213 out of 510) were present between 0.5 and 1.5π , which represents the mitotic position in the continuous cell-cycle phase (Fig 11c). We confirmed our analyses by observing that the expression of the cell cycle gene *Top2a* in the cells of the MENs cluster was highly correlated with this phase (Fig 11d). Next, we found that a key cell cycle regulator gene *Ect2* (epithelial cell transforming 2), which encodes for the guanine nucleotide exchange factor protein ECT2, which activates RhoA in a narrow zone at the cell equator in anaphase during cell division⁹⁹, was expressed in the MENs cluster and its expression was highly correlated with the cell's mitotic position on the Tricycle plot (Fig 11e). We then used antibodies against ECT2 to immunolabel the LM-MP from P21 mice and found expression of this key cell cycle regulator protein in DECORIN-expressing and MHCst-expressing MENs at this age (Fig 11f, g). Thus, the Tricycle-based computational analyses found evidence of cell cycling in the MENs cluster and identified a key cell cycle gene *Ect2* as a marker for putative MENs precursor cells.

Reduced GDNF-RET signaling accelerates ENS aging to cause intestinal dysfunction

Since reduced GDNF or RET levels are associated with intestinal dysfunction in patients⁸³,¹⁰⁰,¹⁰¹, we hypothesized that alterations in GDNF-RET signaling unrelated to those seen with normal aging, would cause dysfunction. To test this hypothesis, we studied lineage proportions and intestinal function in a mouse model of reduced RET signaling. Ret-null heterozygous mice, which have been previously used to study the effect of reduced RET signaling in the adult ENS, have normal ENS structure but altered gut physiology⁸⁷. A similar mouse model with a Ret^{CFP} allele has a CFP reporter inserted at its *Ret* locus rendering it null¹⁰². Ret⁺/CFP (or Ret⁺/-) mice carrying a single copy of the *Ret* gene showed significant reduction in Ret transcript and RET protein expression in the early post-natal murine gut¹⁰². Similarly, we found significantly reduced Ret transcript expression in the adult LM-MP of Ret^{+/+} mice compared to age-matched wildtype Ret^{+/+} mice (Suppl. Fig 10e). In these mice, using antibodies against CFP/GFP, Hu, and MHCst, we confirmed that the NENs marker Ret-CFP, and the MENs marker MHCst were expressed

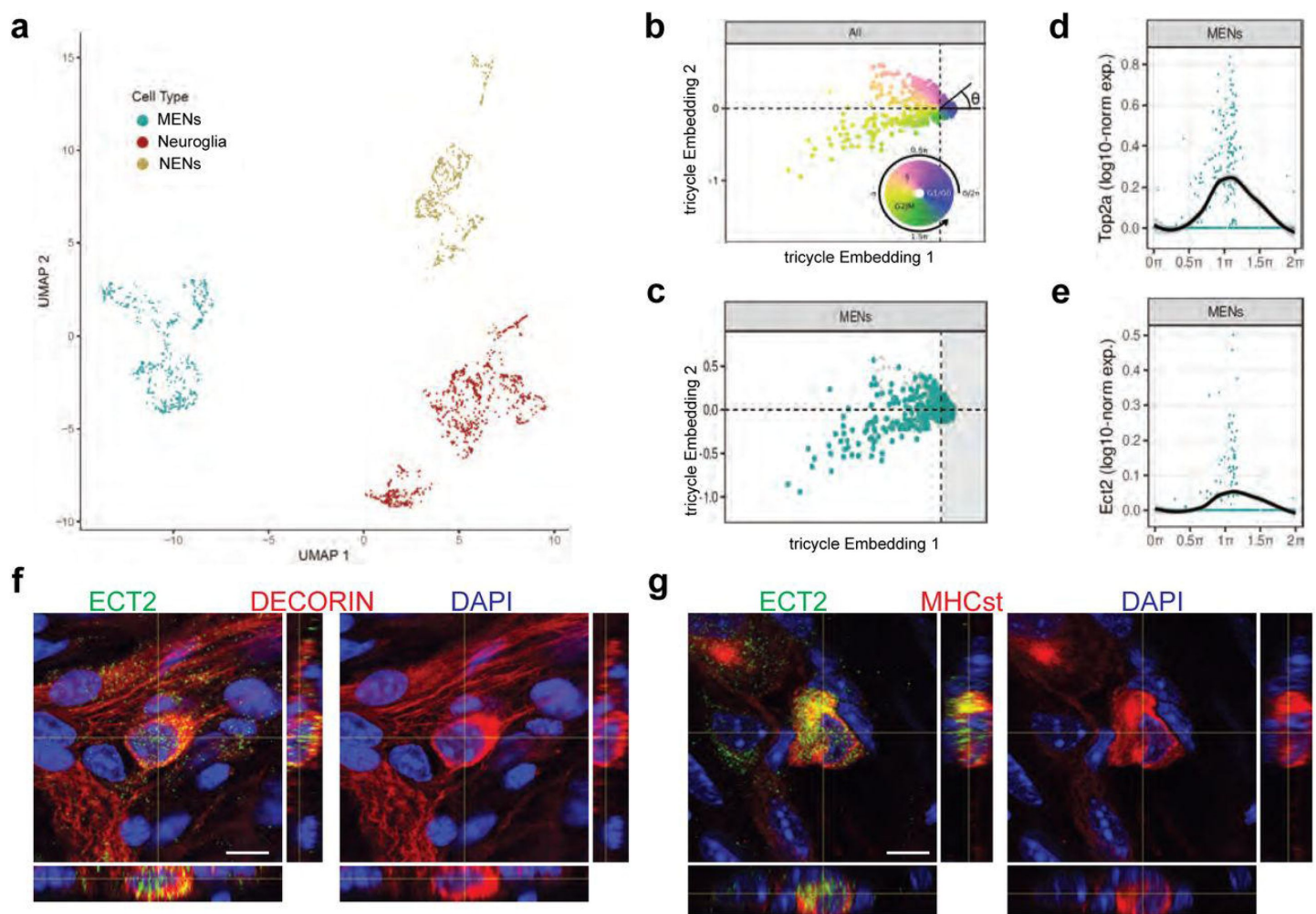


Fig 11

Ect2-expression labels a population of cycling MENs

(a) UMAP representation of the cells of the MENs, NENs, and Neuroglia clusters from scRNAseq of cells from the small intestinal LM-MP tissue of two post-natal day 21 (P21) mice.

(b) Tricicle analyses of ENS cell-types with their cell cycle positions. The continuous cell cycle position (theta) is measured as the angle from the origin. Anti-clockwise representation of cells that are represented between 1.5π and 0.5π space on the embedding are increasingly present within the quiescent G1/G0 cell cycle phase. In contrast, cells that are represented between the 0.5π and 1π space on the embedding are present within the S phase, and those within the 1π and 1.5π space are present within the G2M phase.

(c) Cells within the P21 MENs cluster were projected into Tricicle software. 213 of the 504 P21 MENs were present between the 0.5π and 1.5π space on the embedding and hence were inferred to be undergoing cell cycling.

(d, e) Expression of cell-cycle correlated genes Top2a and Ect2 in MENs shows significant expression of these two genes in cells of the P21 MENs cluster that are present between the values of 0.5π and 1.5π in the Tricicle embedding. Loess curve fittings (black) represent the dynamics of gene expression over the cell cycle (theta).

Orthogonal views of small intestinal myenteric ganglia from a P21 wildtype mouse when immunostained with antibodies against ECT (green) and against MENs markers (f) DECORIN (red) and (g) MHCst (red), shows that ECT2 is expressed in a subset of MENs at this age. Nuclei are labeled with DAPI (blue). Scale bar denotes 10 μ m.

by different neuronal subpopulations (**Fig 12a**). Using the adult Ret^{+/-} mice, we tested the effect of partial Ret loss on ENS lineages at two adult ages: 9 weeks (~P60) and 16 weeks (~P110). Using antibody against CFP/GFP to detect CFP⁺ RET-expressing neurons, we found that Ret^{+/-} mice show a significant reduction in the proportions of Ret-CFP⁺ NENs (**Fig 12b**; 9 weeks: %CFP⁺ neurons: 24.91 ± 5.42 SEM of 837 neurons from 3 mice; 16 weeks: %CFP⁺ neurons: 13.13 ± 0.98 SEM of 1227 neurons from 5 mice; $p = 0.03$, Student's t-test). We observed a corresponding significant increase in the proportions of MHCst⁺ MENs with age in Ret^{+/-} mice (**Fig 12c**; 9 weeks: %MENs: 58.74 ± 7.33 SEM of 644 neurons from 3 mice; 16 weeks: %MENs: 82.84 ± 3.58 SEM of 935 neurons from 5 mice, One-way ANOVA, $p = 0.014$), while control Ret^{+/+} mice showed no significant age-associated change in the proportions of MENs (**Fig 12c**; 9 weeks: %MENs: 43.27 ± 3.24 SEM of 780 neurons from 3 mice; 16 weeks: %MENs: 54.48 ± 4.07 SEM of 1276 neurons from 5 mice, One-way ANOVA, $p = 0.36$), which is consistent with our previous results that MENs proportions in wildtype animals are relatively stable in this time window. The expedited loss of NENs in Ret^{+/-} mice confirms that depletion of endogenous RET signaling in the adult ENS accelerates the aging-associated loss of NENs.

Having previously shown that aging mice have intestinal dysmotility¹⁰³, we tested whether the increased loss of NENs in the Ret^{+/-} ENS, concomitant with the expansion of MENs accelerated ENS aging, causes an early onset of aging-associated intestinal dysmotility. We studied whole gut transit time (WGTT) in a cohort ($n = 8$) of adult Ret^{+/-} mice and their littermate control ($n = 10$) Ret^{+/+} mice over 7 weeks, between 9 and 16 weeks of age. While 9-week adult Ret^{+/-} mice were similar to control Ret^{+/+} mice, WGTT between the two genotypes diverged with age. Consistent with a prior report⁸⁷, 16-week old Ret^{+/-} mice displayed significantly delayed intestinal transit compared to age-matched control Ret^{+/+} mice (**Fig 12d**; WGTT (in min) Ret^{+/+}: 121.4 ± 4.01 SEM; Ret^{+/-}: 157.3 ± 14.62 SEM, $p = 0.048$; Student's t-test).

GDNF reverts aging in the ENS to normalize intestinal motility

Along with others, we have previously shown that aging is associated with slowing of intestinal motility^{104, 105}. We hypothesized that this may be a consequence of the replacement of the juvenile NENs population by MENs and therefore GDNF supplementation, by restoring a more balanced MENs:NENs ratio, may prevent age related changes in motility. We studied 17-month-old male mice (at an age where NENs constitute only ~5% of all myenteric neurons; **Fig 10c**) before and after they received 10 days of intraperitoneal injection of GDNF or saline ($n=5$ for each cohort). While the two cohorts showed no significant difference in their intestinal transit times at baseline (WGTT (in min) Control: 192.8 ± 11.55 SEM; GDNF: 202.4 ± 7.60 SEM, $p = 0.50$, Student's t-test), GDNF treatment caused significant improvement in intestinal transit (**Fig 13a**, WGTT (in min) Control: 175.0 ± 8.89 SEM; GDNF: 101.0 ± 8.91 SEM, $p = 0.0004$, Student's t-test), reaching levels previously observed in healthy mice (**Fig 12d**).

GDNF treatment significantly reduced the proportions of MHCst⁺ MENs (**Fig 13b**; Suppl. Fig 11a, b; Control: %MENs: 88.76 ± 1.48 SEM of 909 neurons from 5 mice; GDNF: %MENs: 74.12 ± 5.98 SEM of 799 neurons from 5 mice; $p = 0.045$; Student's t-test) while increasing the numbers of RET⁺ NENs (**Fig 13c**; Suppl. Fig 11c, d; Control: RET⁺ neurons: 1.35 ± 0.05 SEM in forty-two 40X fields from 4 mice; GDNF: RET⁺ neurons: 3.19 ± 0.56 SEM in forty-one 40X fields from 4 mice, $p = 0.017$; Student's t-test). Consistent with our earlier observation, GDNF treatment did not change the average neuronal numbers in the ileal myenteric ganglia (Control: 17.56 ± 1.82 SEM neurons per ganglia, GDNF: 16.01 ± 1.22 SEM neurons per ganglia, $p = 0.49$, Student's t-test).

The two neuronal lineages in human disease

In addition to the detection of MENs in the adult healthy gut by bioinformatic and immunofluorescence means (**Fig 4**, **5**, **6**, **7**), we next examined the relevance of the two neuronal lineages NENs and MENs in human disease. For this, we tested whether gut dysfunction associated with pathological reductions in NENs-signaling mechanisms are also associated with

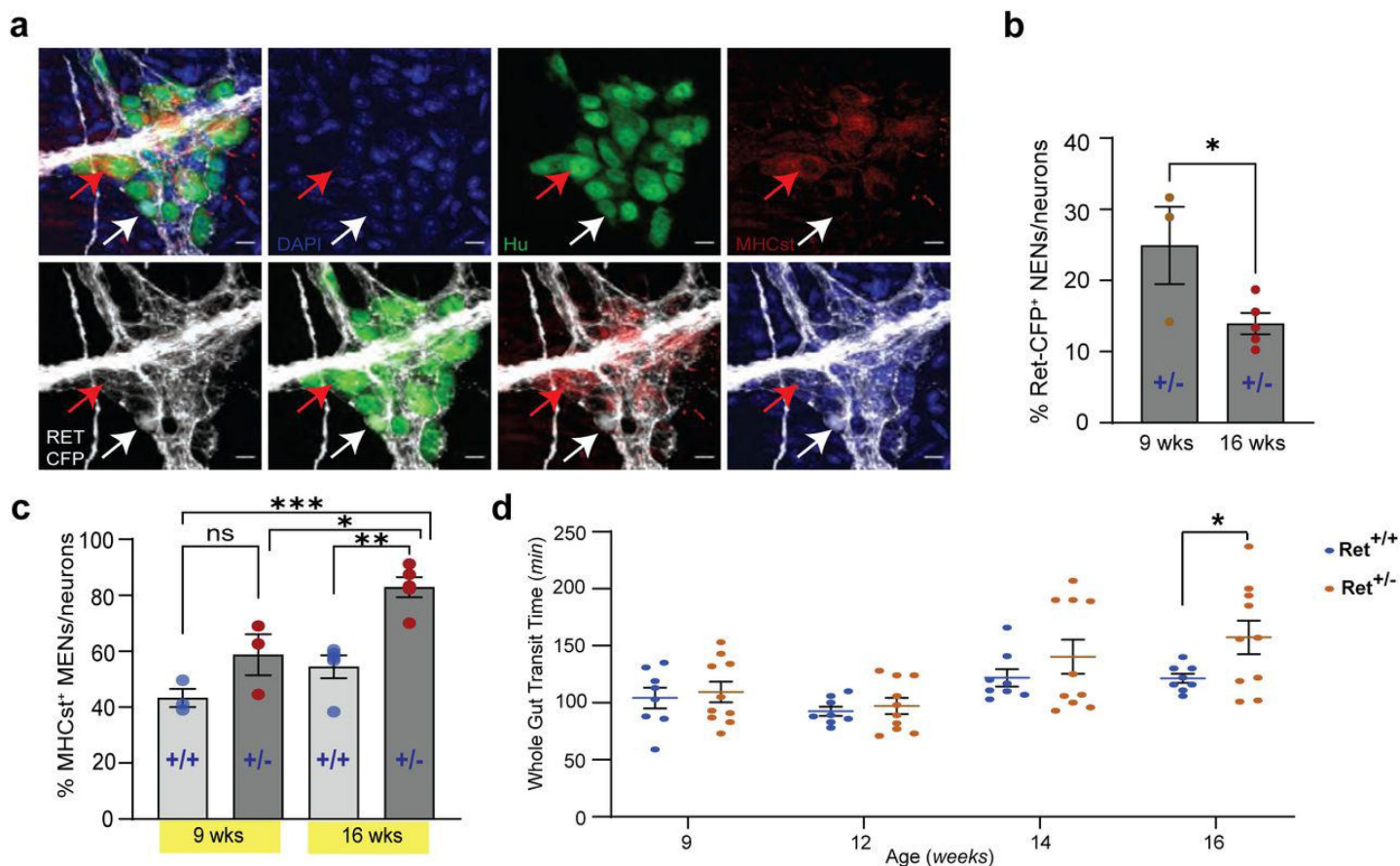


Fig 12

Reduced RET signaling accelerates ENS aging to cause pathology

(a) Hu immunostaining (green) LM-MP tissues from 16-week-old Ret+/CFP (Ret+/-) mouse shows mutually exclusive expression of Ret-CFP (cyan, white arrow) and MHCst (red, red arrow) MENs. Nuclei are stained with DAPI (blue). Scale bar = 10 μ m.

(b) Quantification of Ret-CFP+ neurons from 9- and 16-week-old Ret+/- mice show age-associated loss of Ret-CFP+ neurons. Data represent mean $\pm$ S.E.M. Student's t-test * $p < 0.05$.

(c) Quantification of MHCst+ MENs shows significant increase in their proportions in Ret+/- mice but not in Ret+/+ mice with age. Data represent mean $\pm$ S.E.M. One way ANOVA * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

(d) Measures of whole gut transit time (WGTT) in cohorts of Ret+/- and Ret+/+ mice MENs show significant slowing of whole gut transit of Ret+/- but not Ret+/+ mice with age. Data represent mean $\pm$ S.E.M. One way ANOVA * = $p < 0.05$.

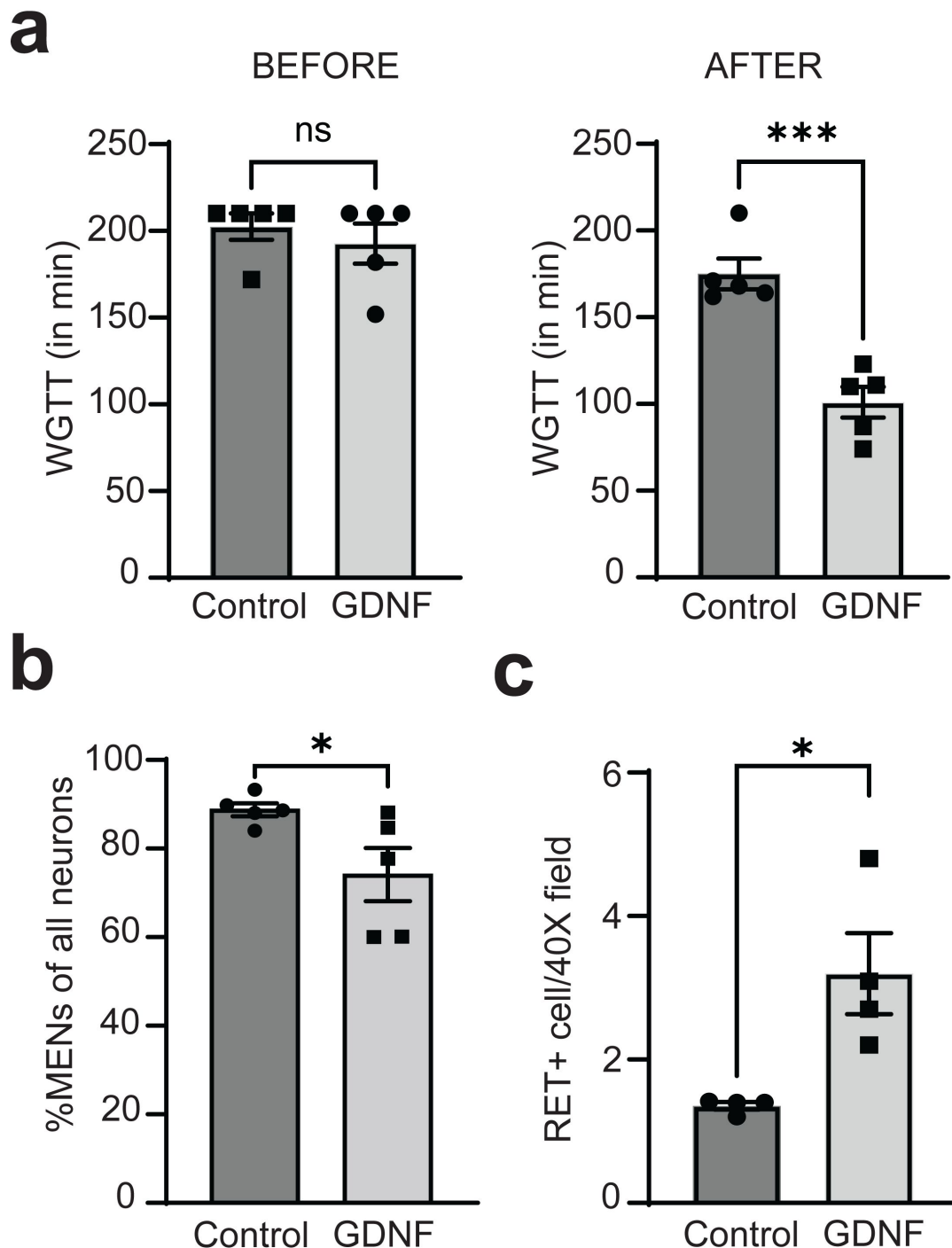


Fig 13

GDNF normalizes altered intestinal motility by increasing NENs proportions in the aging gut.

(a) Measures of whole gut transit time (WGTT) in GDNF (treated with GDNF) and Control (treated with Saline) cohorts of 17-month-old mice taken before the start of treatments and after the end of 10 consecutive days of treatment shows that the two groups are matched in their transit times before treatment, but GDNF treatment significant decreases average transit times when compared to the control cohort. Data represent mean $\pm$ S.E.M. Student's t-test *** = $p < 0.001$.

(b) Quantification of percent MHCst+ MENs per Hu-labeled neurons in myenteric ganglia in the GDNF and Control cohorts shows significant decrease in their proportions in GDNF-treated cohort but not in saline-treated control cohort. Data represent mean $\pm$ S.E.M. Student's t-test ** $p < 0.01$.

(c) Quantification of numbers of RET+ NENs per 40X field views of myenteric ganglia shows significant increase in their numbers in GDNF cohort mice when compared with Control cohort mice. Data represent mean $\pm$ S.E.M. Student's t-test * $p < 0.05$.

increased abundance of MENs. We mined previously generated and publicly available transcriptomic data from intestinal tissues from humans with normal gut motility (controls) and from patients with obstructed defecation (OD) associated with enteric neuropathy.¹⁰³ In this dataset, Kim et al. have previously shown that OD patients have significantly reduced intestinal expression of *Gdnf* and *Ret*.¹⁰³ Again, using our transfer learning tool *ProjectR*,^{72, 106} which allowed us to query bulk-RNAseq data across species to estimate relative use of MENs-specific NMF-patterns generated earlier, we tested whether the usage of MENs-specific NMF-patterns was significantly altered between transcriptomes of OD patients and healthy humans ($n = 3/\text{group}$).¹⁰³ This projection analysis indicated that 2 of the 4 MENs-specific NMF-patterns showed significantly higher usage in OD samples compared to controls (**Fig 14a**, Suppl. Fig 12; Student's t-test, $p < 0.05$), providing evidence that the enteric neuropathy in these patients is associated with a relative increase in MENs-specific transcriptional signatures. We further queried the differential gene expression analysis performed by Kim et al and found that in addition to *Gdnf* and *Ret* (as reported by them) other important NEN genes (*Snap25* and *Nos1*) were also significantly downregulated while the MEN marker genes identified in this study (*Clic3*, *Cdh3*, *Slc17a9*) (**Fig 14b**) were significantly upregulated.

Discussion

Current dogma states that the adult ENS is exclusively derived from neural crest precursors that populate the gut during fetal development.¹⁰⁷ The results of this study indicate a much more complex system, one in which the fetal and early life “juvenile” ENS consisting of neural crest-derived enteric neurons (NENs) is incrementally replaced during maturation by mesoderm-derived enteric neurons (MENs). Eventually, the aging ENS consists almost exclusively of the neurons of the MEN lineage. Using a combination of floxed transgenic reporters bred into multiple lineage fate mapping transgenic mice, this study also provides the first definitive evidence of a significant mesodermal contribution to any neural tissue. Previously, the mesoderm and especially the Lateral Plate Mesoderm (LPM) was known to give rise to diverse cell-types within the viscera, including several in the gut,¹⁰⁸ and our study shows that cells of this embryonic derivation also give rise to ENS neurons. A previous report on a dual origin of the ENS described adult enteric neurons from *Foxa2*⁺ or *Sox17*⁺ precursors¹⁴ and inferred that these were endodermal in origin. However, *Foxa2* and *Sox17* are also expressed by mesoderm-derived cells.^{25–28} By contrast, using two NC-lineage-traced mouse lines (*Wnt1-cre* and *Pax3-cre*), two lineage-traced mouse lines marking mesodermal derivatives (*Tek-cre*, and *Mesp1-cre*), and robust adult mesoderm markers, we identify a population of *Mesp1*-derived MHCst- and MET- expressing adult enteric neurons, which makes up the entire non-NC-derived population of myenteric neurons. These results provide robust evidence that the second source of adult enteric neurons is the mesoderm, and not the endoderm.

Distinct neuronal nature of MENs

While the developmental origins of neurons have been thought to be restricted to the neural tube and the neural crest, many neurons – such as placode- derived neurons, have been known to be derived from non-neural tube, non-neural crest developmental lineages.¹⁰⁹ Similarly, while supposed pan-neuronal markers, such as *Snap25*, are expressed specifically by neurons, not all neurons express *Snap25*.¹¹⁰ Indeed, release of neurotransmitters may occur independently of *Snap25*.^{111–116} suggesting that the expression of this protein is not central to neuronal functions. These present us with important context based on which, one can study the data generated using multiple lines of evidence to infer the neuronal nature of MENs. *Firstly*, we show that MENs are intra-ganglionic cells, which conditionally express reporters under mesoderm-specific lineage fate mapping models and are labeled with antibodies against the neuronal marker Hu. *Secondly*, MENs show expression of important ENS neurotransmitter-encoding or generating proteins CGRP, NOS1, and ChAT. *Thirdly*, with our single cell transcriptomic analysis, where we

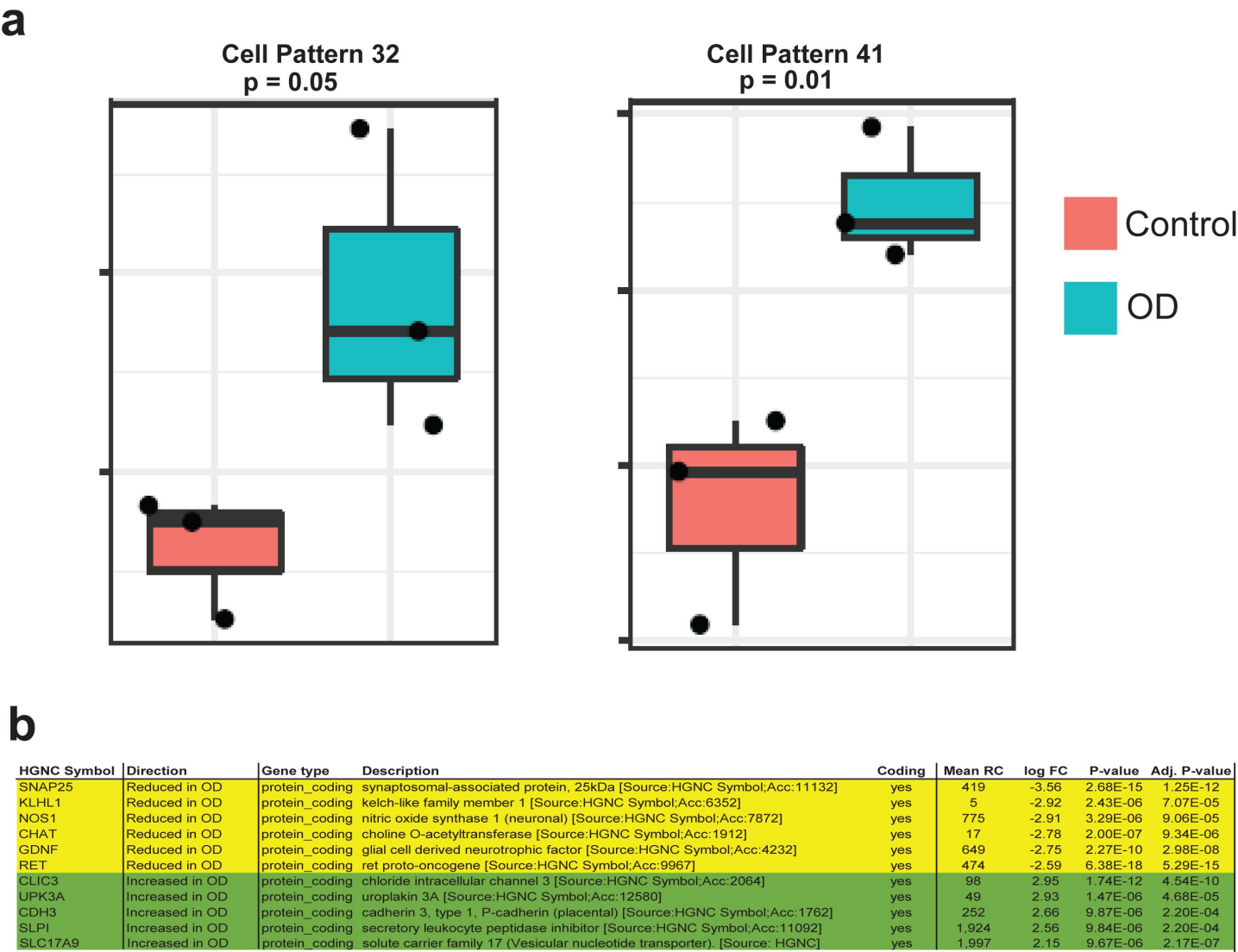


Fig 14

Patients with chronic gut dysmotility show significant shifts in their normal proportions of the two neuronal lineages.

(a) ProjectR-based projection of bulkRNAseq data from intestinal specimens of patients with normal motility and patients with obstructive defecation (OD), a chronic condition of intestinal dysmotility, into the 50 different NMF patterns learnt earlier, shows that the MEN-specific NMF patterns 32 and 41 were significantly upregulated in bulk-RNAseq of OD patients compared to controls. Data represent mean $\pm$ S.E.M. One way ANOVA. Data mined from raw data generated by Kim et al.¹⁰³

(b) OD patients show a significant decrease in the expression of important NENs-associated genes such as Ret, Gdnf, Snap-25, Nos1, Khlh1, and Chat, while showing a significant increase in the expression of important MENs-specific genes such as Clic3, Upk3a, Cdh3, Slpi, and Slc17a9. Data taken from Kim et al.¹⁰³

used the markers *Calcb*, *Met*, and *Cdh3* that we had validated to identify and annotate the MEN cell cluster, we discovered an additional set of neuronal-specific marker genes (*Pde10a*, *Vsnl1*, *Stmn2*, *Stx3*, *Tubb2b*, *Slc17a9*, *Hand2*, *Gpr88*, *Elavl2*, *Ntf3*) which were found to be expressed widely or selectively within MENs. These markers include both novel as well as established enteric neuronal markers^{2, 63, 117}. *Fourthly*, further transcriptomic analyses of the MENs scRNAseq cluster identifies the expression of many other neuronally significant genes – including those encoding for ion channels, neurotransmitter receptors, hormones, calcium channels, etc. – which provide a further glimpse into the diverse neuronal functions of MENs. *Finally*, our analyses shows that MENs express the pan-ENS- expressed gene *Phox2b*, and that our MENs scRNAseq cluster maps to a neuronal cluster in the May-Zhang et al.⁵⁰ database, which we shows expression of ‘mesenchymal’ and ‘neuronal’ markers⁵⁰. Our data, together with our analyses of publicly available data, thus provides evidence on the neuronal nature of MENs. While the co-expression of aforementioned pan-neuronal markers establishes the neuronal nature of MENs, the expression of set of key genes in MENs suggests putative specialized functional roles of these subpopulations when they co-exist with NENs in adults. These include the expression of genes associated with putative sensory (*Calcb*)¹¹⁸, neuromodulatory (*Ntf3*)¹¹⁹, secretory (*Cftr*)¹²⁰, immunomodulatory (*Il18*)², hormone regulating (*Ghrh*)¹²¹, extracellular matrix (ECM)-modulating (*Dcn*), and motility-regulating roles (*Slc17a9* or *VNUT*)¹²². These MEN-enriched or MEN-specific expression profiles suggest that MENs perform various roles in the regulation of diverse gastrointestinal functions. While the current scRNAseq data is restricted in providing meaningful cell clusters that can inform the true functional diversity of MENs, future work will provide detailed analyses and functional characterization of MENs.

Beyond establishing the neuronal nature of MENs, our analyses also help establish the distinct neuronal nature of MENs. Based on the lineage fate mapping experiments with multiple combinations of neural crest and mesoderm lineage fate mapping mice models and based on the expression of mesoderm-specific markers such as *MHCst* and *Decorin*, we identified that MENs are a population of mesoderm-derived cells. *MHCst* is a subset of myosin heavy chain isoforms that has been previously shown to be specifically expressed by a subset of mesoderm-derived muscle cells¹²³. While prior reports do not clarify the nature of the exact epitope and the gene responsible for *MHCst*, the expression of the smooth muscle myosin heavy chain protein *MYH11* specifically in MENs suggests that the epitope detected by the anti-*MHCst* antibody in MENs is of the mesoderm specific protein *MYH11* protein.

Further, our bioinformatic analyses of the human gut cell atlas data generated by Elmentaite et al.⁸¹ also shows the existence of a putative MENs-like cell cluster in the human gut, which shows a similar co-expression of mesenchymal and neuronal markers as observed by us in our MENs cluster and by May-Zhang et al.⁵⁰ in their ‘mesenchymal neuronal’ cluster. Indeed, Elmentaite et al.⁸¹ identified this supposed mesenchymal cluster by the expression of the mesoderm-specific gene *Decorin*. Further, by observing the co-expression of neuronal and mesoderm-specific markers in human gut tissue, we validate the presence of neurons that express mesoderm-specific markers or MENs in adult human tissue.

Detection and identification of MENs in other datasets

Recent studies have significantly increased our understanding of the molecular nature of various classes of enteric neurons. However, these studies differ significantly in their methods and tools used, which have precluded them from detecting or correctly identifying the developmental origins of MENs. While May-Zhang- et al.⁵⁰ observed the presence of a small neuronal cluster that expressed mesenchymal marker genes, their study did not further interrogate the developmental origins of the ‘mesenchymal’ enteric neurons. Drokhyansky et al.⁴⁹ in their unbiased MIRACL-seq analyses and Elmentaite et al.⁸¹ in their human gut cell atlas datasets detected the MENs cluster but could not correctly identify their neuronal nature, possibly owing to the expression of mesenchymal genes in this cluster, and also due to the absent or lower than expected expression of canonical neural markers such as *Ret* and *Snap25*. In contrast to these

studies that performed single cell or nuclear transcriptomic studies that were unbiased by lineage markers, other transcriptomic datasets from Drokhlyansky et al.⁴⁹, Wright et al.²², and Zeisel et al.⁴⁸ were generated using enriched neural crest-derived cells, which contributed to the absence of MENs in these datasets. An important outlier to these studies is Morarach et al.⁵¹ from the Marklund group, which utilized the newly characterized BAC-transgenic line Baf53b-cre to label and flow sort enteric neuronal cells for subsequent single cell RNA sequencing. Our analysis of ileal and colonic tissue from this line shows that it does not comprehensively label all enteric neurons. While this may seem at odds from the Morarach et al.⁵¹ report, this apparent difference in the behavior of this transgenic line can be explained by the fact that in contrast to the established and accepted practice of counting all Hu-immunolabeled neurons employed by us and others²²,⁵⁰,¹²⁴, the Morarach et al. study restricted the enumeration to only include neurons that showed only cytoplasmic and not nuclear expression of Hu. Neuronal Hu proteins (HuB, HuC, and HuD) show both nuclear and cytoplasmic localization.¹²⁵, and since the Morarach et al.⁵¹ study only enumerated cytoplasmic Hu-expressing neurons, their characterization of this transgenic mouse line was performed using only a subset of all neurons. In addition, compared to Drokhlyansky et al.⁴⁹, Wright et al.²², and May-Zhang et al.⁵⁰ whose protocols for neuronal enrichment were based on flow sorting neuronal nuclei, the Marklund group (in both Zeisel et al.⁴⁸ and Morarach et al.⁵¹) performed flow sorting of late post-natal enteric neuronal cells, which can cause significant stress on neuronal cells. These factors, together with the BAC transgenic nature of the Baf53b-cre line, may have contributed to the lack of MENs in the Morarach et al.⁵¹ data. Further, all neuronal cells described in the Morarach et al.⁵¹ study express Snap25 (which we show is not expressed by all adult enteric neurons), and mostly map to known neural crest-derived cell clusters identified in Zeisel et al.⁴⁸. This suggests that the rich transcriptomic atlas of enteric neurons generated by deep transcriptomic profiling of Baf53b-cre-derived neurons carried out by Morarach et al.⁵¹ is restricted only to the NENs, which comprise of half of all adult enteric neurons. Thus, prior experimental data could not establish the true developmental identity, correct annotation, and transcriptomic definition of a population of mesoderm-derived neurons or MENs in murine and human tissue.

By contrast, we were able to correctly identify the MENs cluster in unbiased scRNAseq of adult murine LM-MP cells and use it to both identify and validate the expression of MENs-specific markers in the adult murine LM-MP tissue, as well as use bioinformatic analyses to identify the MENs in publicly available murine data from May-Zhang et al.⁵⁰ and human data from Elmentaite et al.⁸¹. This allowed us to show the expression of MENs markers MHCst, MET, DCN, and SLC17A9 in many enteric neurons in adult humans, suggesting that the mesoderm-derived ENS may be a feature common to mice and humans alike. MENs in both species can readily be identified by their expression of MENs-specific markers, thus providing a convenient tool to further identify and study these neurons.

Development of MENs and MENgenesis

The presence of small population of MENs in the gut at the post-natal age day 10 (P10) and their expansion during maturation suggests that key timepoints in their development correspond with the post-natal development of the ENS. Importantly, since MENs populations are expanding at this age, performing scRNAseq-based bioinformatic analyses of the juvenile LM-MP cells not only provided us with the computational evidence of significant cell cycling in the MENs cluster at this age, but also provided us with the information on Ect2, a cell-cycle marker expressed by cycling putative MENs precursor cells. Ect2 transcripts were found expressed by cycling cells within the MENs cluster at the P21 age, which also showed significant enrichment in the expression of the cell-cycle gene Top2a. Except for a cluster of cells annotated as proliferating glial cells that show expression of the cell cycle gene Top2a, Ect2 gene expression is not detected in other murine myenteric neural crest-derived cells in the scRNAseq study performed by Zeisel et al.⁴⁸ at the P21 juvenile age. Our anatomical observation on the expansion of the MEN population in the adolescent gut along with the computational evidence of cell cycling in the MENs cluster at this

age, and the detection and validation of Ect2 gene expression in a subset of MENs provide evidence of significant MENgenesis during the juvenile phase. ECT2 expression was found in MHCst- and DCN- expressing myenteric cells at this age, suggesting that many of these are proliferating MEN- precursor cells, and may not be terminally differentiated functional neurons.

The rapidly expanding MEN population is 10-11 days old in the P21 post-natal gut (as they originate in the gut just before P10 post-natal age) and thus, the age at which these cells partake in regulation of diverse gastrointestinal functions is yet unknown. This important issue precludes us from using the two MENs transcriptomic datasets to compare how MENs functions change with age. Prior studies have reported on the differing birthdates of various neural crest-derived enteric neuronal sub-populations in the developing fetal gut^{9, 51, 126}. Similar studies need to be performed in the future to understand the birthdates of the various subpopulations that comprise MENs, and to ascertain when do they become functional. A recent study by Parathan et al.¹²⁴ highlighted important neuronal and neurochemical changes in the adolescent gut. Further studies on how the ENS matures and ages thus need to be performed in the context of NENs and MENs to truly understand how shared and distinct functions of these two lineages change with maturation and age. A crucial aspect of this work will require further identifying the molecular markers for the Ect2-expressing proliferating MEN progenitor cells. Our earlier work showed that Nestin-expressing neural progenitor cells are neural crest-derived cells that contribute to steady state neurogenesis in the adult ENS¹⁹, thus suggesting that MENs are derived from Ect2- expressing non-Nestin⁺ precursor cells.

Use of scRNAseq to identify MENs

The use of single cell and single nucleus transcriptomic approaches provides an important tool that has been recently used to perform molecular taxonomy of fetal and post-natal enteric neurons and glial cells^{22, 48, 50, 51}. In addition to performing deep sequencing that allows for cluster-based molecular taxonomy, these tools can also be utilized for identification of novel cell types, where prior information on their presence and marker expression is unknown^{52, 98, 127-129}. Compared to the other studies that utilized this tool to study the ENS, we used the single cell transcriptomics to both confirm the presence of MENs and identify more MEN-specific markers; as well as to use the MEN-specific transcriptomic signature to query their presence in other murine and human datasets. In contrast to its use for performing molecular taxonomy, which requires deep sequencing of an enriched cell population, our strategy of opting for a lower read depth (shallow sequencing) is efficient, practical, and economical for discovering and identifying novel celltypes⁵². In addition, even if a gene is being expressed at a medium - low level, there is a certain probability that it will not be detected by scRNAseq in both deep and shallow sequencing approaches. This is important since the older v2.0 10X Genomics chemistry (used for our 6-month dataset) captures only 10-14% of the cellular transcriptome, and that from the newer v3.1 chemistry (for our P21 data) captures only a third of the transcriptome from a suspension of homogenous single cells^{130, 131}. While a partial solution to this issue is to increase sequencing depth, beyond a certain point, this strategy leads to diminishing returns as the fraction of PCR duplicates increases with deeper sequencing. While getting insights into cellular heterogeneity of MENs is important for furthering our understanding of how they regulate diverse gut functions, the transgenic mice that can specifically label only MENs or all enteric neurons to allow for their enrichment are currently not available^{22, 48-51, 71}. Without the availability of MENs- specific transgenic models, the scRNAseq strategy employed by us is the only currently available tool for querying the transcriptome of MENs. These reflect significant bottlenecks that have disallowed the comprehensive and an in-depth characterization of all enteric neurons in all the current studies on the adult ENS.

Expression of MET and RET by MENs and NENs

Since our scRNAseq data highlighted the lineage-specific nature of the expression of Ret and Met in the adult ENS, we studied whether these genes regulated the origin, expansion, and maintenance of the neuronal populations that express them. The expression of MET by MENs in the mature adult ENS is consistent with an earlier study by Avetisyan et al. that reported that a deletion of Met in the Wnt1-cre expressing NC-lineage did not alter the abundance of MET⁺ neurons in the adult ENS⁴⁴. If Met was expressed solely by neural crest-derived cells, then the loss of MET by enteric neurons would have been expected since the conditional deletion of Met gene in this floxed Met transgenic animal has been observed to cause a significant or comprehensive loss of MET protein expression in other organs^{132–135}. While at first glance, observations of the mutually exclusive nature of RET and MET provided by us and by Avetisyan et al.⁴⁴ in the adult gut might not agree with Zeisel et al.'s⁴⁸ observed co-expression of Ret and Met transcripts in NENs at the P21 developmental stage, our observation that a small proportion of NENs in the P21 gut express MET helps provide an explanation. Our P21 data and the data from Zeisel et al.⁴⁸ are consistent with one of the observations made by Avetisyan et al.⁴⁴, who showed the existence of a population of fetal neural crest-derived enteric neurons that are co-dependent on GDNF and HGF and hence would express both RET and MET. In contrast to these observations made in the fetal and juvenile ENS, the anatomical observations made by Avetisyan et al.⁴⁴ in the adult ENS (where MET immunoreactivity in the adult enteric neurons is exclusive of Ret expression) suggests that the population of fetal-derived Ret and Met co-expressing neurons is lost after maturation. Compared to the P21 scRNAseq data from Zeisel et al.⁴⁸ that shows that all Met-expressing neural crest-derived neurons co-express Calcb (CGRP), Ret, and Chat, Avetisyan et al.'s anatomical data in the adult gut shows that despite accounting for a third of all enteric neurons and expressing CGRP, MET-expressing adult enteric neurons do not co-express Ret and only a small proportion of these MET⁺ neurons co-express ChAT. Thus, Avetisyan et al.'s data agrees with our data on the adult ENS which shows exclusive expression of RET and MET by MENs and NENs, respectively, and that while MENs consist of a large proportion of CGRP-expressing MET⁺ neurons, they contain only a small population of ChAT-expressing neurons. Along with Avetisyan et al.'s data, our data suggests that the adult small intestinal myenteric plexus contains a significant proportion of neurons that do not immunolabel with antibodies against NOS1 or ChAT. This observation is consistent with earlier data from Parathan et al.¹²⁴ who showed that ~35–40% of small intestinal myenteric neurons from 6-week-old mice did not immunolabel with antibodies against NOS1 or ChAT.

Since they do not express the neural crest-marker RET, MENs are the only neuronal population that respond to HGF alone. In this study, we show that MENs are dependent on HGF-MET signaling in a manner analogous to the requirement of GDNF-RET signaling by NENs. While our study does not discount that the exogenous HGF supplementation in juvenile mice may alter proportions of RET and MET-co-expressing neurons within the NENs subpopulation, our report focuses on HGF's ability to drive the expansion of MENs population in the ENS. Similarly, Ret haploinsufficiency-mediated loss of the NEN lineage causes a proportional increase in the MEN lineage, while maintaining overall neuronal numbers. Conservation of neuronal number, despite significant shifts in the proportions of the two neuronal lineages suggests the presence of yet unknown quorum sensing signaling mechanisms between the two lineages that reciprocally regulate their populations to maintain the structure of the post-natal ENS. This again implies the existence of a yet undefined precursor cell responsible for the expansion of the MEN population in a manner analogous to what we have previously described for NENs¹⁹.

Relevance to aging and human disease

While it is known that Gdnf expression in the mature gut is significantly downregulated^{44, 87, 96, 136}, the functional consequences of this loss have been unclear⁸⁷. We found that reduced GDNF-RET signaling drives the age-dependent loss of NENs as this loss can be slowed or reversed by GDNF supplementation and accelerated by further reduction of Ret expression. In aging

animals, GDNF-driven resuscitation of NENs was associated with a functional normalization of age-associated delay in intestinal transit. Our results identify a novel role for GDNF in maintaining or resuscitating the canonical NEN population, while preserving overall enteric neuronal numbers. In the last few years, studies have focused on identifying juvenile protective factors (JPFs), the loss of which correlates with or drives maturation- and aging-associated phenotypes¹³⁷. In this context, GDNF may therefore qualify as a JPF or a *senolytic* as its presence maintains the dominance of NENs in maturing ENS, corresponding to a juvenile phenotype; and its re-introduction promotes and resuscitates the genesis of NENs in adult and aging gut to restore healthy gut function. The exact nature of the cells that respond to GDNF re-introduction and generate NENs is yet unknown, but it can be hypothesized that these may include Nestin⁺ enteric neural stem cells and/or GDNF-responsive Schwann cells¹⁹, ¹³⁸. In addition to its effect on resuscitating NENs, acute administration of GDNF also augments peristaltic motility by increasing neuronal activity²², ¹³⁹. Since the aging gut has a very small number of RET-expressing GDNF-responsive NENs, we assume that GDNF-driven normalization of intestinal motility would first increase the proportions of RET-expressing NENs and then augment their activation. This further emphasizes the importance of using GDNF or similar RET agonists in the treatment of age-associated intestinal dysmotility.

While Avetisyan et al. showed that MET expression highly correlated with CGRP-expression in the adult murine myenteric plexus⁴⁴, MET co-expression by all MHCst⁺ human enteric neurons in our data and by all PGP9.5⁺ neurons in human enteric neurons by Avetisyan et al.⁴⁴ suggests that MET-expression in the human ENS may not be restricted to CGRP-expressing IPANs. In addition, recent data shows that our understanding of the nature of markers that can correctly identify IPANs in the murine and human ENS is still evolving⁵⁰, ¹⁴⁰, suggesting that ascribing function to neurons using a single marker may be incorrect.

The detection of MENs cluster in the post-natal and adult scRNAseq data in the gut cell atlas generated by Elmentaite et al.⁸¹, their increasing representation with age, and the subsequent validation of the MEN-specific markers MHCst, MET and SLC17A9 in a subset of adult human ENS neurons shows that our findings may be of clinical importance. Many gastrointestinal motility functions are compromised with advancing age, resulting in clinically significant disorders¹⁴¹. Although the exact mechanisms will need to be worked out, our results indicate that a MENs-predominant ENS is associated with significant differences in gut motility. The progressive nature of the change in enteric neuronal lineages with age may have pathological implications for the elderly gut when the balance is overwhelmingly in favor of MENs. Understanding the forces that regulate parity between these two different sources of neurogenesis therefore holds the promise of arresting or reverting progressive loss of gut motility with increasing age. Our results may also have implications for the pathogenesis of disordered motility unrelated to aging, as downregulation of *Gdnf* and *Ret* expression has been associated with diverticular disease and obstructed defecation in adult patients¹⁰⁰, ¹⁰³, ¹⁴². GWAS analyses further showed that *Gdnf* gene was in the eQTL associated with increased incidence of diverticular disease¹⁴³. It is in this context that the identification of SNAP-25 as a NENs marker gene and the transcriptomic pattern analyses of patients with chronic obstructed defecation are significant. Expression of *Snap25* is upregulated by GDNF and is significantly downregulated in gut tissues of patients with diseases associated with significant reduction in GDNF-RET signaling (diverticular disease and obstructed defecation)⁸³, ¹⁰⁰, ¹⁰³. Thus, while earlier thought to be a pan-neuronal⁴⁹, ¹⁴⁴, establishing the identity of SNAP-25 as a NEN lineage-restricted marker provides us not only with an important tool to query proportions of NENs in murine and human tissue but also suggests the NENs-limited nature of prior observations based on *Snap25* transgenic animals⁸², ¹⁴⁴. Gut dysmotility disorders that present with conserved ENS neuronal numbers¹⁰⁰ have puzzled investigators on the etiology of the disease. Our results suggest an explanation based on NENs-to-MENs lineage shifts that conserve overall enteric neuronal numbers with variable effects on function.

In conclusion, using multiple lines of evidence, we show that the ENS of the juvenile and mature mammalian gut is dominated by two different classes of neurons that have distinct ontogenies and trophic factors, associated with functional differences. The shift in neuronal lineage may represent a functional adaptation to changes in nutrient handling, microbiota or other physiological factors associated with different ages. Further research in the functional differences between the neuronal lineages and factors that regulate the parity between these two nervous systems in humans during the lifetime will be important to advance our understanding of the adult ENS and the treatment of age-related and other pathological disorders of gut motility.

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Author contributions

S.K., and P.J.P. conceived the study; S.K., M.A., M.L., L.A.G. and P.J.P. designed the research studies; S.K., L.B., C.Z., A.S., A.B., and M.S. conducted the experiments; S.K., Z.H., M.K., J.L., L.B., S.B., E.V., J.S., S.N., M.S., G.L., and L.A.G. acquired and analyzed the data; S.K., and P.J.P. drafted the manuscript.

Methods

Animals

Experimental protocols were approved by The Johns Hopkins University's Animal Care and Use Committee in accordance with the guidelines provided by the National Institutes of Health. Presence of vaginal plug was ascertained as 0.5 days post-fertilization and this metric was used to calculate age of mice. Only male mice were used for the studies detailed in this report. The *Wnt1*-cre:*Rosa26*-tdTomato lineage-traced line was generated as detailed before by breeding the B6.Cg-Tg(*Wnt1*-cre) with the Ai14 transgenic mouse line (Jax #: 007914) containing the *Rosa26*-tdTomato transgene.^{19, 145, 146} The *Wnt1*-cre line used in this study is the well validated line for studying neural crest derivatives previously used by us and others^{18, 19, 22, 48, 147}, and not the *Wnt1*-cre2 line used by Drokhlyansky et al.⁴⁹. We again validated the fidelity of this line

by observing whether any aberrant recombination occurred in our *Wnt1-cre:Rosa26-tdTomato* line by studying the intestinal mucosal tissue and found that the line behaved expectedly to label neural crest- derivatives and epithelial cells (Suppl. Fig 1i). *Pax3-cre:Rosa26-tdTomato* lineage-traced line was generated by breeding the Ai9 transgenic mouse line (Jax #: 007909) with the *Pax3-cre* transgenic mouse (Jax #: 005549). The *Wnt1-cre:Hprt-tdTomato* mouse was generated by breeding our aforementioned *Wnt1-cre* transgenic mouse line with the *Hprt-tdTomato* transgenic mouse line (Jax #: 021428, kind gift from Prof. Jeremy Nathans). *Mesp1-cre:Rosa26-tdTomato* mice were generated by breeding the *Mesp1-cre* transgenic mice¹⁴⁸ (gift from Dr. Chulan Kwon, JHU) with the Ai14 transgenic mice. *Mesp1-cre:Rosa26-GFP* mice were generated by breeding the *Mesp1-cre* transgenic mice with the *Rosa26-EGFP* transgenic mouse line (gift from Dr. Akira Sawa, JHU). Neither the *Rosa26-tdTomato* (Ai14) line nor the *Rosa26-EGFP* line showed any reporter expression in the absence of a cre driver (Suppl. Fig 1j, k). *Ret+/CFP* mice (MGI:3777556) were inter-bred to get a colony of adult *Ret+/+* and *Ret+/CFP* mice. *RetCFP/CFP* mice died at or before term. *Tek-cre:Hprt-tdTomato* mice were generated by breeding *Tek-cre* transgenic mice (also known as *Tie2-cre*; Jax #: 004128) with the *Hprt-tdTomato* transgenic mouse line. 17-month- old male C57BL/6 mice from the aging mouse colony of the National Institute of Aging were procured for the GDNF-treatment experiment.

Human Tissues

Human tissues were obtained under IRB protocol IRB00181108 that was approved by Institutional Review Board at the Johns Hopkins University. Pathological normal specimens of human duodenum and colon were obtained post-resection. Tissues were obtained from adult donors and were de-identified such that the exact age, gender, and ethnicity of the donors was unknown.

Tissue preparation

Mice were anesthetized with isoflurane and sacrificed by cervical dislocation. A laparotomy was performed, and the ileum was removed and lavaged with PBS containing penicillin-streptomycin (PS; Invitrogen), then cut into 1-cm-long segments and placed over a sterile plastic rod. A superficial longitudinal incision was made along the serosal surface and the LM-MP was peeled off from the underlying tissue using a wet sterile cotton swab¹⁹ and placed in Opti-MEM medium (Invitrogen) containing Pen-Strep (Invitrogen). The tissue was then laid flat and fixed within 30 minutes of isolation with freshly prepared ice cold 4% paraformaldehyde (PFA) solution for 45 minutes in the dark to preserve fluorescence intensity and prevent photo-bleaching. All LM-MP tissues post-isolation were fixed within 30 minutes of their isolation. After the fixation, the tissue was removed and stored in ice cold sterile PBS with Pen-Strep for immunofluorescence staining and subsequent microscopy.

For human tissues, duodenal tissue from adult human patients (n = 3 patients), who did not have any prior history of chronic intestinal dysmotility, that was removed by Whipple procedure was obtained. A colonic sample from a pathologically normal colonic resection from an adult donor suffering from colon carcinoma who similarly did not have prior history of chronic intestinal dysmotility was also obtained. The resected tissue was placed in ice cold Opti-MEM medium (Invitrogen) containing Pen-Strep (Invitrogen). The mucosal and sub-mucosal tissue was dissected out in the medium under light microscope and the muscularis layer containing myenteric plexus tissue was obtained. The tissue was laid out between two glass slides and fixed overnight in ice cold 4% PFA after which it was removed and stored in ice cold sterile PBS with Pen-Strep for immunofluorescence staining, optical clarification, and subsequent microscopy.

Immunohistochemistry

For murine tissue: The fixed LM-MP tissue was washed twice in ice-cold PBS in the dark at 16°C. The tissue was then incubated in blocking-permeabilizing buffer (BPB; 5% normal goat serum with 0.3% Triton-X) for 1 hour. While staining for antibodies that were mouse monoclonal, 5%

normal mouse serum was added to the BPB. The tissue was then removed from the BPB and was incubated with the appropriate primary antibody at the listed concentration (Supplementary Table 1) for 48 h at 16°C in the dark with shaking at 55 rpm. Following incubation with primary antibody, the tissue was washed three times (15-min wash each) in PBS at room temperature in the dark. The tissue was then incubated in the appropriate secondary antibody at room temperature for 1 hour while on a rotary shaker (65 rpm). The tissue was again washed three times in PBS at room temperature, counterstained with DAPI to stain the nuclei, overlaid with Prolong Antifade Gold mounting medium, cover-slipped, and imaged.

Immunostaining for ChAT was performed using fixed and unpeeled murine tissues from adult male *Wnt1-cre:Rosa26-tdTomato* mice. The intestinal segments were obtained by perfuse fixing the mouse with ice-cold PBS and then freshly prepared 4% PFA solution. Once the intestine was harvested, the ileum was flushed with cold HBSS with 0.4 mol/L N-acetyl-L-cysteine and then PBS to remove luminal contents. After, the tissue was fixed in 4% PFA overnight. The fixed tissue was then immersed in 2% Triton-X 100 solution for 2 d at 15°C for permeabilization. The tissue was immunostained with antibodies against Hu (ANNA1) and against ChAT (rabbit, Abnova). The primary antibody was then diluted (1:100) in the dilution buffer (0.25% Triton X-100, 1% normal goat serum, and 0.02% sodium azide in PBS) and incubated for 1 day at 15°C. An Alexa Fluor 647-conjugated goat anti-rabbit secondary antibody (1:200, Invitrogen) and an Alexa Fluor 488-conjugated goat anti-human secondary antibody (1:200, Invitrogen) were then used to reveal the immunopositive structure. Finally, the labeled specimens were immersed in FocusClear solution (CelExplorer) for optical clearing before being imaged by laser confocal microscopy using a Zeiss LSM 510 META. The LSM 510 software and the Avizo 6.2 image reconstruction software (VSG) were used for the 2D and 3D projection of the confocal micrographs. The Avizo software was operated under a Dell T7500 workstation and the “Gaussian Filter” function of Avizo was used for noise reduction of the micrographs.

Colchicine treatment: For CGRP immunostaining, mice were injected with Colchicine at a concentration of 5 mg/Kg body weight 16 hours (overnight) before they were sacrificed. The mice were housed singly during this time and adequate gel packs were provided. Food and water were provided *ad libitum*. On the following day, the mice were sacrificed, and their LM-MP tissues were harvested as detailed above.

For human tissue: The fixed muscularis layer containing myenteric plexus tissue was removed from ice cold PBS and incubated in blocking-permeabilizing buffer (BPB; 5% normal goat serum, 5% normal mouse serum with 0.3% Triton-X) for 4 hours. The tissue was then removed from the BPB and was incubated with the appropriate primary antibody at the listed concentration (Supplementary Table 1) for 5 days at 16°C in the dark with shaking at 55 rpm. Following incubation with primary antibody, the tissue was washed five times (15-min wash each) in PBS at room temperature in the dark. The tissue was then incubated in the appropriate secondary antibody at 16°C in the dark with shaking at 55 rpm for 2 days. The tissue was again washed in dark for five times in PBS that contained DAPI at room temperature. After the final wash, the tissue was suspended in tissue clarification buffer CUBIC¹⁴⁹ for 1 hour at 4°C in the dark after which it was overlaid with Prolong Antifade Gold mounting medium, cover-slipped, and imaged. Briefly, the CUBIC optical clarification buffer was made by mixing 2.5 g of urea (25% by wt), 2.5 g of N, N, N', N'-tetrakis (2-hydroxy-propyl) ethylenediamine (25% by wt), 1.5 g of Triton X-100 (15% by wt) in 35 ml of Distilled Water. The solution was shaken till the ingredients were dissolved and yielded a clear viscous solution.

Microscopy

Except for ChAT microscopy, all other imaging was done by using the oil immersion 63X objective on the Leica SP8 confocal microscope and by using the oil immersion 40X objective on the Olympus Fluoview 3000rs confocal microscope with resonance scanning mode. For thick tissues,

such as human tissues, the Galvano mode of the Olympus Fluoview 3000rs microscope that enabled higher resolution imaging and averaging was used. Images obtained were then analyzed using Fiji (<https://fiji.sc/>).

Live tissue imaging of the mouse gut tissue was performed by harvesting small intestinal tissue from an adult Wnt1-cre:*Rosa26*-tdTomato mouse and immediately putting it in OptiMEM solution. The tissue was then immediately put on a chamber slide containing OptiMEM and imaged in live tissue culture conditions of the EVOS M7000 microscope under the 20X objective.

Enumeration of neurons

Enumeration of tdTomato⁺ and tdTomato⁻ neurons was performed on tissue with native tdTomato fluorescence. The native tdTomato fluorescence in fixed tissue was intense, thus there was no need to increase fluorescent signal using anti-RFP/tdTomato antibodies.

Enumeration of enteric neurons to study alterations to ENS neuronal numbers was performed by following the well-established protocol^{150–153} of counting the numbers of neurons in myenteric ganglia and comparing them between animals for statistically significant differences. Identification of myenteric ganglia was performed according to our pre-determined method published earlier¹⁹. Briefly, contiguous clusters of neurons were defined as a ganglion and the total numbers of neurons within these clusters were enumerated as numbers of myenteric neurons per ganglion. As a rule, clusters of 3 neurons or more were deemed to consist of a ganglion and our enumeration strategy did not count extra-ganglionic neurons. We imaged ~10 ganglia per tissue for our enumeration and each group studied had $n \geq 3$ mice. Identification and enumeration of neurons and detection of co-localization was performed manually by trained laboratory personnel. **Fig 1d** comprises of the data from 6 P60 mice, which includes data from the 3 mice that were used for the development experiment presented in **Fig 8c**.

Protein isolation and detection

After the LM-MP tissue was isolated, it was weighed and placed in a sterile 1.5 ml microfuge tube. 1X RIPA buffer (Cell Signaling Technology) with Halt Protease Inhibitor Cocktail (Thermo Scientific) at 5X concentration, Phosphatase Inhibitor Cocktails II and III (Sigma-Aldrich) at 2X concentrations were added to the tissue lysate buffer. Tissue was disrupted using 1.0 mm silica beads in Bullet Blender 24 (Next Advance) for 5 mins at highest setting. The lysate was incubated at 4°C with shaking for 30 mins, centrifuged at 14,000rpm for 20 mins and the supernatant was taken and stored in -80°C in aliquots. Protein concentration was estimated using Bradford assay solution (Biorad) following the manufacturer's protocol. For immunoblotting, 40 µg of protein was loaded per well of 4%-20% gradient denaturing gel (Biorad). Protein marker used was Precision Plus Dual Color standards (Biorad). After fractionating the proteins, they were blotted onto ImmunBlot PVDF membrane (Biorad) overnight at 4°C at 17V for 12-16 hours. After blotting, membrane was blocked with Odyssey TBS blocking buffer (Li-Cor) for 1 hour at room temperature with shaking. Incubation with primary antibodies were carried out at 4°C with shaking for 24 hours. Following binding, the blot was washed 4 times with TBS-T (Tris Buffered Saline with 0.5% Tween) for 15 mins each with shaking at room temperature. Secondary antibody incubation was carried out in dark at room temperature for 1.5 hours with shaking. The blot was then washed 4 times for 15 mins each and imaged on Odyssey CLx system (Li-Cor).

Part of the aforementioned protocol was also followed for detecting HuB protein (recombinant Human ELAVL2, 1-346aa, from Biosource; Catalogue number MBS205995). The protein was loaded in two amounts (0.5 µg and 1.25 µg) in separate lanes of a 4-20% gradient denaturing gel (Biorad) along with protein marker Precision Plus Dual Color standards (Biorad). After fractionating the proteins, they were blotted onto ImmunBlot PVDF membrane (Biorad) overnight at 4°C at 17V for 12-16 hours. After blotting, membrane was blocked with Odyssey TBS blocking buffer (Li-Cor) for 1 hour at room temperature with shaking. Incubation with primary antibodies were carried out at

4°C with shaking for 24 hours. Following binding, the blot was washed 4 times with TBS-T (Tris Buffered Saline with 0.5% Tween) for 15 mins each with shaking at room temperature. Secondary antibody incubation was carried out in dark at room temperature for 1.5 hours with shaking. The blot was then washed 4 times for 15 mins each and imaged on Odyssey CLx system (Li-Cor).

Antibody used to detect RET was a well validated antibody used previously for detecting RET in ENS¹⁵⁴ and was further validated by us using a western blot on total proteins isolated from murine small intestinal LM-MP (Suppl Figure 8e). Antibodies used are detailed in the Supplementary Table 1.

RNA isolation and quantitative detection of specific transcripts

The isolated tissue was stored in RNALater Solution (Ambion). RNA was isolated using RNeasy Mini Kit (Qiagen) following manufacturer's protocol. RNA quantification was carried out using Epoch Microplate Spectrophotometer (BioTek). cDNA synthesis was carried by SuperScript IV VIL0 Master Mix (Invitrogen). Quantitative Real-time PCR was carried out using Taqman Gene Expression Master Mix (Applied Biosystems) and Roto-Gene Q (Qiagen). The probes used are listed in Supplementary Table 1.

Single Cell RNA sequencing and analyses of LM-MP tissues

Single cell preparation from murine ileal LM-MP tissues: Ileal LM-MP tissues from male littermate C57/BL6 wildtype mice were isolated by peeling as previously described¹⁹. Succinctly, mice were anesthetized with isoflurane and sacrificed by cervical dislocation. A laparotomy was performed and the small intestine was removed and lavaged with PBS containing penicillin-streptomycin (PS; Invitrogen). The ileum was then isolated which was then cut into 2-cm-long segments and placed over a sterile plastic rod. A superficial longitudinal incision was made along the serosal surface and the LM-MP was peeled off from the underlying tissue using a wet sterile cotton swab and placed in Opti-MEM medium (Invitrogen) containing PenStrep (Invitrogen). The tissues were then dissociated in Digestion Buffer containing 1 mg/ml Liberase (Sigma-Aldrich) in OptiMEM. Tissues from mouse 1 were dissociated in the Digestion buffer containing Liberase TH and tissues from mouse 2 were dissociated in the Digestion buffer containing Liberase TL. Dissociation was performed at 37°C for 30 minutes on a rotary shaker, after which the cells were centrifuged at 200g for 7 minutes, and the pellet was resuspended in ice cold sterile PBS. The cell suspension was passed through a 40 µm cell sieve and the resulting filtered cell suspension was again centrifuged at 200g for 7 minutes. This process of cell centrifugation and filtration was repeated two more times, after which the cells were resuspended in 1 ml ice cold sterile PBS. The repeated steps of serial cell washes and filtration removed clumps and debris and the viability of the resulting cell suspension was estimated to be >90% using Trypan Blue dye test. The cells were then processed through 10X Genomics Chromium V2.0 system (for the 6-month-old murine tissues) and 10X Genomics Chromium V3.1 system (for the P21 murine tissues) according to the manufacturer's suggested workflow. The processing was done at the GRCF Core Facility at the Johns Hopkins University. The pooled libraries were sequenced on an Illumina HiSeq 2500 (for 6-month-old tissues) and Illumina Novaseq (for P21 tissues) to an average depth of 3.125×10^8 reads per sample library. The sequencing was performed at the CIDR core facility at the Johns Hopkins University.

For processing data from scRNAseq on 6-month-old murine LM-MP tissues: Pre-processing of FASTQs to Expression Matrices: FASTQ sequence files were processed following a Kallisto Bustools workflow compatible with downstream RNA velocity¹⁵⁵. References required for pseudo-alignment of transcripts were obtained using the `get_velocity_files` (functionality of BUSpaRSE (<https://github.com/BUSTools/BUSpaRse>)), with "L=98" for 10X Genomics v2.0 sequencing chemistry. Reads were pseudo-aligned to an index built from Ensembl 97 transcriptome annotation (Gencode vM22; GRCm38). Across two samples processed, a total of 578,529,125 reads were successfully pseudo-aligned. Barcodes within a Hamming distance of one to known 10X Genomics v2.0 barcodes were corrected. Reads were classified as "spliced" or "unspliced" by their

complement to the target list of intronic sequences and exonic sequences, respectively, and subsequently quantified separately into expression count matrices. Spliced counts are used for all analyses.

Single cell gene expression analysis: scRNA-seq count matrices were analyzed using Monocle3. 11,123 high-quality cells were identified as meeting a 200 UMI minimum threshold with a mitochondrial read ratio of less than 20%; droplets that did not meet these criteria were excluded from the analysis. Mitochondrial counts were determined as the sum of reads mapping to 37 genes annotated to the mitochondrial genome. All genes with non-zero expression were included for downstream analysis. Raw counts were first scaled by a cell-size scaling factor and subsequently log10 transformed with a pseudo-count of 1. Normalized values are used in place of raw counts unless otherwise noted.

Prior to UMAP dimensionality reduction, batch effects between the two biological replicates were assessed and corrected via the mutual nearest neighbors (MNN) algorithm as implemented by Batchelor in Monocle3¹⁵⁶. 15 clusters of cells in the UMAP embedding were identified by Leiden community detection. 30 marker genes for each cluster were identified based on greatest pseudo R^2 values and used for supervised annotation of cell types by searching UniProt, Allen Cell Atlas and through literature search with Pubmed.

NC-derived cell clusters were identified by expression of NC markers Ret and Sox10. MEN cluster was identified by its expression of CGRP-coding Calcb, Met, and Cdh3. The pan-MENs protein marker MHCst was identified by labeling with an antibody S46 which labels all members of the MHCst family⁴¹. Since the antibody does not identify a single gene product, MHCst immunostaining could not be used to identify a specific gene marker for use in the annotation of the MEN cluster. For further analysis into the MENs population, the full LM-MP dataset was subset to include only the 2,223 cells annotated as MENs. These cells were re-processed as above, but with a reduced PCA dimensionality of $k=20$ as input for the UMAP embedding. 5 clusters of cells in the UMAP embedding were identified by Leiden community detection ($k=10$, resolution = $5e-4$).

P20 LM-MP scRNA processing and analysis: Pre-processing of FASTQs to Expression Matrices: FASTQ sequence files were processed following a Kallisto Bustools workflow compatible with downstream RNA velocity. References required for pseudo-alignment of transcripts were obtained using the `get_velocity_files` (functionality of BUSpaRSE (<https://github.com/BUSpaRSE>)), with “L=91” for 10X Genomics v3.1 sequencing chemistry. Reads were processed following the same steps as the six month LMMP dataset. Spliced counts are used for all analyses.

Single cell gene expression analysis: scRNA-seq count matrices were analyzed using Monocle3. 11,264 high-quality cells were identified as meeting a 600 UMI minimum threshold with a mitochondrial read ratio of less than 20%; droplets that did not meet these criteria were excluded from the analysis. Mitochondrial counts were determined as the sum of reads mapping to 37 genes annotated to the mitochondrial genome. All genes with non-zero expression were included for downstream analysis. Raw counts were first scaled by a cell-size scaling factor and subsequently log10 transformed with a pseudo-count of 1. Normalized values are used in place of raw counts unless otherwise noted.

NC-derived and MENs cell clusters were identified by expression of established marker genes. Further, the identity of MENs was confirmed by a projection analysis using MENs-specific patterns learned in the six month dataset. To investigate differences in division potential within the ENS, the data was subset to NENs, neuroglia, and MENs for computational cell cycle analysis (NENs: $n=526$; neuroglia: $n=844$; MENs: $n=510$). Continuous scores for cell cycle position ($0-2\pi$) were calculated via Tricycle by projecting each cell into the default reference space. The relevance of

these scores was confirmed by profiling the scores' correlation with expression of genes with known variation over the cell cycle. Lastly, each cell was unambiguously characterized as “non-cycling” ($0-0.5\pi$ or $1.5-2\pi$) or “cycling” ($0.5-1.5\pi$) by binarizing Tricycle scores.

External datasets: Spliced and unspliced count matrices for mesenchymal subset of the Gut Cell Atlas were obtained from <https://www.gutcellatlas.org/81>, and existing annotations were used when available. SnRNAseq counts matrices from May-Zhang et al.⁵⁰ were downloaded from GEO GSE153192. Bulk RNAseq dataset from the Human Obstructed defecation study by Kim et al.¹⁰³ was obtained from GEO GSE101968.

Pattern discovery and ProjectR analyses: Pattern discovery was utilized to identify sets of co-expressed genes that define cell-type specific transcriptional signatures. The normalized expression matrix was decomposed via non-negative matrix factorization (NMF) as implemented in the R package *NNLM*, with $k=50$ and default parameters. Cell weights for each pattern were grouped by assigned cell-type and represented by heatmap. Pattern vectors were hierarchically clustered by a Euclidian distance metric, implemented in *ComplexHeatmap*¹⁵⁷. These patterns were then tested on the bulk RNA-Seq expression matrix for the Human Obstructed Defecation study.¹⁰³ The log2 expression ($\log_2(\text{rpkm} + 1)$) from this study was projected onto the NMF patterns using *projectR*⁷². Students' t tests were performed on the projection weights from the Control and OD groups to test for differences between them. For the projection of datasets generated by May-Zhang et al.⁵⁰ and Elmentaite et al.⁸¹, log10-transformed normalized counts were projected.

Analysis of May-Zhang et al. dataset

Raw UMI count matrices for the single-nucleus RNA sequencing of enteric neurons performed by May-Zhang et al.⁵⁰ from the ileum of adult mice were obtained from GEO (GSE153192). The *DropletUtils* package (v1.10.3)^{158, 159} was used to compute barcode ranks, knee, and inflection point statistics for all the samples. All barcodes in the 10X genomics libraries with total UMI greater than the inflection point were considered to be cell-containing droplets and retained for further analysis. The *inDrop* libraries were filtered to retain all barcodes with UMI greater than 200. The filtered matrices were combined to form a *SingleCellExperiment* object.

Cell-specific scaling factors accounting for composition biases were computed using *computeSumFactors* (*scran* v1.18.7)¹⁶⁰. The raw UMI counts were then scaled using the scaling factors and log 2 transformed after addition of a pseudo count of 1 using *logNormCounts* (*scater* v1.18.6)¹⁶¹. Batch effects between the samples were corrected using *fastMNN* (*batchelor* v1.6.3)¹⁵⁶.

Unsupervised clustering was performed using a graph-based clustering technique. The graph was obtained using *buildSNNgraph* (*scran* v1.18.7)¹⁶⁰ and the *louvain* algorithm implemented in the *igraph* package (v1.2.7)¹⁶² was used to identify communities. Differential expression testing was performed between all pairs of clusters using *findMarkers* (*scran* v1.18.7)¹⁶⁰. One-sided Welch's t-test was used to identify genes that were upregulated in a cluster as compared to any others in the dataset. Benjamini-Hochberg method was applied (to combined p-values) to correct for multiple testing and the genes were ranked by significance. The top ranked genes were used as marker genes for the clusters. The log fold change from the pairwise comparison with the lowest p-value was used as the summary log fold change.

The log normalized counts from this dataset were then projected into the NMF patterns learned on our scRNAseq data. This was performed using transfer learning implemented in *projectR* (v1.6.0). The projection weights corresponding to the four MEN-specific patterns were then clustered using graph-based clustering and visualized by UMAP (*uwot* v0.1.10) (<https://github.com/jlmeville/uwot>).

Whole gut transit time analyses

Whole-gut transit time (WGTT) for every mouse was analyzed by the method using the carmine red protocol¹⁹. Mice were placed in individual cages and deprived of food for 1 hour before receiving 0.3 mL 6% (wt/vol) carmine solution in 0.5% methylcellulose by oral gavage into the animal's stomach. The time taken for each mouse to produce a red fecal pellet after the administration of carmine dye was recorded in minutes. The experiment was terminated at 210 minutes post-gavage and the WGTT of any mice that did not expel the red dye at the termination was marked at the value of 210 min. The mean difference in whole gut transit time (in minutes) between both the Ret^{+/+} and Ret^{+/-} mice cohorts, and the GDNF and Saline-treated Control cohorts were analyzed statistically.

In vivo Injections

GDNF injection: Similar to prior report that gave sub-cutaneous injections of GDNF to post-natal mice⁹⁶, we took 6 littermate 10 day old (P10) male Wnt1-cre:*Rosa26*-tdTomato mice and divided into two subgroups, GDNF and Control. Each mouse in the GDNF group was injected sub-cutaneously with 50 μ l of 2mg/ml of GDNF (Peprotech Catalogue #: 450-44) every other day, while the Control group was injected with 50 μ l of sterile saline. The mice were given 5 doses and then sacrificed on P20, after which their LM-MP tissues were isolated as detailed above. The tissues were then immunostained with antibodies against Hu and imaged. In a separate experiment, adult (P60) mice were also injected sub-cutaneously with GDNF (100 μ l of 100 μ g/ml of GDNF). The mice were given 5 doses over a course of 10 days and then sacrificed on P70, after which their LM-MP tissues were isolated as detailed above. For studying the effect of GDNF on aging mice, two cohorts of 17-month-old male C57BL/6 mice (n=5 mice/cohort) were obtained from the aging colony of the National Institute of Aging. Before the start of dosing, the whole gut transit time was assayed. The animals were then injected daily sub-cutaneously either with 100 μ l of saline (Control) or 100 μ l of GDNF (500 μ g/ml) for 10 consecutive days, after which the mice were sacrificed, and their LM-MP tissues were isolated as detailed above. The tissues were then immunostained with antibodies against Hu and imaged.

HGF injection: Similar to prior report that gave sub-cutaneous injections of HGF to post-natal mice, we took 6 littermate 10 day old (P10) male Wnt1-cre:*Rosa26*-tdTomato mice and divided into two subgroups, HGF and Control. Each mouse in the HGF group was injected sub-cutaneously with 100 μ l of 2mg/ml of HGF (Peprotech Catalogue #:315-23) every other day, while the saline group was injected with 100 μ l of sterile saline. The mice were given 5 doses and then sacrificed on P20, after which their LM-MP tissues were isolated as detailed above. The tissues were then immunostained with antibodies against Hu and imaged.

Statistics

Data was analyzed using Graphpad Prism 8.3.1 and R using Unpaired Students t-test, Simple Linear Regression, and Ordinary One-Way ANOVA.

Data

All raw data are provided in Supplementary Table 2 and 3. We imaged atleast 10 ganglia per tissue for our enumeration and each group studied had $n \geq 3$ mice. Raw single cell RNA sequencing data is archived on the NCBI GEO server and can be accessed under the accession numbers GSE156146 and GSE213604.

Code

Code generated for the scRNA/snRNA analyses is available at https://github.com/jaredslosberg/6month_ENS/.

We wish to thank Prof. Lior Pachter at Caltech for his help with responding to the reviewer's comments.

Suppl Figure 1: Presence and absence of tdTomato reporter expression in the myenteric ganglia of adult neural crest lineage-traced mice

(a) Hu-immunostained Wnt1-cre:*Rosa26*-tdTomato LM-MP tissue, where tdTomato-expression (red) marks neural crest-derived cells, and Hu (green) labels myenteric neurons, we observed the presence of higher intensity fluorescence (red arrows) and lower intensity fluorescence (orange arrows) in various tdTomato⁺ neurons. The myenteric ganglia also contained Hu⁺ neurons that did not express tdTomato (green arrow) and these neurons alone were deemed to be non-neural crest-derived neurons. Scale bar denotes 10 μ m.

(b) Reporter non-expressing Hu-immunolabeled neurons (green, green arrows) can be observed both through the expression of the tdTomato reporter (red), as well as on antibody labeling of the tdTomato reporter (gray).

(c) Freshly harvested small intestinal tissue from Wnt1-cre:*Rosa26*-tdTomato mice when imaged under 20X magnification of a live tissue fluorescence microscope shows the presence of cells that express tdTomato (red) and those that don't.

(d) Cells showing hyper-aggregation of the tdTomato reporter can be found in freshly harvested full-thickness small intestinal myenteric plexus in tissues imaged under 20X magnification of a live tissue fluorescence microscope.

(e) Myenteric ganglia from fixed LM-MP tissue of adult Wnt1-cre:*Rosa26*-tdTomato mice show show hyper-aggregation of tdTomato (white arrow) when imaged using a confocal microscope. Nuclei are stained with nuclear marker DAPI (blue). Scale bar denotes 10 μ m.

(f) Cells showing hyper-aggregation of tdTomato (red, red arrow) show no detectable nuclear labeling with DAPI (blue) or of pan-neuronal marker Hu (green, green arrow). Scale bar denotes 10 μ m.

In addition, interrogation of two other neural crest-specific lineage-traced mouse lines confirms our observations with Wnt1-cre:*Rosa26*-tdTomato mice. **(g)** In Wnt1-cre:*Hprt*-tdTomato adult male mice, tdTomato expression (red) is present only in a subset of Hu-immunolabeled (green) neurons (red arrows) while it is not detected in other Hu-expressing neurons (green). Similarly,

(h) only a subpopulation of Hu⁺ adult enteric neurons (green) is derived from the neural crest, as seen by its co-expression of tdTomato (red, red arrows) in the Pax3-cre:*Rosa26*-tdTomato mice while other neurons that do not express tdTomato (white arrows) are inferred to be not derived from the neural crest. Scale bar denotes 10 μ m.

(i) Orthogonal views of small intestinal villus from a Wnt1-cre:*Rosa26*-tdTomato mouse that shows presence of tdTomato-expressing (red) neural crest-derived fibers inside the villus but a complete lack of tdTomato-expression by intestinal epithelial cells. Nuclei are labeled with DAPI (blue). Scale bar denotes 10 μ m.

(j) Representational image of the myenteric plexus from a *Rosa26*-tdTomato mouse without any cre driver showing that in absence of cre recombinase to drive tdTomato expression, there is a lack of tdTomato expression in this layer. Nuclei are labeled with DAPI (blue). Scale bar denotes 10 μ m.

(k) Representational image of the myenteric plexus from a *Rosa26*-EGFP mouse without any cre driver showing that in absence of cre recombinase to drive EGFP expression, there is a lack of EGFP expression in this layer. Nuclei are labeled with DAPI (blue). Scale bar denotes 10 μ m.

(l) Western blot image showing detection of two different loading amounts of the recombinant HuB protein by the rabbit polyclonal antibody against HuC/D (abcam). Molecular Weight marker with defined band sizes indicated on the right.

Suppl Figure 2: Tek-expressing and Mesp1-expressing mesodermal lineage contributes to adult enteric neurons.

(a) Adult small intestinal LM-MP layer from a male Tek-EGFP mouse, where EGFP expression (green) labels Tek-expressing cells was immunostained with specific antibodies against the pan-neuronal marker PGP9.5 (red) and imaged under a confocal microscope. The Tek-EGFP expression did not label any cells within the myenteric plexus marked by PGP9.5. Nuclei are labeled with DAPI (blue). Scale bar denotes 10 μ m.

(b) We observed the presence of higher intensity fluorescence (red arrows) and lower intensity fluorescence (green arrow) in various tdTomato⁺ cells in a section of heart tissue from a 6-month-old *Mesp1*-cre:*Rosa26*-tdTomato adult male mouse, where tdTomato (red) labels *Mesp1*-derived cells of the mesodermal lineage that includes cardiomyocytes and vasculature. Nuclei are stained by DAPI (blue). Scale bar denotes 10 μ m.

(c) Adult small intestinal LM-MP layer from a 6-month-old male *Mesp1*-cre:*Rosa26*-tdTomato mouse, where tdTomato (red) cells are mesoderm-derived cells, was immunostained with specific antibodies against the pan-neuronal marker Hu (green) and imaged under a confocal microscope. tdTomato-expressing cells outside the ganglia (red arrows) were brighter than tdTomato⁺ neurons inside the ganglia (green arrows). Nuclei are labeled with DAPI (blue). Scale bar denotes 10 μ m.

(d) Adult small intestinal LM-MP layer from a 2-month old male *Mesp1*-cre:*Rosa26*-EGFP mouse, where EGFP cells are mesoderm-derived, was immunostained with specific antibodies against the reporter EGFP (green) and the pan-neuronal marker Hu (red) and imaged under a confocal microscope. A population of Hu-immunolabeled neurons were also labeled with antibodies against EGFP (green arrows) while other neurons were not (red arrows), suggesting the derivation of EGFP⁺ neurons from the *Mesp1*-expressing mesoderm. **(e)** No primary control for the Anti-GFP immunostaining showed very weak EGFP expression in the fixed LM-MP tissue from these animals. Nuclei were labeled with DAPI (blue). Scale bar = 10 μ m. Consistent with the observations made with the tdTomato reporter in this transgenic line, the *Mesp1*-cre:*Rosa26*-EGFP animals also show a brighter reporter signal in the vasculature and a dimmer reporter signal in the myenteric neurons.

(f) MHCst⁺ (cyan) labels all tdTomato⁺ (red) Hu⁺ (green) adult neurons (white arrows) but not the tdTomato⁻ neurons (yellow arrow) in the mesoderm-specific Tek-cre:*Hprt*-tdTomato lineage-traced lines. Nuclei are stained with DAPI (blue). Scale bar = 10 μ m.

Suppl Figure 3: Validation of MENs-specific markers by immunohistochemistry and confocal microscopy

Small intestinal LM-MP from several adult 5-month-old male *Wnt1-cre:Rosa26-tdTomato* mice, where tdTomato-expression (red) labels neural crest-derived cells, was used to ascertain and validate the expression of the novel MENs marker and of known neuronal marker genes.

(a) The myenteric ganglion shown in [Fig 2h](#) is presented here as a merged image (where MHCst immunostaining and tdTomato-expression are shown together) and with MHCst immunostaining without tdTomato signal to better appreciate the presence of MHCst-expressing neurons in the ganglia. MHCst-specific antibody S46 (green) was found to label tdTomato non-expressing cells within the myenteric ganglia as well as cells outside of the myenteric ganglia. Neural crest-derived cells within the myenteric ganglia expressed tdTomato. Nuclei are labeled with DAPI (blue). Scale bar denotes 10 μ m.

The immunostaining of the MENs-markers elucidated through scRNAseq analyses: **(b)** CDH3, **(c)** AEBP1, **(d)** SLPI, **(e)** CFTR, **(f)** NT-3, **(g)** CLIC3, **(h)** FMO2, **(i)** IL-18, **(j)** MYL-7, and **(k)** SLC17A9 (all immunolabeled cells in these panels are green; green arrows) was found to be localized to or enriched in cells without tdTomato expression, while the tdTomato⁺ cells were found to show not to express these MENs-specific markers (red arrow). In addition, we also immunolabeled the myenteric ganglia from this transgenic mouse line with known neuronal markers (gray) and with antibodies against the pan-neuronal marker Hu (green) to show that the neuronal markers Elavl2 or HuB **(l)**, GPR88 **(m)**, HAND2 **(n)**, PDE10A **(o)**, TUBB2B **(p)** and VSNL1 **(q)** were expressed by both tdTomato⁺ (red arrow) and tdTomato⁻ Hu⁺ neurons. Orthogonal views of images where 3D image z-stacks were obtained are provided for better visualization of marker expression in the 3D space. Nuclei in these tissues were labeled with DAPI (blue). Scale bar denotes 10 μ m.

Suppl Figure 4: scRNAseq metrics

(a) MENs are significantly larger in size as compared to the NENs, as the mean Feret's Diameter of the *Wnt1-cre:Rosa26-tdTomato*- MENs is significantly more than that of the *Wnt1-cre:tdTomato*⁺ NENs. Student's t-test; ** p value < 0.01.

(b, c) UMAP representation of sequenced cells generated from 10X Genomics Chromium-derived libraries from the two mouse samples, taken from separate littermate adult male mice, shows similar representation of their cells across the various clusters.

(d) Quasiviolin plot of expressed genes provide a comparison of important MEN-specific and neuronal genes between the NENs and MENs clusters in the adult ileal May-Zhang et al. dataset and the NENs, Neuroglial, and MENs clusters in our scRNAseq dataset.

(e) Top genes that are pattern drivers for the four MEN-specific NMF patterns along with their gene weights.

(f) The four MEN-specific patterns were together used to label a group of cells (True) that together showed increased usage of the MEN-specific patterns, when compared to other cell types (False) in the adult ileal scRNAseq dataset from May-Zhang et al.⁵⁰

(g) Top-expressed genes in the group of cells **(f)** that showed higher utilization of the MEN-specific patterns, compared to other cells in the dataset.

(h) Representation of the four MEN-specific NMF patterns in the UMAP of our data and the projection of the May-Zhang et al.⁵⁰ murine ileal dataset and the entire gut mesenchymal single cell RNA seq dataset by Elmentaite et al.⁸¹ into the MEN-specific patterns highlights cell clusters that show higher usage of the four NMF patterns. Bottom row shows increasing usage of these patterns with age in the entire mesenchymal gut cell atlas data.

Suppl Figure 5: Expression of neuronally significant genes by MENs

Sparklines plot of gene expression in various cell clusters, as revealed by single cell transcriptomics of diverse LM-MP cells from small intestinal tissue of two 6-month-old mice, show that expression of some genes is enriched in the MENs cluster, when compared to the other major clusters of cells in the dataset. Lower to higher expression in the plot is represented by yellow to blue gradient. Subsequent analyses shows that proteins coded by these genes are neuronally significant.

Suppl Figure 6: Expression of mesodermal marker MYH11, and reporter expression under the Baf53b-cre transgenic line in post-natal myenteric neurons

(a) Orthogonal views of a myenteric ganglion of an adult *Wnt1-cre:Rosa26-tdTomato* mouse, where tdTomato (red) is expressed by neural crest-derived cells, shows that immunolabeling with a MYH11-specific antibody (green) labels tdTomato non-expressing cells. Nuclei are labeled with DAPI (blue). Scale bar denotes 10 μ m.

(b) Representative image of the myenteric plexus layer from a *Myh11-cre^{ERT2}:Rosa26-YFP* adult male mouse, which was dosed with tamoxifen for two consecutive weeks, and which when immunostained with antibodies against YFP/GFP (green) and the pan-neuronal marker Hu (red), shows the presence of YFP-expressing neurons (green arrows) in the myenteric ganglia that also contains YFP negative neurons (red arrow). Nuclei are labeled with DAPI (blue). Scale bar denotes 10 μ m.

(c) Immunostaining of healthy human adult duodenal tissue with antibodies against the pan-neuronal marker PGP9.5 (green) and MYH11 (red), shows that in the circular muscularis layer of the tissue, MYH11 labels the smooth muscle and not the PGP9.5 immunostained nerve fibers and cell bodies. Scale bar denotes 10 μ m.

(d) Immunostaining of healthy human adult duodenal tissue with antibodies against the pan-neuronal marker PGP9.5 (green) and MYH11 (red), shows that in the myenteric ganglia, MYH11 immunolabels a subset of PGP9.5-expressing myenteric neurons (white arrows). Scale bar denotes 10 μ m.

(e) Representative image of a myenteric ganglion from a *Baf53b-cre:Rosa26-tdTomato* adult male mouse, where tdTomato (red) is expressed by Baf53b expressing cells or those derived from Baf53b-expressing cells, when immunostained with antibodies against the pan-neuronal marker Hu (green), shows the presence of neurons that express tdTomato (white arrows) along with neurons that do not express tdTomato (green arrows). Nuclei are labeled with DAPI (blue). Scale bar denotes 10 μ m.

(f) Graphical representation of the proportions of tdTomato-expressing Hu⁺ myenteric neurons in the LM-MP tissue of two male P21 Baf53b-cre:*Rosa26*-tdTomato mice shows that myenteric ganglia from ileum, proximal colon, and distal colon contain significant proportions of neurons that do not express tdTomato under the Baf53b-cre transgenic line.

Suppl Figure 7: Putative mesoderm-derived enteric neurons (MENs) are present in adult human myenteric ganglia.

(a, b) The mesodermal specific markers, MET and MHCst are expressed by a subpopulation of human enteric neurons. Myenteric ganglia from normal duodenal tissue of 2 different adult human subjects express the pan-neuronal marker Hu (green), with distinct subpopulations positive and negative for MET (blue) and MHCst (red). MET and MHCst-expressing neurons (red arrows) in the human ENS are assumed to be mesoderm-derived enteric neurons (MENs) and those not expressing these MENs markers (green arrows) are presumably NENs. Nuclei are labeled with DAPI (gray). Scale bar = 10 μ m.

(c) The mesodermal specific marker DECORIN is expressed by a subpopulation of human enteric neurons. Myenteric ganglia from normal human duodenal tissue when immunostained with antibodies against the pan-neuronal marker Hu (green) and DECORIN (red) shows the presence of DECORIN-expressing myenteric neurons, which are assumed to be MENs. Nuclei are labeled with DAPI (blue). Scale bar = 10 μ m.

(d) Distribution of the various mesenchymal clusters of the post-natal healthy gut cell atlas data (generated by Elmentaite et al.⁸¹) by donor age. For the age of each donor, the proportion of cells annotated as belonging to each cluster is shown. Proportions of Cluster 1 are observed to expand with age.

Suppl Fig 8: Sub-clustering MENs in the scRNAseq dataset generated from 6-month-old mice.

(a) Sub-clustering the MENs cluster from our scRNAseq dataset into 5 distinct clusters **(b)** was found to be not based on the expression of cluster-specific marker genes.

Suppl Fig 9: HGF and GDNF ratios in maturing and adult murine gut.

(a) Ratio of HGF:GDNF values in fluorescence intensities of their western blots (normalized to their own house-keeping protein Beta Actin) from the small intestinal LM-MP of post-natal mice at different ages during their maturation.

(b) Ratio of HGF:GDNF expression values from the full thickness small intestinal tissue of adult mice taken from the Tabula muris database⁹⁴.

(c) Analyses of human plasma proteome from 1,025 individuals was performed by Sathyan et al.⁹⁵ and the correlation of various plasma proteomes with aging was calculated. Of the genes that showed significant correlation with age ($p < 0.05$), we found that the HGF levels in human

plasma correlated positively with age (above 0), while the levels of GDNF and RET correlated negatively with age (below 0).

Suppl Fig 10: Identification of the MENs cluster in the P21 scRNAseq data and expression of Ret in NENs and in the Ret heterozygous mice.

(a) UMAP representation of all the cell clusters generated from scRNAseq performed on all cells from the P21 murine LM-MP.

(b) Projection of scRNAseq from the P21 murine LM-MP cells into the four MEN-specific NMF patterns generated using scRNAseq on 6-month-old murine LM-MP cells shows higher usage of the four NMF patterns in the putative MENs cluster in the P21 dataset (black arrow).

(c) Expression of the genes *Ret* and *Met* in the MENs and NENs clusters in the scRNAseq of P21 LM-MP cells shows that while *Met*-expression is detected in both MENs and a subset of NENs, the *Met*-expressing NENs subset shows significant expression of *Ret*, while the MENs do not express *Ret*.

(d) Sparklines plot of gene expression of various neuronally significant genes in the scRNAseq data from Zeisel et al., which was generated using flow sorted *Wnt1-cre:Rosa26-tdTomato*⁺ cells from a P21 mouse, shows that *Met* expression is observed in the clusters ENT6, 8, and 9 which also show expression of CGRP-expressing gene *Calcb*, the neural crest-specific gene *Ret*, Choline acetyltransferase-expressing gene *Chat* and the pan-neuronal marker PGP9.5- expressing gene *Uchl1*.

(e) Fold change in transcript abundance of the gene *Ret* in the small intestinal LM-MP tissue from *Ret* wildtype (*Ret* WT) and *Ret* heterozygous (*Ret* Het) littermate adult mice shows that *Ret* transcripts are significantly less abundant in the tissue of *Ret* Het mice, when compared to those from *Ret* WT mice. *Ret* transcript expression in every sample was normalized to the expression of the house keeping gene *Hprt*. Student's t-test; * p value < 0.05.

Suppl Fig 11: Effect of GDNF treatment on MHCst-expressing MENs and RET-expressing NENs in aging mice

Representative image of MHCst (red) and Hu (green)-immunostained myenteric ganglia of **(a)** saline-treated control and **(b)**. GDNF-treated 17-month-old mice show MHCst⁺ (yellow arrows) and MHCst- neurons. Note the reduction in MHCst-immunostaining on inter-ganglionic fibers in the 17-month-old mice when treated with GDNF, compared to control mice. Nuclei are stained with DAPI (blue). Scale bar indicates 10 μ m.

Representative image of RET-immunostained (green) myenteric ganglia of **(c)**. saline-treated control and **(d)**. GDNF-treated 17-month-old mice. Nuclei are stained with DAPI (blue). Scale bar indicates 10 μ m.

(e) Western blot image of a 4-20% gel performed using the RET antibody on total protein lysate from small intestinal LM-MP layer from post-natal mice shows the expected band between 150 and 250 kDa of RET protein.

Suppl Fig 12: Projection of the bulk RNA sequencing data of intestinal tissue from Control and Patients with Obstructed Defecation into our murine scRNAseq-derived NMF patterns using *projectR*.

Transcriptomic data from intestinal tissues of patients with normal intestinal motility and those with obstructed defecation (OD) was procured from GEO (GSE101968). Non-negative matrix factorization (NMF), as implemented in the R package *NNLM* (<https://github.com/linxihui/NNLM>), was performed on the murine scRNAseq data using $k=50$ and default parameters; and cell weights for each pattern were grouped. Using *projectR*, the $\log_2$ expression ($\log_2(\text{rpkm} + 1)$) from the human bulk RNA sequencing data from control and OD patients were projected into the murine scRNAseq-derived NMF patterns. The mean projection weights from Control and OD groups were tested for statistically significant differences using Students' t tests.

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Joint Public Review:

In this manuscript, the authors challenge the fundamental concept that all neurons are derived from ectoderm. The key points of the authors argument are as follows:

1. Roughly half of the cells in the small intestinal longitudinal muscle-myenteric plexus (LM-MP) that express a pan-neuronal marker do not, by lineage tracing, appear to be derived from the neural crest.
2. Lineage tracing and marker gene imaging suggest that these non-neural crest derived neurons originate in the mesoderm, leading to their designation as mesodermal-derived enteric neurons (MENS).
3. Single-cell sequencing of LM-MP tissues confirms the mesodermal origin of MENS.
4. MENS progressively replace neural crest derived enteric neurons as mice age, eventually representing the bulk of the EN population.

There is broad agreement among the reviewers that the identification and description of this cell population is important, and that the failure of these cells to be labeled by neural crest lineage tracers is not artifactual. The work with transgenic lines is convincing that some presumptive neurons in the enteric nervous system (ENS) likely originate from an alternative source in the postnatal intestine and that this population increases in aging mice.

There is, however, ongoing disagreement between the authors and reviewers about whether the authors' provocative and potentially paradigm-changing proposal that these are neurons of mesodermal origin has been established. While the authors believe they have addressed the reviewers' concerns in multiple rounds of review (much of this prior to submission), the reviewers remain unconvinced and continue to request additional data and analyses.

A key premise of the preprint review system is that the best interests of science are not served by endlessly litigating disagreements around papers by either compelling the authors to do extensive and expensive additional experiments that they do not believe to be necessary or by treating the authors' claim as established in the face of continued skepticism. Accordingly the editor believes it is time to present this work, which everyone agrees contains important observations and valuable data, along with the following editor's synthesis of the reviewers' concerns and author responses about the question of these cells' origins. We encourage anyone interested in the details to review the already posted reviews and authors' response.

The following key issues have been raised during review:

- * Is the lineage tracing and marker gene expression data definitive as to mesodermal origin?
- * Are the cells analyzed in the genomic experiments the same as those identified in the lineage tracing experiments?
- * Does the genomic data establish that the sub-population of cells the authors focus on are of mesodermal origin?
- * Are there alternative explanations for the lineage tracing and genomic observations than a mesodermal origin?
- * Is the lineage tracing and marker gene expression data definitive as to mesodermal origin?
- *

The proximal evidence that the authors present for a mesodermal origin of the non-NC derived cells is presented in Figure 2, which establishes the presence, via lineage tracing of Tek⁺ and Mesp1⁺ (and therefore mesoderm derived) and Hu⁺ (and therefore neuronal) cells. The fraction of lineage labeled cells in each case (~50%) corresponds roughly to the fraction of cells that do not appear to be NC derived.

The reviewers raise several technical questions about the lineage tracing experiments, including issues of incomplete labeling, ectopic labeling and toxicity. The authors have addressed each of these with data and/or citations, and the editor believes they have demonstrated, subject to the broader limits of lineage tracing experiments, that there are Hu⁺ cells in the tissue that are derived from cells that do not express NC markers and that do express mesodermal markers.

One reviewer raised the question of whether these cells are neurons. This appears to the editor to be a valid question, in that specific neuronal activity of these cells has not been established. But the authors' argument is persuasive that their Hu⁺ state would have led them to be designated neurons and that changing that designation based on not being derived from NC is circular. However the possibility that, despite this accepted designation, these cells are not functionally neurons should be noted by readers.

* Are the cells analyzed in the genomic experiments the same as those identified in the lineage tracing experiments, and does this data establish mesodermal origin? *

To provide orthogonal evidence for the presence of mesodermally derived enteric neurons, the authors carried out single-cell sequencing of dissociated cells from hand-dissected longitudinal muscle - myenteric plexus (LM-MP) tissue. They use standard methods to identify clusters of cells with similar transcriptomes, and designate, based on marker gene expression, two clusters to be neural crest derived enteric neurons (NENs) and mesoderm derived enteric neurons (MENs). However the reviewers raised several issues about the designation of the cells MENs, and therefore their equation with the cells identified in lineage tracing.

While the logic behind specific choices made in the single-cell analysis is not always clear in the manuscript, such as why genes not-specific to MENs were used to identify the MEN cluster and how genes were selected for subsequent analysis (although both issues are explained better in the authors' response to reviewers), they in the end identify a single large cluster that has the characteristics of MENs (it expresses both neuronal and mesodermal markers) that is (by immunohistochemistry) broadly associated with the previously described tissue MENs.

The standard methods for the delineation of clusters in single-cell sequencing data (which the authors use) are stochastic and defy statistical interpretation, and the way these data and analyses are used is often subjective. The editor shares the reviewers' confusion about aspects of the analysis, but also finds the authors' assertions that they have described a cluster of cells that express both neuronal and mesodermal genes, and that this cluster corresponds to the tissue MENs described in lineage tracing, to be broadly sound.

The biggest weakness in the single-cell data and analysis - identified by all reviewers - is the massive overrepresentation of MENs relative to NENs. The authors' explanation - that some cells are more sensitive to manipulations required to prepare cells for sequencing - is certainly well-represented in the literature and is therefore plausible. But it isn't fully satisfactory, especially because it undermines the notion that the MENs and NENs are functionally equivalent (though one could argue in response that increased fragility of NENs is why they are progressively replaced by MENs).

There are many additional questions about the single cell analysis that are difficult to resolve with the data in hand. I think everyone would agree that an ideal analysis would have more cells, deeper sequencing, and comprehensive validation of the identity of each cluster of cells. But given the time and expense required to carry out such experiments, we cannot demand them, and must take the data for what they are rather than what they could be. And in the end, it is the editors' view that these data and analyses bolster the authors' claims, without conclusively establishing them. That is, these data should neither be dismissed nor, on their own, considered definitive.

* Are there alternative explanations for the data than that they are mesodermally derived neurons? *

As discussed above, the reviewers generally agree that the lineage tracing experiments are careful and well-executed, and the authors have provided data that demonstrates that the data are highly unlikely to be due to either incomplete or ectopic lineage marking. The reviewers raise several possible alternative hypotheses, some based on the literature and some based on the genomic data. The authors discuss each in detail in their response. The editor would note that, at this stage in the history of single-cell analysis, the criteria for using single cell sequencing data to establish cell type and cell origin are not well established, and that neither the presence nor absence of specific sets of genes in single cells should not, for both technical and biological reasons, be considered dispositive as to identity.

* Additional aspects of paper: *

There are additional intriguing aspects of the paper, especially the increase in the number of MENs relative to NENs over time, suggesting functional replacement of one population with the other, and some evidence for and speculation about what might be regulating this evolution. However these are somewhat secondary points relative to the central question at hand of whether the authors have discovered a population of mesodermally derived neurons.

* Editor's summary and comment: *

The editor believes it is a fair summary to say that the authors believe they have gone to great lengths to provide multiple lines of evidence that support their hypothesis, but that these reviewers, while appreciating the potential importance of the authors' discovery of an unusual cell type, are not yet convinced of its origin.

In an ideal world, the authors, reviewers and editor would all ultimately agree on what claims the data presented in a paper supports, and indeed this is what the traditional journal publishing system tries to achieve. But the system fails in cases like this where no consensus between authors and reviewers can be reached, as it neither makes sense to "accept" the paper and imply that it has been endorsed by the reviewers, nor to "reject" it and keep the work in peer review limbo.

There is certainly enough here to warrant the idea and the data and arguments behind it being digested and considered by people in the field. It may very well be that the authors - who have spent years working on this problem and likely know more about this population of cells than anyone on Earth - are right that they have discovered something that changes how we think about the development of the nervous system. To the extent the reviewers are representative, people are likely to need additional data to be convinced. But it is time to put that to the test.

Reviewer #1 (Public Review):

The manuscript by Kulkarni et al proposes a new cellular origin of ENS, which is increased with age and therefore may be associated with the gradual decline of gut function. The study

is based on an initial observation that many enteric neurons do not seem to retain tdTomato expression in Wnt1Cre-R26-Tom mice, suggesting a loss of neurons that are replaced by a non-neural crest source. Further detection of reporter expression within the ENS of Tek and Mesp Cre-lines indicated a mesodermal origin of the new enteric neurons. Mesodermally derived neurons (MENS) were associated with Met, while neural crest derived neurons (NENS) expressed Ret. GDNF could decrease occurrence of MENS (defined as tdTomato-negative cells), while HGF had the opposite effect. Age-associated decline in gut transit was alleviated with GDNF treatment, while Ret heterozygote mutants had an increase of MENS. Overall, the study suggests that neural crest derived neurons are replaced by mesodermal-derived neurons that lead to an overall reduction in GI-physiology and that manipulation of the balance between the two types of neurons could have beneficial effects of age-associated gut malfunction. Generation of neurons from non-ectodermal sources would be a paradigm shift not only in the ENS, but in the Neuroscience field as a whole. The presence of mesenchymal marker genes in subsets of cells of the ENS in native gut tissue is convincing and the lack of retained fluorescent reporter expression in ENS from the many neural and Cre drivers used is indeed clear.

The current state of the manuscript is though not conceivable as it has unsound interpretation of data at many places, most importantly there is no firm connection between the MENs identified in tissue and the scRNA cluster annotated as MENs. "scRNA-seq-MENs" show very little expression of the bona fide neuron markers used to detect "tissue-MENs" including *Elavl4* and the overall proportions of "scRNA-seq-MENs" in the tissue is very far from that of "tissue-MENs". Hence, the claims that "tissue-MENs" equals "scRNA-seq MENs" could be excluded or their interpretation discussed in an unbiased manner. Marker expression of "scRNA-seq MENs" are suggestive of mesothelial cell identities, not ENS cells. Even the annotation of scRNA-seq profiles denoted as neural-crest derived enteric neurons (NENS) is highly questionable as 25% of the cells display bona fide lymphatic epithelial cell markers and no neuronal markers.

Reviewer #2 (Public Review):

In this study, the authors propose the possibility that some neurons in the enteric nervous system (ENS) originate postnatally from a non-ectodermal source. This possibility is investigated using a combination of transgenic lines, single cell RNA-sequencing (scRNA-seq), and immunofluorescence. Initially the authors identify a subset of neurons within myenteric enteric ganglia that are not lineage-labeled by canonical neural-crest derived cre-LoxP strategies. In their analysis, the group seeks to show that these neurons have an origin distinct from neural crest-derived progenitors that are known to initially colonize the developing gut. The team uses multiple cre lines (both Wnt1-cre and Pax3-cre) as well as several distinct reporter lines (Rosa-tdTomato, Rosa-EGFP, Hprt-tdTomato) to demonstrate that the lack of labeling by neural crest cre transgenes is consistent across several tools and not due to any transgene or reporter line artifact. Based on prior analysis that suggests some neurons in the ENS might be arising from a mesodermal lineage, the authors evaluate the possibility that mesoderm could contribute neurons to the ENS by evaluating expression of Tek-cre and Mesp1-cre tagged cell types in myenteric ganglia. The work with transgenic lines is convincing that some ENS neurons originate from an alternative source in the postnatal intestine and that this population increases in aging mice.

The authors apply single cell RNA-sequencing to identify additional markers of these non-neural crest enteric neurons. They rely on dissociation of laminar gut muscle preparations, stripped from the outside of the adult intestine, that contain many cell types including smooth muscle, vasculature, and enteric ganglia. In the analysis of this scRNA-seq data, the authors focus on a cluster of cells in the resulting UMAP plots as being the MENs cluster based on labeling of this cluster with three genes (*Calcb* (CGRP), *Met*, and *Cdh3*). Based on expression of these marker genes there are a very large number of MENs and very few

neural crest-derived enteric neurons (NENs) seen in the UMAPs. It is not clear why this difference in cell numbers has occurred. The early lineage tracing data shown with cre transgenes (Figures 1 and 2) shows relatively equal numbers of NENs and MENs in confocal imaging studies, yet in the RNA-seq UMAPs thousands of MENs are displayed while very few NENs are present. There is the possibility that the authors have identified a cell cluster as MENs that does not coincide with the *Mesp1-cre* or *Tek-cre* lineage labeled neurons observed within enteric ganglia of the laminar gut muscle preparations. The authors state that they have "used the single cell transcriptomics to both confirm the presence of MENs and identify more MEN-specific markers", however there is not a direct relationship made in this study between the MENs imaged and the cells profiled by single cell RNA-sequencing.

In their analysis the authors note a difference in the percentage of enteric neurons labeled by the neural crest lineage tracer line, *Wnt1-cre*, relative to the total neurons labeled by the pan-neuronal marker *HuC/D* with age of the mice studied. They undertake a temporal analysis of the percentage of *Wnt1-cre* labeled neurons over total *HuC/D* neurons over the lifespan and note a decrease of *Wnt1-cre* labeled neurons with age. Further, the team assessed levels of growth factors that are known to promote proliferation and survival of NENs (GDNF-Ret signaling) versus factors known to promote growth of mesoderm (HGF) with age and document a decrease in GDNF-Ret signaling while HGF levels increase with age. The authors propose that the balance between these two signaling pathways is responsible for the shift in proportions of NENs versus MENs in aging animals.

Some of the conclusions of this paper are supported, but several additional analyses are needed to reach the outcomes that the authors infer:

1. Because the scRNA-seq data generated in this study derives from mixed cell populations present in laminar gut muscle preparations, there is a gap between the image data shown for the mesodermal cre lineage tracing and the MENs clusters the authors have selected in their single cell RNA-seq analysis. The absence of direct transcriptional profiling of cells labeled by *Mesp1-cre* or *Tek1-cre* expression prevents the authors from definitively connecting their in situ lineage labeling with specific clusters in the single cell RNA-seq analysis.
2. Differential gene expression is the standard approach for identifying markers of a particular cluster and yet this is lacking in this study, and the rationale for why some genes were prioritized as markers of MENs is missing from the manuscript. Reanalysis of the authors posted single cell RNA-seq data found that genes integral to calling MENs (marker genes) were detectable in the data. *Met*, *Cdh3*, *Calcb*, *Elavl2*, *Hand2*, *Pde10a*, *Vsnl1*, *Tubb2b*, *Stmn2*, *Stx3*, and *Gpr88* were all expressed in very few cells and at low levels. Given this, how were these genes chosen to be marker genes for MENs, especially given the low sequencing depth utilized?
3. The authors rely on *Phox2b* as a marker for all ENS cells, including MENs. However, reprocessing of the authors posted single cell RNA-seq data finds that *Phox2b* is not detected in any of the cells in the MENs cluster and it's only expressed in very few cells of the neuroglia cluster. This discrepancy between the data the authors have generated and what is widely known about *Phox2b* expression in the ENS field must be explained as the absence of *Phox2b* message suggests there is an issue with reliance on low-depth scRNA-seq data for reaching the stated conclusions.
4. The authors have not considered potential similarities between their MENs and other developing ENS lineages, like enteric mesothelial fibroblasts reported by Zeisel et al. 2018, and further analysis is needed to show that MENs are indeed a distinct cell type. Top marker genes of the author's MENs clusters were expressed more often in the clusters that were left out of Morarach et al 2021's E15.5 and E18.5 datasets because those clusters were mostly *Phox2b*-negative on UMAPs. This lack of *Phox2b* expression

matches the characteristic of the MENs clusters' Phox2b-negative status in the authors single cell dataset. It is important to note that the Morarach dataset consists of Wnt1-cre lineage labeled (originating from neural crest) flow sorted cells. This is of import as it implies that Phox2b-negative cells ARE present within the Wnt1-cre lineage labeled population, an aspect that is relevant to this study's data analysis.

5. Upon reprocessing of the authors MENs-genesis dataset with integration by sample as the authors describe, Met expression is evident within the cluster of NENs on the resulting UMAP plot and yet the authors rely on this gene as a marker of MENs. Whether Met expression is restricted to MENs should be resolved because the authors state it is exclusive to MENs and they subsequently investigate this gene across lifespan. Because it is not clear that Met is absent from neural crest derived enteric neurons this caveat complicates the interpretations of the present study.
6. The authors apply MHCst immunofluorescence to mark MENs, but do not show any RNA expression for the MHCst transcripts in their single cell data. How did the authors come to the conclusion that MHCst IHC would be an appropriate marker for MENs? This rationale is missing from the text.

Reviewer #3 (Public Review):

In this manuscript, the authors challenge the fundamental concept that all neurons are derived from ectoderm. Specifically, they aim to show that while the early ENS arises embryologically from neural crest (NENs), with age it is slowly replaced by mesoderm-derived neurons (MENs). This claim is based on an array of transgenic reporter mice, immunofluorescence, and transcriptomics. They further propose that the transition from NENs to MENs is regulated by a changing balance in GDNF-RET versus HGF-MET signaling, respectively.

This is a provocative and potentially paradigm-changing proposal, but the data presented and the interpretation of that data fall short of establishing it.

1. MENs share more common characteristics with fibroblasts. The authors interpret this as representing neurons with fibroblast characteristics. Why not fibroblasts with neuronal characteristics? The ability to express neurotransmitter receptors and calcium channels is common in fibroblasts, but that isn't sufficient to characterize a neuron. For example, many cell types express neurotransmitters (CGRP in ILCs, Penk in fibroblasts). Expressing one of the Hu proteins (Elavl2) probably isn't enough to call these "neurons," especially when neurons usually express Elavl3-4 (HuC/D). Including calcium imaging and showing presence of action potentials would strengthen the argument that these are in fact neurons.
2. The scRNA-seq is unconvincing. There are several technical issues and the analysis omits important information required to make an unbiased assessment.
 - a. One issue in the interpretation is that MENs are shown by IHC to constitute half the neuronal population, with NENs making up the other half. The authors state that they performed an unbiased approach, sequencing all cells in the muscularis. If it were truly unbiased, then why do they detect a 28-fold increase in MENs in the single cell data? This does not reflect the IHC findings and points to an issue in technique that needs to be addressed.
 - b. Cell populations annotated by the author are confusing. The "unknown" population expresses many genes that are epithelial markers. This is puzzling because the authors state that they only sequenced the muscularis. This leads to questions regarding the initial samples and whether they were dissected appropriately or contaminated by another population.

c. The authors report a population of ICCs at P21 which is not identified at 6-months. Closer inspection of their data shows bona fide ICC markers, *Ano1* and *Kit*, in their SMC cluster at 6-months, with failure to identify ICC clusters, raising questions about whether they have identified a new cell type.

d. While the authors critically examine other scRNA-seq datasets and claim that those groups mislabeled their populations, the above does not instill confidence in their ability to counter the unified literature.

1. MENs are identified based on genes that could be related to neurons, including calcium channels, neurotransmitter receptors, etc. It is worth noting that mesenchymal cells, ICCs, and smooth muscle also possess these characteristics. Therefore, it hard to justify why these MENs are considered "neurons." The authors should perform an analysis to examine homology between clusters in order to show which clusters the MENs are more similar to, neurons or otherwise.

2. Several issues raise questions about the quality of the scRNA-seq data, making interpretations very difficult:

- a. MENs are identified to have higher UMI counts than other cells, which the authors interpret as the cells being bigger than others. If this is the case, why is this only observed in the P21 dataset and not at 6 months. Notably, high UMIs are also a sign of doublet contamination.

- b. Authors include data from RBCs. As they do not have a nucleus, RNA abundance is low as expected. However, markers for RBCs include smooth muscle specific markers, *MYH11* (an MEN marker) and *Acta2*. The presence of these markers can indicate high levels of "ambient RNA" which enters droplets from other cells lysed during digestion. Interestingly, MENs appear to cluster close to RBCs.

- c. In light of the above possible evidence of doublet contamination and high levels of ambient RNA, the markers of MENs need to be reconsidered. MENs are stated to express markers that were previously (up until this manuscript) accepted markers of intestinal mesothelium (*Ukp3b*, *Krt19*, *WT1*), smooth muscle cells (*Myh11*), and fibroblasts (*Dcn*, *C3*, *Col6a1*), raising the possibility that MENs are an erroneous cluster containing RNA from all these cell types.

1. The MEN population appears to be the largest cell population in the gut, which is unprecedented. The authors compare their scRNA-seq data to several other studies that have not made similar observations. Such analysis of other datasets is used to inform on the new data being generated. In the current manuscript, however, this takes the reverse approach and the authors analyze other data based on the assumption that they all mislabeled the MEN population.

- a. In their assessment of Drokhyansky et al., the authors claim that their mesothelium annotation is wrong despite expressing known mesothelial markers. This includes the gene *Ukp3b* which is a bona fide mesothelial marker in the gut but is also expressed by "MENs." They proceed to analyze the Elmentaite et al. dataset and state that their "transitional fibroblast" population are actually MENs. That paper also has a population of *Ukp3b*+ mesothelial cells and it is unclear why those are not actually MENs like in the Drokhyansky et al. study.

- b. The authors often refer to the study of May-Zhang et al. and their cluster annotated as "mesenchymal neurons" in the gut. It should be known that the original authors never made this claim. Rather, they acknowledge that the clusters in their study with poor correlation to neuronal profiles exhibit strong predictions for mesenchymal and vascular/immune cell types. They state: "We considered the possibility that these clusters might be non-neuronal." If

these are "mesenchymal neurons" then the same logic would indicate that there are vascular neurons and immune cell neurons, and therefore this does not make a very compelling case.

1. A weakness of this study is that a lot of the data relies on reporter gene expression. The authors need to acknowledge several weaknesses of this approach. First, Wnt1-tdT recombination may be incomplete or one can have "Cre mosaicism" and therefore the lack of tdT is not sufficient evidence to say that those neurons are not neural crest-derived. Second, one can have off-target or leaky Cre expression, leading to low-level tdT expression, as seen in many of the images in this study. Third, Cre can exhibit toxicity and this may be more problematic in older mice given the long-term continuous expression of Cre (He et al, *Am J Pathology*, 2014;184:1660; Loonstra et al, *PNAS*, 2001;98:9209; Forni et al, *J Neurosci*, 2006;26:9593; Rehmani et al, *Molecules*, 2019;24:1189; Gillet et al, *Sci Rep*, 2019;9:19422; Stifter and Greter, *Eur J Immunol*, 2020;50:338).

Author Response:

We thank eLife for carrying out the peer review of our preprint. In this letter, we will provide a response to the eLife assessment, and the editor's public review, and will also address the major points raised in the peer-review of our study.

First, we wish to inform the readers that including this review, our manuscript has now been reviewed 5 times. These have included three reviews at an earlier journal, a review at eLife under the older model, and the current review at eLife under the new model. In an effort to provide transparency and increase the reader's confidence in our study, all the prior reviews and our rebuttals to them have been uploaded to Biorxiv and are publicly available for all readers to peruse [1]. These reviews will show that we have responded comprehensively with additional data, and analyses over the last 3 years. Of the current reviewers, Reviewer #1 (who was also Reviewer #1 at the earlier journal) has reviewed our manuscript all 5 times. At the prior journal, an additional Reviewer (#2) carried out 3 cycles of review – and we responded fully and comprehensively to all the issues and comments of that Reviewer. It is our understanding that the prior Reviewer #2 did not respond to the review request from eLife, after which eLife recruited two new Reviewers (current Reviewers #2 and #3), who have now reviewed our work twice – once under the older model and now again under the newer model.

Next, to ease readability, we will respond to the review in three parts. Part A will be dedicated to the editors' public review. Part B will be dedicated to the response to eLife assessment, and we will respond to the reviewers' comments in Part C.

Part A: Response to editor's public review: We thank the editor for his nuanced and fair read of our data and our inferences, and of the multiple back-and-forth cycles of reviews and rebuttals. The editor's public review highlights key points put forth in our data, and succinctly discusses the evidence provided for our claims. Here, we respond to each of these highlights.

(i) The editor agrees that subject to the broader limits of lineage fate-mapping experiments, which are universal for every prior and current study of vertebrate development, we have provided sufficient evidence for the presence of a population of cells within the myenteric ganglia, which shows mesodermal and not neural crest derivation, and which expresses the pan-neuronal marker Hu among other neuronal and mesenchymal/mesodermal markers.

Given that the current accepted annotation for enteric neurons depends on their expression of pan-neuronal markers (which we show are expressed by MENs), expression of neurotransmitter-encoding genes and proteins (such as CGRP, NOS1, ChAT, etc, which we

show are expressed by MENs), and their localization within the enteric plexuses (we show evidence of intra-ganglionic localization of MENs in the myenteric plexus), our data suggests that in describing MENs, ours is the first report describing the presence of a mesoderm-derived neuronal population in a significant neural tissue. By virtue of the continual expansion of the MENs population with maturation and aging, we show evidence that MENs contributes to the post-natal maturation and aging of the enteric nervous system (ENS), and by reducing the proportions of MENs in aging tissue, we can rejuvenate the ENS to normalize gut function in aging mice.

(ii) The editor comments on whether beyond the accepted norm of their intraganglionic localization and expression of pan-neuronal markers, MENs can be described as functional neurons. We agree that in our manuscript, we did not test how MENs function. This is expressly because the current report is the first step in the study of MENs and does not aim to understand how MENs regulate various gut functions. In this response however, we wish to put forth a few arguments that would clarify some of the existing evidence on the functional nature of MENs as well as the current state of knowledge on ENS functions. These would help the readers understand the current evidence on the functional nature of MENs, and in addition, why it would be premature to expect MENs to exhibit canonical neuronal behavior.

a. MENs generate neurotransmitters and neuropeptides: Enteric neurons release various neurotransmitters, and their ability to generate important neurotransmitters such as nitric oxide (NO) and acetylcholine depends on their expression of enzymes Nitric Oxide Synthase 1 (NOS1) and choline acetyltransferase (ChAT). Our work shows that sub-populations of MENs express these important neurotransmitter-generating enzymes (Fig 3). Further, our data also shows that MENs express CGRP, which is an important neuropeptide for regulating various gut functions (Fig 3). These important data show that at the protein level, many MENs have the same cellular machinery as that of NENs that can help carry out regulation of important gut functions.

b. MENs have been shown to be functional in a prior study: Recently, enteric neurons have been shown to carry out significant immunomodulatory functions. These have included the expression of cytokines such as IL-18, which regulates intestinal barrier (as shown by Jarret et al. [2]), and CSF1, which regulates macrophage recruitment [3]. Jarret et al shows that the enteric neuron-derived IL-18 regulates immunity at the mucosal barrier. We show that the IL-18 – expressing enteric neurons are MENs (Fig 4), and thus, the data from Jarret et al [2] provides evidence that MENs are indeed functional in the in vivo environment.

c. We do not quite know how many enteric neurons work at the electrophysiological level: Canonical vertebrate neurons exhibit resting membrane potentials (RMP) in the range of -70 to -80 mV, and during neuronal activation, an increase in membrane potential beyond the threshold of -55 mV activates their action potential [4]. By contrast, past and recent studies have shown that the average RMP of rodent and human enteric neurons is significantly more positive than -70 mV (for human ENS: -48 ± 8 mV, for mouse ENS: -46 ± 6 mV for S neurons, -56 ± 5 mV for AH neurons) [5, 6]. These data suggest that enteric neurons show significant departures from canonical neuronal behaviors and thus, expecting MENs to adhere to canonical neuronal behavior – when most of the ENS does not adhere to expected norms – would be incorrect.

d. A neuron is not defined by its ability to generate an action potential: Neuronal behavior does not require the presence of action potentials, as observed in the neurons in *C. elegans* [7], much in the same way that the presence of action potentials is not restricted to neurons as it occurs in nonneuronal cells, including in enteroendocrine cells of the mammalian gut [8]. Thus, the presence or absence of action potentials cannot be the basis for adjudicating whether or not a neurotransmitter-expressing cell in a neural tissue is a functional neuron.

(iii) The Editor, after reading the extensive prior and recent correspondence between the authors and the reviewers on whether the cells analyzed in the transcriptomic experiments are the same as those observed in tissues (called tissue MENs by a reviewer), opined that he found “the authors’ assertions that they have described a cluster of cells that express both neuronal and mesodermal genes, and that this cluster corresponds to the tissue MENs described in lineage tracing, to be broadly sound”.

We are enthused by the Editor’s opinion, as we had previously argued that our data connecting the transcriptomic data to tissue MENs is robust on the basis of extensive immunohistochemical validations of marker genes found in our single cell transcriptomic analyses. The Editor notes some confusion on why some marker genes not specific to MENs were used for the analyses and further points to the prior rebuttals we have posted on Biorxiv [1], where detailed clarifications on the choice of marker genes have been made. In the interest of readability, we direct the readers to these prior rebuttals at Biorxiv for more details. Succinctly, we initially tested canonical neuronal genes by immunolabeling (such as NOS1, ChAT, CGRP, etc) in NENs and MENs before performing single cell transcriptomic experiments. After performing the transcriptomic experiment, we next chose to validate neuronal and mesenchymal genes that were found expressed in the MENs cluster (such as DCN, SLPI, IL-18, NT-3, etc). Finally, in previous cycles of review, on the reviewer’s insistence, we included data on the expression of a host of neuronal genes and their encoded proteins (including Vsnl1, Pde10a, etc) to provide further evidence of neuronal identity of MENs.

While without a significantly large cluster of NENs, it is impossible to know in our transcriptomic data, whether a gene expressed by MENs would be similarly expressed by NENs, it is important to note that lack of detection of a gene in the single cell experiments cannot be inferred as lack of its expression in those cells, and hence, our inferences on whether any marker gene was exclusively expressed by neurons of a particular lineage were determined by immunohistochemistry. Additionally, we wish to reiterate and inform the readers that our study provides detailed analysis of prior work by May-Zhang et al [9], where they have described a small cluster of Phox2b-expressing cells from the murine myenteric plexus that shows the expression of neuronal and mesenchymal markers. Our analyses shows that the transcriptomic profile of MENs matches the molecular signature of these cells. In the longitudinal muscle – myenteric plexus layer, only glial cells and neurons express Phox2b [10], suggesting that this cluster sequenced by May-Zhang et al are cells of the myenteric plexus. We provide evidence that the majority of the MENs were left unsequenced by MayZhang et al and that this minimized the representation of MENs in their data (Fig 5). These data together provide important confirmation of our argument that the transcriptomic MENs point to no other cell type but the tissue MENs.

(iv) The Editor opines that a weakness in our current data is the significant overrepresentation of MENs in the single cell experiment, while also noting that our “explanation - that some cells are more sensitive to manipulations required to prepare cells for sequencing - is certainly well-represented in the literature and is therefore plausible....But it isn’t fully satisfactory”. In our prior arguments (as well as in Part C), we have provided explanations based on prior observations that the issues of disproportionate representation of cell types are a technical limitation of the single cell transcriptomic methodology, which is prevalent in other experimental conditions for ENS (including the gut cell atlas study by Elmentaite et al [11]), and for other cell types in various organs. Due to this limitation, proportions of cells in the single cell space should not be inferred as their proportions in tissues. We also agree with the Editor that owing to the low representation of NENs, our data does not allow for a detailed comparison of the similarities and differences between the neurons of the two lineages, and that “an ideal analysis would have more cells, deeper sequencing, and comprehensive validation of the identity of each cluster of cells.” While in this study our aim was to describe the existence of MENs and not to perform an in-depth

characterization of their sub-populations, we agree that this is the logical next step in creating a better understanding of the true diversity of ENS neurons. To that, we are currently evolving the methodologies to allow for a deeper and a more comprehensive analyses and validation of the various MENs populations, and study how they differ from NENs. We aim to publish these data in our next study.

(v) We agree with the Editor's assessment on our transcriptomic data that "these data and analyses bolster the authors' claims, without conclusively establishing them. That is, these data should neither be dismissed nor, on their own, considered definitive." We have only used our single cell transcriptomic data to provide additional support for our claims (which are based on extensive lineage fate mapping and immunohistochemical analyses) and are not using these as a stand-alone definitive proof of a mesodermal origin. The data from the transcriptomic experiments were used to learn additional molecular markers, whose expression in MENs in tissue could be tested by immunohistochemistry. With this methodology, we provide data on the coexpression of neuronal and mesenchymal markers by MENs, and test by computational analyses whether similar neuronal population exists in other murine and human transcriptomic datasets.

In addition, we completely agree with the Editor that "at this stage in the history of single-cell analysis, the criteria for using single cell sequencing data to establish cell type and cell origin is are not well established, and that neither the presence nor absence of specific sets of genes in single cells should not, for both technical and biological reasons, be considered dispositive as to identity." We are very mindful of this limitation of these analyses and hence have continually ensured that our study only uses transcriptomic data of postnatal MENs to define a preliminary molecular signature of MENs, and not to infer developmental origins of MENs.

(vi) We thank the Editor for his summary and for highlighting that despite using multiple lines of evidence to support our hypothesis, the current reviewers are not yet convinced of the mesodermal origin of MENs. Our study utilizes well established tools for lineage fate-mapping (which are the only tools that currently are widely disseminated and accepted in the field of developmental biology) to show that MENs are not derived from the (Wnt1-cre, Pax3-cre -expressing) neural crest and instead are derived from the (Mesp1-cre, Tek-cre -expressing) mesoderm. The reviewers agree that by using multiple lines of evidence, we have established that our results of lineage fate-mapping are real and not due to any artifact. With this rationale, the reviewers would agree that MENs observed in tissue do not show evidence of derivation from neural crest while showing evidence of derivation from the mesoderm. Despite this, we cannot ascertain the scientific rationale for why despite agreeing with our lineage fate-mapping methods and analyses, the reviewers remain unconvinced as to the developmental origins of MENs. We do not know what other experiment would pass the reviewers' muster to definitively annotate the mesodermal origins of MENs.

We wish to highlight that a recent study in ctenophores, where the investigators show evidence of a syncytial neural net [12], shows that much of the dogmatic view of how neurons are supposed to work is being overturned and newer paradigms that support broader interpretations for the definitions of neurons and how they regulate functions are being established. Our work on the developmental origins of a large population of neurons of the ENS, which is regarded as a primordial and conserved neural tissue, should be viewed in a similar vein.

Part B: Response to eLife assessment: Ours is the first report on the mesodermal derivation of a large population of neurons in a significant nervous system in mammals. We show that this population of neurons, called MENs, is molecularly distinct from the canonical neural crest-derived lineage of neurons, and that the post-natal ENS shows evidence of increasing presence of MENs in the maturing and aging ENS. We show that the two neuronal lineages are sensitive to their own growth factors, which can be used to manipulate their proportions in tissue, and thereby provide a potential rejuvenating therapy for age-associated intestinal

dysmotility. We also show that on the basis of MENs' marker expression, MENs may be present in the human ENS, and that disproportionate changes in their proportions are associated with chronic gut dysmotility disorders. Our work has profound implications in the multiple fields, including those of enteric and peripheral neurobiology, developmental biology, medicine, and aging. We are thankful that the eLife assessment found that we provide sufficient evidence for this important work.

Part C: Response to Reviewers: Here, we wish to note that all the comments of the reviewers have been sufficiently addressed in prior reviews. All prior reviews, and our extensive rebuttals are available at our preprint for the readers' perusal [1]. In this response, we wish to succinctly address some comments that have continued to emerge in this round of peer-review.

(i) We wish to highlight that the Reviewers 1 and 2 agree that our lineage-fate mapping experiments are correct and the results are not a result of any artifact. In addition to the additional reviewer in the prior reviews at an earlier journal, whose comments were addressed in full, we have a total of three reviewers who agree that our results on lineage fate-mapping are robust. Reviewer 3 comments on the possibility of 'cre mosaicism' or the deleterious issues with long-term expression of cre. Our prior rebuttals have dealt with this comment at length, but succinctly, our results are (a) based on extensive cre and floxed reporter controls for both the lineages, and (b) replicate observations made by other labs – including the Pachnis, the Heuckeroth, and the Southard-Smith labs to provide confidence that these are not due to any artifacts in cre or reporter gene expression. Finally, cre in the two lineage fate mapping systems (Wnt1-cre and Mesp1-cre) is only developmentally expressed and thus, there is no reasonable possibility that our results would be impacted by long-term expression of cre. Thus, our results and inferences on lineage fate mapping, which is central to our annotation of the two distinct developmental lineages, correctly describe the developmental origin of MENs.

(ii) By using extensive immunolabeling for (~21) markers that were learnt from our transcriptomic experiments, we provide evidence of the firm connection between the cluster of cells we annotated as MENs in the single cell transcriptomic experiments and the MENs we observe in tissues. Thus, we have performed more validation for these neurons than any other studies that have traditionally used 2 - 3 markers to validate a cell cluster in the ENS.

In addition, by providing evidence of the expression of pan-neuronal marker Hu and other ENS markers that include NOS1, ChAT, CGRP, etc and ~40 neuronally significant genes, we have established the neuronal nature of MENs. With regards to annotation of MENs as neurons, we expected and understand the confusion in the field with our discovery of mesoderm-derived neurons that coexpress neuronal and mesenchymal markers. We wish to put forth the following arguments for the readers to consider.

a. The annotation of Hu-expressing cells within the myenteric ganglia has been traditionally accepted as an enteric neuron. In those terms, by virtue of their intra-ganglionic presence and expression of Hu (and our data shows that Hu antibodies do not discriminate between the three neuronal isoforms of Hu) and other neuronal markers such as NOS1, ChAT, and CGRP, MENs should be annotated as neurons. We had addressed the semantic nature of this question in our last rebuttal (review #3, reviewer 1), which is available on the preprint [1].

b. As the molecular data on MENs suggests that they have significantly different biology, it would not be unreasonable to expect that their neuronal behavior may be quite different. This is underscored by the fact that we observe many MENs to lack the expression the protein SNAP25, whose presence is thought to be central to canonical neuronal behavior. We also cite evidence that neurons without SNAP-25 expression occur in the CNS neurons as well. In light of these discoveries, gauging the biology and neuronal behavior of MENs is a significant

undertaking as it cannot be assumed that the behavior of MENs will be similar to that of NENs.

c. It is not logical to say that “Expressing one of the Hu proteins (Elavl2) probably isn't enough to call these “neurons” especially when neurons usually express Elavl3-4 (HuC/D)” especially when there are currently no antibodies to discriminate between the three neuronal gene products.

d. While at the outset it maybe an easy proposition to suggest that we provide evidence of neuronal activity in MENs by calcium flux or by electrophysiological means, it is important to know that calcium flux exists in all cells of the gut wall, including in smooth muscles, enteric glia, neurons and thus studying calcium flux will not provide definitive proof of neuronal behavior in MENs. Further, we reiterate from Part A of this response letter that “neuronal behavior does not require the presence of action potentials, as observed in the neurons in *C. elegans* [7], much in the same way that the presence of action potentials is not restricted to neurons as it occurs in non-neuronal cells, including in enteroendocrine cells of the mammalian gut [8]. Thus, the presence or absence of action potentials cannot be the basis for adjudicating whether or not a neurotransmitter-expressing cell in a neural tissue is a functional neuron.”

(iii) Our identification and validation of the molecular identity MENs using single cell transcriptomic experiments helps us establish the congruency of our cell cluster with a similar cluster enteric neurons previously observed by the SouthardSmith lab in their analyses. Thus, similar to our observations on the lineage-fate mapping models, observations on our transcriptomic data are also in-line with the observations made by other labs in the field.

(iv) To address any remaining confusion in the minds of the reviewers and of the readers about the correct methodology for interpreting single cell transcriptomic data and the limitations of this technique, we wish to reiterate that:

a. Single cell or nucleus RNA sequencing methods are biased towards sequencing transcripts that are abundant relative to all other transcripts for that individual cell (detection and amplification bias). Thus, while the same transcript may be equally expressed at an absolute level in two different cells, it will be more readily sequenced and detected in the cell where the transcript is relatively more abundant.

b. Correct interpretation of single cell/nucleus transcriptomic data relies on an understanding that not all transcripts of a cell can be sequenced and detected, and thus absence of the expression of transcripts in a cell does not imply absent gene expression. Together this shows the fallacy of an argument often put-forth by the reviewers that a lack of detection of a gene transcript (for e.g. *Phox2b*) in MENs in a scRNAseq experiment should be inferred as a lack of expression of this transcript, even though we provide evidence of the expression of *PHOX2B* protein in MENs, and the expression of this transcript in the MENs in the data from the Southard-Smith lab.

c. scRNAseq is not a technique where annotation of a previously unknown cluster should be biased by the detection of expression of one or two genes, and instead establishing identity or conferring novel annotation of that cluster is defined by co-expression of several genes which must be validated in tissue.

d. It is well known that enzyme-based dissociation methods are unequally tolerated by diverse cell types, which is known to cause over- or underrepresentation of several cell types in scRNAseq (Uniken Venema et al.[13], who showed that dissociation method drives detection and abundance of cells sequenced; Wu et al.[14], showed the existence of similar dissociation bias in the kidney; Tiklova et al.[15] showed that specific subpopulations of *Dat*-expressing neurons in the developing mammalian brain were underrepresented in

scRNAseq). The Gut Cell Atlas study (Elmentaite et al.[16]) was not able to detect NENs in the adult intestinal tissue. The lack of detectable canonical enteric neurons (NENs) in the adult tissue in their study should not be viewed as an absence of NENs in those tissues, and with the same logic, a restricted abundance of NENs and a larger abundance of MENs in our dataset cannot and should not be viewed as a reliable indicator of their actual proportions in tissues. The aim of our study is not to provide a comprehensive molecular atlas for all cells that reside in the LM-MP tissue layer, but to use the information in this atlas to identify a cell cluster that best describes MENs, and then use additional tools to validate this information.

e. Without extensive validation by immunohistochemical or other means, detection of transcripts of a particular gene ‘Z’ (which is known to be expressed in cell type ‘X’) in a particular cell cluster ‘A’ of a single cell transcriptomic dataset does not directly imply that cell cluster ‘A’ points to cell type ‘X’. Thus, the detection of transcripts of the gene *Wt1* (which is known to be expressed in mesothelial cells) in MENs, in itself does not mean that the MENs cluster comprises of mesothelial cells. It simply suggests that in addition to its expression in mesothelial cells, *Wt1* gene is also expressed by MENs – an inference which is supported by data that show the expression of *LacZ* in myenteric ganglia cells in the *WT1*-cre transgenic mouse (Wilms et al 2005 [17]).

(v) Our study has performed two scRNAseq studies, first to establish the distinct molecular signature of MENs, and second to provide transcriptomic evidence of MENs-genesis. In the last and current review, Reviewer 2 opines that we should perform an additional single cell RNA sequencing experiment just to show that the MENs cluster is represented in the mesoderm-enriched transcriptomic data. There is no doubt that owing to the expression of various mesodermal-markers that we show are expressed by MENs (both transcriptomically in scRNAseq and at the level of proteins in tissues), the cluster of MENs is mesodermal in origin. Thus, we have already provided evidence and met a higher burden of proof on the mesodermal identity of MENs, and thus, we do not consider the costly scRNAseq experiment proposed by the reviewer a definitive experiment that would justify the time or the cost.

(vi) Our prior rebuttals have provided the reviewers with evidence that shows that our study has used standard bioinformatic pipelines to analyze our data, and our inferences of the transcriptomic data are sound and well validated by additional methods.

(vii) Many comments of the reviewers that required textual edits were already carried out after the prior review at eLife. While a revised version of our manuscript was submitted to eLife for the current review, it is unfortunate that the reviewers have not updated many of their comments. For the sake of brevity, we will not be responding further to the comments that we have already addressed at length in prior rebuttals or in form of textual edits.

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