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REVIEW

Enteric Neuromics: How High-Throughput "Omics" Deepens Our Understanding of Enteric Nervous System Genetic Architecture

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SUMMARY

Novel high-throughput techniques like single cell RNA sequencing expand our understanding of the enteric nervous system. This review integrates high-throughput findings to further characterize established functional subtypes of enteric neurons and glia and how enteric gene expression patterns change during disease.

Recent accessibility to specialized high-throughput "omics" technologies including single cell RNA sequencing allows researchers to capture cell type- and subtype-specific expression signatures. These omics methods are used in the enteric nervous system (ENS) to identify potential subtypes of enteric neurons and glia. ENS omics data support the known gene and/or protein expression of functional neuronal and glial cell subtypes and suggest expression patterns of novel subtypes. Gene and protein expression patterns can be further used to infer cellular function and implications in human disease. In this review we discuss how high-throughput "omics" data add additional depth to the understanding of established functional subtypes of ENS cells and raise new questions by suggesting novel ENS cell subtypes with unique gene and protein expression patterns. Then we investigate the changes in these expression patterns during pathology observed by omics research. Although current ENS omics studies provide a plethora of novel data and therefore answers, they equally create new questions and routes for future study.
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High-throughput "omics" research investigates molecular information on a large and comprehensive scale. The flexibility and resolution of omics technologies continue to increase while cost decreases,[1] making omics methods increasingly accessible and attractive to basic and clinical researchers. This has led to a rapid growth in the number of published studies using omics approaches to understand the enteric nervous system (ENS). The ENS is embedded within the gut wall and provides local control of gastrointestinal functions through intrinsic neurocircuitry and integration with multiple cell types in the gastrointestinal tract and other organs.[2] The ENS is composed of neurons and glia with generally well-known electrophysiological properties, anatomic features, and protein markers.[2–5] However, much of the complexity of the ENS remains unknown and would benefit from developing a deeper understanding of cellular heterogeneity, functional attributes of cells and cellular networks, and genes that contribute to disease.

Omics technologies are helping to disentangle complexity within the ENS on a scale that was previously inaccessible. The advent of single cell RNA sequencing (scRNA-seq) now allows characterizing heterogeneity between individual cells,[1] and cellular genomic libraries are available to explore the cellular makeup of the ENS in fine resolution. We begin this review by summarizing the "pre-omics" understanding of the cellular makeup of the ENS and describe omics strategies used to study the ENS. Then we focus on how omics data expand known ENS cell diversity and cellular changes in gastrointestinal disease (Figure 1). We conclude by discussing strengths and challenges of current ENS omics data and future directions for the field.

Pre-omics Understanding of ENS Cellular Makeup

Classification of Enteric Neurons

Enteric neurons are traditionally classified by their morphology, electrophysiological properties, and neurotransmitter expression. Whereas initial descriptions were based on guinea pigs,[2–4] additional comparative data in mice provided murine-specific ENS characterization.[6] Enteric neuron morphology was initially described by A. S. Dogiel[7] and has been characterized by imaging techniques that include intracellular dye filling, silver staining, retrograde tracing, immunohistochemistry, and electron

Abbreviations used in this paper: **ACh, acetylcholine; AH, after-hyperpolarization; CGRP, calcitonin gene-related peptide; ChAT, choline acetyltransferase; DNBS, dinitrobenzene sulfonic acid; ENS, enteric nervous system; GDNF, glial cell line–derived neutrophilic factor; HSCR, Hirschsprung disease; IBS, irritable bowel syndrome; IBD, inflammatory bowel disease; IFAN, intestinofugal/viscerofugal afferent neuron; IL, interleukin; IPAN, intrinsic primary afferent neuron; NPY, neuropeptide Y; scRNA-seq, single cell RNA sequencing; SST, somatostatin; VIP, vasoactive intestinal peptide.**

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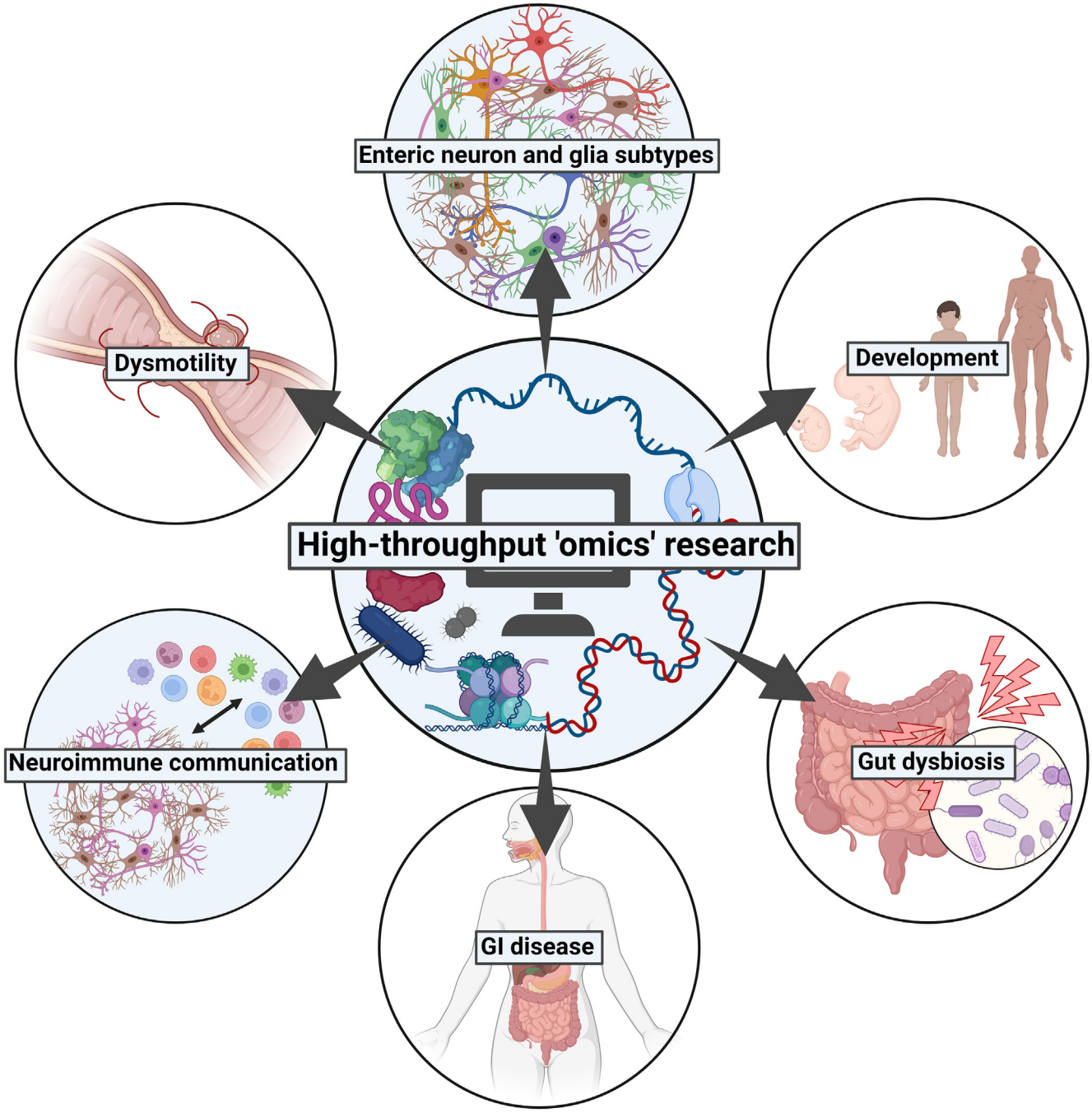

Figure 1. Deepening the understanding of enteric nervous system function and disease through high-throughput omics approaches. This review focuses on how data obtained by high-throughput approaches such as single cell sequencing deepen our understanding of cell identity, mechanisms of intercellular communication, and disease processes in the enteric nervous system. Created with BioRender.com.

microscopy. Neuronal cell bodies are typified by the shape and number of axons and dendrites in addition to where these processes project. Neuronal morphology is more complex and clearly defined in larger species such as pigs and humans. Thus, although several Dogiel subtypes can be identified in these mammals, only Dogiel type I and II morphologies are observed in mice.[4,6] Type I neurons have flat, elongate, and irregular cell bodies with a single axon and numerous short dendrites, whereas type II neurons have smoother and larger cell bodies with multiple long axons. Type I neurons project and communicate with adjacent ganglia in the plexus and musculature, whereas type II neurons communicate with neurons throughout the gut wall, within and between ganglia, and the mucosa.[4] Neurons

are also classified by electrophysiological properties, which have been mainly characterized in guinea pigs[8,9] and mice.[10] Two main types of enteric neurons are categorized as having either synaptic- or AH (after hyperpolarization)-type electrophysiological properties that differ on the basis of action potential speed and magnitude, the length of AH potentials, and tetrodotoxin sensitivity. Synaptic-type neurons typically display Dogiel type I morphology and include interneurons and motor neurons, whereas AH-type neurons typically display Dogiel type II morphology and are considered sensory neurons.[4]

Defining the neurochemical coding of enteric neurons was a significant advancement in identifying neuronal subtypes and understanding how enteric neurons communicate with one another and target cells. Enteric neurochemical coding has been defined by multiple approaches including immunohistochemistry in combination with retrograde tracing, electrophysiology, and pharmacology. Integrating these biomolecular data with morphologic and electrophysiological properties is the basis for current definitions of enteric neuron subtypes, which include motor neurons, interneurons, and sensory neurons. Although these definitions are based largely on studies in guinea pigs, many of the core features of enteric neuron subtypes are conserved between mice and humans. Excitatory and inhibitory motor neurons reside in the myenteric plexus and innervate the circular and longitudinal muscle of the intestine. Motor neurons are defined by Dogiel type I morphology in guinea pigs,[2–4] but many have an unclear morphology in mice, characterized by small or medium-sized cell bodies without obvious dendrites.[6] Excitatory motor neurons are cholinergic and release acetylcholine (ACh) but can also release tachykinins. Inhibitory motor neurons are nitrergic and release nitric oxide in addition to vasoactive intestinal peptide (VIP) and purines.[2–4,6] Excitatory motor neurons express choline acetyltransferase (ChAT) and/or vesicular acetylcholine transporter in guinea pigs and mice; however, although both circular and longitudinal muscle-projecting excitatory motor neurons also express tachykinins in guinea pigs, tachykinins are not always expressed by the latter in mice. All inhibitory motor neurons in both guinea pigs and mice express nitric oxide synthase and VIP, whereas those innervating circular muscles can also express neuropeptide Y (NPY).[4,6] Secretomotor/vasodilator neurons in guinea pigs have 3 known subtypes categorized as non-cholinergic VIP^+ neurons, $ChAT^+$/calretnin $(Calb2)^+$ neurons, and $ChAT^+/NPY^+$ neurons.[2–4] In mice these submuscosal neurons are categorized into 2 non-cholinergic and 1 cholinergic subtype(s). Both non-cholinergic secretomotor and vasodilator neurons express VIP and NPY, whereas secretomotor neurons also express tyrosine hydroxylase. Cholinergic secretomotor neurons express ChAT, calcitonin gene-related peptide (CGRP), and somatostatin (SST).[11]

At least 4 types of interneurons are present in the small intestine of guinea pigs and mice. Ascending interneurons are cholinergic and also use tachykinins.[2–4,6] These neurons are involved in local motility reflexes.[3,4] Subtypes of descending interneurons involved in local motility reflexes include an ACh^+/nitric oxide synthase$^+$ subtype that is VIP^+ in guinea pig but not mouse and an ACh^+/serotonin$^+$ subtype that is involved in secretomotor reflexes.[2,4,6] A third type of descending interneuron is ACh^+/SST^+ and is involved in small intestinal migrating myoelectric complexes. Whereas the other two interneuron subtypes are characterized by Dogiel type I morphology in guinea pig and mouse, this third subtype is characterized by distinct filamentous dendrites.[3,4,6]

Intrinsic primary afferent neurons (IPANs) regulate intrinsic reflex pathways of the intestine and are involved in chemosensation and mechanosensation. IPANs have Dogiel type II morphology and AH-type electrophysiology,[3,4] and most express ChAT and CGRP. In guinea pigs IPANs also express tachykinins and isolectin B4.[2,4] IPANs can be identified in mice, humans, and pigs by neurofilament (*Nefm*) staining[6] and by advillin expression in mice, albeit the latter is expressed by other neuronal subtypes as well.[12] Intestinofugal/viscerofugal afferent neurons (IFANs) reside in the myenteric plexus and project to prevertebral ganglia where they synapse with post-ganglionic sympathetic neurons. These cells contribute to intestinal functions that involve integration with other gastrointestinal organs. IFANs are rare (<1%) and typically display a Dogiel type I morphology (occasionally type II) in guinea pigs and mice. IFANs use ACh and VIP signaling but also express cholecystokinin, gastrin releasing peptide, and opioid-related peptides.[2,4,6,13]

Classification of Enteric Glia

Enteric glial heterogeneity and functions were covered extensively in a recent review[14] and will not be reiterated here. Current glial subtypes are defined on the basis of morphology and anatomic location in the gut wall and may include differences in marker expression and response to various transmitters.[5,15] Canonical markers used to identify enteric glia include glial fibrillary acidic protein, S100B, Sox10,[5,15,16] and *Plp1*.[16] However, expression of glial markers within a single cell varies over time and is reflective of their current state.[15,16] Therefore, whether expression patterns are indicative of different glial subtypes or ongoing cellular dynamics is unclear.

Omics in the Enteric Nervous System

The technical details of current omics techniques and the strengths and challenges of applying these techniques to biomedical research are discussed in detail elsewhere.[1,17,18] Here we briefly introduce omics techniques used in ENS research. Genomics identifies variation in DNA sequence, primarily using genome-wide association studies. Genome-wide association studies genetic code from diseased humans to identify genetic mutations (specifically single nucleotide polymorphisms) that may confer disease risk. Sequencing the entire genome or coding exome can also identify mutations. Transcriptomics identifies and quantifies RNA expression. Transcriptomics initially used microarray platforms but now primarily consists of sequencing (RNA-seq). Typically RNA-seq focuses on which genes are expressed and how their expression level changes. However,

this method can also identify noncoding RNAs such as microRNAs or long noncoding RNAs that influence transcription of coding genes. Proteomics quantifies protein abundance, modification, and interaction. Compared with transcriptomics, proteomics captures a related but separate understanding of gene expression.[17]

Altered pipelines of these fundamental omics modalities are used to attain omics data from specialized sources. For instance, DNA sequencing can specifically target variation in the bacterial 16s rRNA gene to taxonomically identify organisms within the gut microbiome. Transcriptomic studies can capture gene expression signatures from specified cells of interest by combining RNA-seq with cell-specific isolation strategies. These strategies range from using genetic driver mouse lines and performing cell sorting protocols to post hoc computational analyses focusing on known cell-specific pathways. One of the most recent of these is scRNA-seq, which measures gene expression within individual cells.[1] ScRNA-seq is the primary technique used in ENS research to further resolve subtypes of enteric neurons and glia by grouping individual cells into clusters based on overall shared gene expression patterns. Similarly, proteomics

Table 1. Omics Dataset Metadata and Review Criteria: Methods Used and Species/Gastrointestinal Regions Examined in ENS Omics Datasets, by Reference Number

Section	Omics method		Species		Region	
Cell subtype markers	scRNA-seq RNA-seq	20–24,31,32 21	Mouse Human	20–24 20,21,23,31,32	Colon Small intestine	20,21,23,31,32 20–24
Compares regions	scRNA-seq RNA-seq	20,21,23 21,30,34	Mouse Human	20,21,23,30,34 20,21,23	Colon Small intestine	20,21,23,30,34 20,21,23,30,34
Compares species	scRNA-seq RNA-seq	20–23,39 21,25,41	Mouse Human Zebrafish	20,21,23,25,39 20,21,23,39 41	Colon Small intestine Cell culture	20,21,23,41 20,21,23,25,41 39
Compares sexes	scRNA-seq RNA-seq	20–23 21	Mouse Human	20–23 20,21,23	Colon Small intestine	20,21,23 20–23
Dysmotility	scRNA-seq RNA-seq WES MALDI-TOF MS	23 43–45 42 49	Mouse Human Rat	23,43 23,42,44,45 49	Colon Small intestine	23,44,45,49 23,43
Development	scRNA-seq RNA-seq WES Microarray	22,54–57,59 25,41,50,58 42 52,53	Mouse Human Zebrafish	22,25,52–54 42,55–59 41,50	Colon Small intestine Cell culture	41,50,52,53,55–58 22,25,41,50,52,53,55–58 54,59
Neuroimmune communication	scRNA-seq RNA-seq Microarray	20,33 60,61,70,72 71	Mouse Human Rat	20,33,60,61,70 20,71 72	Colon Small intestine Cell culture	20,70,72 20,33,60,61,72 71
Dysbiosis	scRNA-seq RNA-seq GWAS LCMS 16S rRNA-seq	65 30,34,77 82 73 74–76,78–81	Mouse Human Rat	30,34,81,65,73–76,78–80 75,82 77	Colon Small intestine	30,34,65,74,76–81 30,34,73,77
Gastrointestinal disease markers	scRNA-seq RNA-seq Microarray GWAS LCMS	20,23 45,70 53,89 86,87 83,90	Mouse Human	20,23,53,70 20,23,45,83,86,87,89,90	Colon Small intestine	20,23,45,53,70,83,89,90 20,23,53

GWAS, genome-wide association study; LCMS, liquid chromatography-mass spectrometry; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; RNA-seq, RNA-sequencing; 16S rRNA-seq, 16S rRNA gene sequencing; scRNA-seq, single-cell RNA-sequencing; WES, whole exome sequencing.
Review search criteria: Full-text primary research articles were selected from the PubMed database using the following search terms:
("neurons"[MeSH Terms] OR "neuroglia"[MeSH Terms] OR "Ganglia, Spinal"[MeSH Terms] OR "Enteric Nervous System"[MeSH Terms] OR "Colon/innervation"[MAJR] OR "dorsal root ganglia"[All Fields] OR "neuron"[Title/Abstract] OR "enteric glia"[All Fields] OR "glia"[Title/Abstract]) AND ("computational biology"[MeSH Terms] OR "sequence analysis"[MeSH Terms] OR "high throughput"[Title/Abstract] OR "sequencing"[All Fields] OR "next generation"[All Fields]) AND ("gastrointestinal diseases"[MeSH Terms] OR "gastrointestinal tract"[MeSH Terms] OR "Gastrointestinal Microbiome"[MeSH Terms] OR "gastrointestinal"[Title/Abstract] OR "bowel"[Title/Abstract] OR "gut"[Title/Abstract]) NOT Review[Publication Type].
From these results articles were screened for using high-throughput 'omics' methods in the enteric nervous system or referencing enteric nervous cells. A few newer articles were selected outside this due to backlog in MeSH classification.

technology can target specified subsets of proteins such as host and/or microbial metabolites based on their physical and chemical properties.[17,19] The details of omics methods used by the ENS studies discussed in this review are summarized in Table 1.

Using Omics to Define Cellular Subtypes in the Enteric Nervous System

Genetic Markers of ENS Cell Subtypes

Data available from several prominent scRNA-seq studies of the ENS vastly expand the ability to investigate ENS heterogeneity.[20–24] Here we highlight collective findings across these data that identify potential novel cellular markers. Although further examination is required to truly establish whether these markers identify ENS cellular subtypes, these data support additional complexity in the current neurochemical coding of enteric neurons and glia. Our interpretation of synthesized findings is summarized in Figure 2 and Table 2. Some correlations in this table that are not mentioned in the text were based on Wright et al[23] or simply list all clusters the original authors designate as putative neuronal subtypes. Also of note, the dataset from Morarach et al[22] represents an extended capturing of enteric neurons of the same age and region as the dataset presented in Zeisel et al,[24] and thus Morarach et al is primarily discussed below. However, work from Zeisel et al[24] is retained to discuss glia.

Motor Neurons

At least 2–5 subtypes of putative excitatory motor neurons were identified in single cell transcriptional studies.[20–24] Pre-omics guinea pig data suggest longitudinal muscle-innervating excitatory motor neurons express calretnin (*Calb2*), whereas those innervating circular muscle do not.[4] On the basis of this expression pattern, cell clusters ENC 1-2 from Morarach et al[22] innervate longitudinal muscle, whereas ENC 3-4 innervate circular muscle. However, pre-omics data in mouse suggest circular muscle-innervating neurons may also express calretnin[6] and complicates this alignment. This murine pattern of *Calb2* expression aligns with excitatory clusters in May-Zhang et al,[21] where cluster 0 innervates longitudinal muscle and cluster 3 innervates circular muscle. Regardless of functional classification, some novel biomarkers are shared across excitatory motor neuron subtypes and may support further functional subtyping with future investigation. These include combinations of *Gfra2, Oprk1, Htr4,* and *Piezo1.* However, *Piezo1* is also expressed by inhibitory motor neurons, and *Gfra2* is also expressed by IPANs and SST^{+} interneurons; thus, specific combinations of these markers may be required to identify excitatory neuronal subtypes.[20–23]

Although broad functional excitatory subtypes within scRNA-seq clusters are still unclear, these datasets suggest a rarer excitatory motor neuron variant with novel markers. In May-Zhang et al[21] and Morarach et al[22] this cell type exists within Cluster 3 and ENC4, respectively. These cells express high levels of the 5-HT2B receptor gene *Htr2b* in addition to genes encoding a calcium binding protein (*Necab2*), an enzyme that catalyzes the last step in the biosynthesis of Lewis X antigen (*Fut9*), and a transcription factor involved in inducible gene transcription during immune responses (*Nfatc1*). *Fut9* and *Nfatc1* were also expressed by the Chat 3 neuron cluster defined by Wright et al.[23] This cluster could correspond to PEMN2 in Drokhlyanksy et al[20] because of higher enkephalin (*Penk*) expression compared with other putative excitatory motor neurons, as also seen in other datasets.[21,22,24] Functional validation of these expression markers may shed light on the existence of this peculiar variant.

At least 2–4 inhibitory motor neuron subtypes were identified in single cell transcriptional studies based on *Nos1* and *Vip* expression.[20–24] Interestingly, Drokhlyansky et al[20] identified 7 inhibitory neuron clusters, PIMN 1–7. Although all these clusters express *Nos1*, PIMN 1–4 express higher levels of *Vip,* suggesting relative *Vip* expression as a means of stratifying subtypes. Pre-omics murine data suggest inhibitory motor neurons innervating circular muscle may also express neuropeptide y (*Npy*), whereas those innervating longitudinal muscle do not.[6] This supports clustering from May-Zhang et al[21] and Morarach et al[22] where cluster 2 and ENC8 innervate circular muscle, whereas cluster 1 and ENC9 innervate longitudinal muscle, respectively. Potential new co-markers for inhibitory motor neurons include argininosuccinate synthase 1 (*Ass1*),[21,23] *Gfra1*, the receptor for glial cell line-derived neurotrophic factor (GDNF),[23] and *Etv1*.[23,24] However, *Ass1* is expressed by other neurons as well,[20,22] and *Gfra1* and *Etv1* are also expressed in putative interneurons and/or IPANs.[22] Perhaps only co-expression of all these markers is specific to inhibitory motor neurons.

Drokhlyansky et al[20] identified 2 clusters that potentially correspond to secretomotor/vasodilator neurons, PSVN 1 and PSVN 2. These clusters were characterized as secretomotor/vasodilator neurons on the basis of expression of the non-prototypical marker glucagon-like peptide 2 receptor (*Glp2r*). However, PSVN 1 expresses relatively low levels of *Chat* and is possibly non-cholinergic, whereas PSVN 2 expresses relatively higher *Chat* and may be a cholinergic secretomotor neuron. A putative sensory neuron cluster PSN 4 expresses *Sst* and *Calcb,* the beta form of CGRP, and therefore may also identify cholinergic secretomotor neurons. Other studies did not sequence submucosal plexus tissue and therefore did not describe secretomotor/vasodilator neurons.[21–23]

Catecholaminergic Neurons

Pre-omics research supports catecholaminergic neurons in gut that signal via dopamine or norepinephrine/noradrenaline and express tyrosine hydroxylase (*Th*). Noradrenergic signaling within the ENS is thought to be solely from extrinsic neuronal projections, whereas dopaminergic neurons reside within the ENS.[25–27] Dopaminergic enteric neurons are rare neurons that develop relatively late (after embryonic day (E) E18) and express dopamine active transporter (*Dat*) and the dopamine metabolite DOPAC in addition to *Th*.[25,26] These neurons are important regulators of gastrointestinal motility[27] and are involved in motor circuitry, but what specific types of neurons express dopamine is unclear. Interestingly, scRNA-seq studies identify

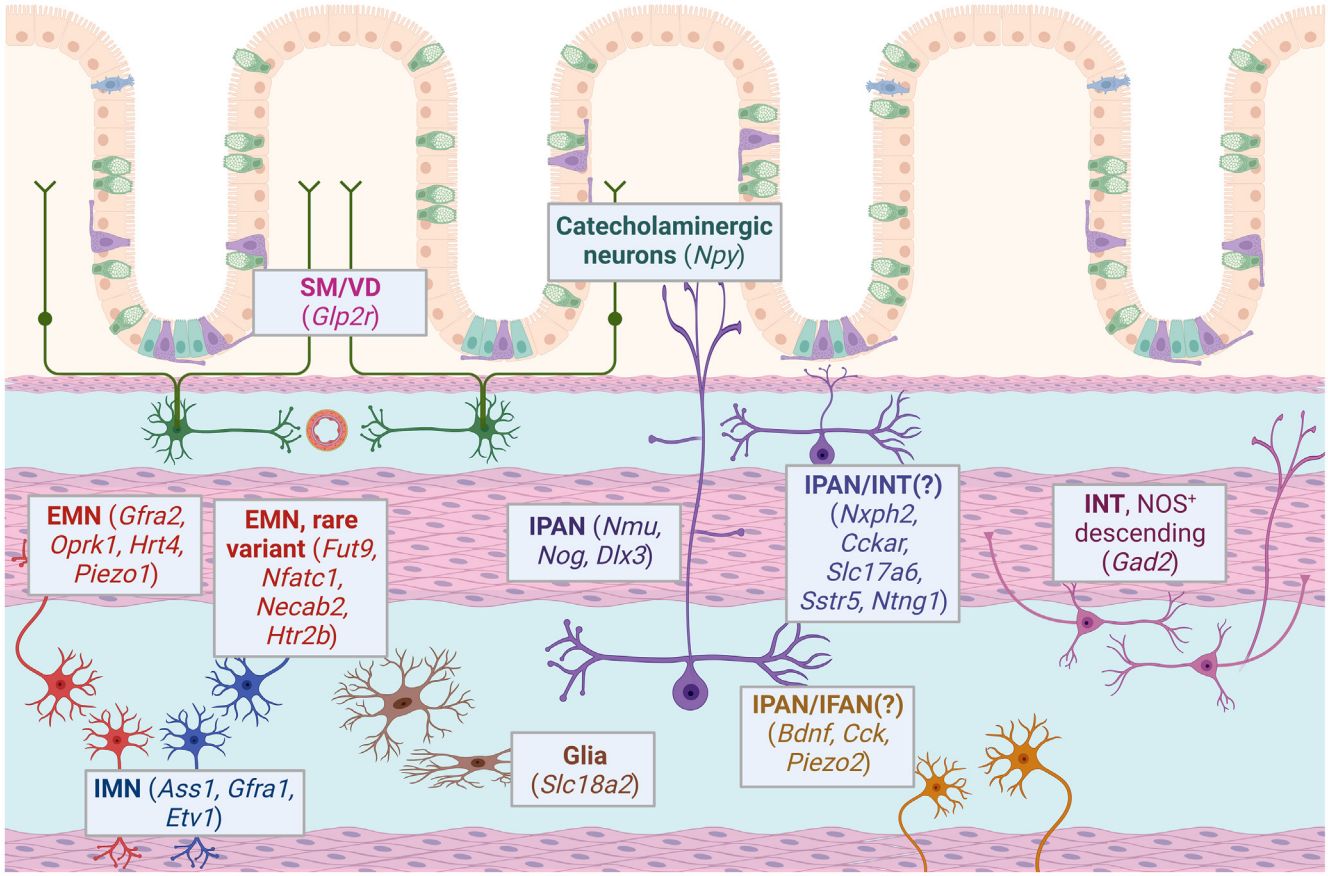

Figure 2. Putative markers of ENS cell subtypes. Novel genetic markers of enteric neuron and glia subtypes from scRNA-seq research. Future genetic and functional research could validate these molecules as important functional or developmental requirements for ENS cell subtypes. EMN, excitatory motor neuron; IFAN, intestinofugal afferent neuron; IMN, inhibitory motor neuron; INT, interneuron; IPAN, intrinsic primary afferent neuron; SM/VD, secretomotor/vasodilator neuron. Created with BioRender.com.

clusters with *Th* expression, but these clusters do not express *Dat* and often also express dopamine beta-hydroxylase (*Dbh*)[20,21] and therefore may actually be noradrenergic neurons. Whether this truly identifies intrinsic noradrenergic neurons or is contamination from extrinsic fibers is unclear, but we will refer to these as catecholaminergic neurons here. Regardless, these data suggest subtypes of motor neurons, interneurons, and sensory neurons are catecholaminergic. This includes PSVN1-2, PIN1, and PIMN3 from Drokhlyanksy et al,[20] cluster 9 from May-Zhang et al,[21] ENC11 from Morarach et al,[22] and Nos1 cluster 2 in E17.5 embryonic mice samples from Wright et al.[23] Many of the clusters also express *Npy* and thus could use this as a co-expression marker. On the basis of prior investigations of enteric dopaminergic neurons they may also co-express combinations of *Ebf1, Meis2, Etv1, Satb1, Klf7,* and *Sox6.*[25] However, these expression markers were determined during ENS development from gastric tissue and may not reflect mature neuronal expression patterns.

Interneurons

Interneurons are more difficult to define by gene signatures in scRNA-seq studies, likely in part because of the overlap of their established markers with other neuron types. Despite this, between 2 and 5 subtypes of putative interneurons were proposed.[20–24] May-Zhang et al[21] and Morarach et al[22] suggest interneuron subtypes that align with current functional classifications. One subtype (cluster 10[21] and ENC10[22]) corresponds to *Nos1*$^+$ descending interneurons. These clusters express *Nos1* and glutamate decarboxlyase (*Gad2*) in addition to various levels of *Chat*. Somatostatin/*Sst*$^+$ descending interneurons may correspond to cluster 9[21] and ENC5.[22] Sensory neuron cluster PSN 4 from Drokhlyansky et al[20] is the main cluster that expresses *Sst* (very little is expressed in their defined interneuron clusters) and therefore may also correspond to this interneuron. The serotonergic/5-HT$^+$ descending interneuron subtype includes cluster 6s[21] and a subset of ENC12[22] characterized by co-expression of serotonin transporter Sert (*Slc6a4*), monoamine vesicle transporter *Vgat2,* and/or dopa decarboxylase (*Ddc*). The ascending interneuron subtype identified by May-Zhang et al[21] (cluster 3s) was characterized by high *Chat* and *Tac1* with potential co-expression of *Calb2*. These are also expressed in possible interneurons ENC4 in Morarach et al.[22]

One cell type is conserved among datasets but classified as both a potential interneuron[20,23] or potential IPAN.[21,22] These cells co-expressed combinations of markers *Nxph2*,

Table 2. Putative Enteric Neuron and Glia Subtypes and Co-Expression Markers

No. of ENS cell subtypes proposed by scRNA-seq

	Drokhlyansky et al[20]	May-Zhang et al[21]	Morarach et al[22]	Wright et al[23]	Zeisel et al[24,a]
Species	mouse | human	mouse	mouse	mouse	mouse
Age	adult	adult	PN21	E17.5 | adult	PN21
Region	ileum, colon | colon	duodenum + ileum + colon	small intestine	intestine | colon	small intestine
No. of EMN	3, 3–5 | 4	2	4	1 | 1–2	—
No. of IMN	2, 4–7 | 5	2	2	0–2 | 0–2	—
No. of INT	2, 2–3 | 2	4	2–5	2–3 | 4–6	—
No. of IPAN	3, 3–4 | 1	2	3	1 | 1–2	—
No. of Glia	3 | 3–6	—	—	— | 4	7

Shared neurochemical markers of putative ENS cell subtypes

Cell subtype (pre-omics murine markers)	Drokhlyansky et al[20]	May-Zhang et al[21]	Morarach et al[22]	Wright et al[23]	Zeisel et al[24,a]	Novel putative co-markers
EMN, circular muscle (*Chat, Tac1, ±Calb2*) EMN, longitudinal muscle (*Chat, Calb2, ± Tac1*)	PEMN 2 PEMN 1,3-5	Cluster 3 Cluster 0	ENC3-4 ENC1-2	Chat 2, Chat 3 Chat 1	ENT6 ENT4-5	*Gfra2, Oprk1, Htr4, Piezo1*
IMN, circular muscle (*Nos1/2, Vip, ± Npy*) IMN, longitudinal muscle (*Nos1/2, Vip*)	PIMN 1-7 PIMN 1-7	Cluster 2 Cluster 1	ENC8 ENC9	Nos 1-2 Nos 1-2	ENT2-3 ENT2-3	*Ass1, Gfra1, Etv1*
INT, descending (*Nos1/2, Chat*)	PIN 1-3	Cluster 10	ENC10	Chat 4		*Gad2*
INT, descending (Sst, *Chat, ± Calb2*	PSN 4	Cluster 9	ENC5			
INT, descending (*Chat,* 5-HT related genes)	PIN 1-3	Cluster 6s	ENC12 (subset)		ENT7 (subset?)	
INT, ascending (*Chat, Tac1, ± Calb2*)	PIN 1-3	Cluster 3s	ENC4		ENT6	
IPAN (*Chat, Nefm, Calca/b, Calb1, ± Calb2*)	PSN 1	Cluster 5	ENC6	Calcb	ENT9	*Nmu, Nog, Dlx3*
Secretomotor, non-cholinergic (*Vip, Npy, Th, ± Calb*) Vasodilator, non-cholinergic (*Vip, Npy, Calb, Th*$^-$) Secretomotor, cholinergic (*Chat, Calca/b, Sst, Calb*)	PSVN 1 PSVN 1 PSVN 2, PSN 4					*Glp2r*
Catecholaminergic neurons (*Th*)	PSVN 1-2, PIN 1, PIMN 3	Cluster 9	ENC11	Nos1 cluster 2		
IFAN (*Cck*)	PSN3	Cluster 7s	ENC7		ENT8	
Glia (*Sox10, S100β, Gfap, Plp1*)	Glia 1-3			Glia1-4	ENTG1-7	*Slc18a2*

EMN, excitatory motor neuron; IFAN, intestinofugal afferent neuron; IMN, inhibitory motor neuron; INT, interneuron; IPAN, intrinsic primary afferent neuron.
[a]Zeisel et al[24] include a dataset collected from the same region and stage as Morarach et al.[22]

Cckar, *Slc17a6*, *Sstr5*, and *Ntng1*.[20–24] These clusters are highly heterogeneous and likely represent multiple neuronal subtypes merged in one semi-identified entity. This is supported by their expression of *Nxph2*, which is part of the neurexophilin family and modulates synaptic plasticity. Functional ablation by these suggested markers may help resolve the identities of these cells.[22]

Intrinsic Primary Afferent Neurons

Between 1 and 4 cell clusters are proposed to correspond to IPANs on the expression of CGRP gene *Calcb,* coding for CGRPβ. Although *Calcb* is the primary CGRP gene expressed by and considered an IPAN marker,[20–24] its paralog gene *Calca* (coding for CGRPα) is expressed in a subset of IPAN clusters in murine juvenile intestine and adult colon,[20,22,28] and thus its expression may be age- or region-dependent. One potential *Calca*$^+$/*Calbc*$^+$ IPAN subtype co-expresses combinations of neuromedin U (*Nmu*), noggin (*Nog*), and homeobox *Dlx3*, corresponding to PSN 1,[20] cluster 5,[21] ENC6,[22] and Calcb.[23] Another putative *Calca*$^+$/*Calbc*$^+$ IPAN subtype expresses a combination of brain-derived neurotrophic factor (*Bdnf*), mechanosensitive ion channel *Piezo2,* and *Cck*. Drokhlyansky et al[20] suggest this is a single subtype (PSN3), whereas Morarach et al[22] identified a *Bdnf*$^+$*Piezo2*$^+$ subtype (ENC12) and a separate cluster (ENC7) as *Bdnf*$^+$*Cck*$^+$.

Although CGRP genes are expressed in IPANs and therefore a putative means of identifying IPAN clusters, these genes are also expressed by secretomotor/vasodilator neurons and therefore may confound IPAN clustering.[11] Morarach et al[22] reported 3 putative IPAN types by clustering: ENC6, ENC7, and ENC12. They subsequently investigated the morphologies of these subtypes, delineating true IPANs by Dogiel type II morphologies and neuronal projections that traveled through layers of the gut wall. They determined that ENC6 and a subset of ENC12 appeared morphologically as IPANs, whereas ENC7 was characterized as atypical IPANs or perhaps even intestinofugal neurons. Because the ENC12 subset expresses *Piezo2*, these may be mechanosensitive IPANs. This work demonstrates further validation of omics subtype classifications is necessary and suggests some of the other proposed IPAN subtypes may not be true IPANs or be atypical IPANs or unique subtypes.

Only 2 datasets categorize clusters as intestinofugal neurons/IFANs. Identification of these clusters was based on *Cck* expression as a known marker of guinea pig IFANs.[4] In addition to ENC7 identified by Morarach et al,[22] May-Zhang et al[21] identified cluster 7s as intestinofugal. In addition, PSN 3 from Drokhlyansky et al[20] may also represent IFANs because these cells express *Cck*. Cluster 7s co-expresses *Slc24a3* and *Carmn* in mice and humans. *Carmn* is a long noncoding RNA critical for cardiac muscle development and pathologic remodeling.[29] Although *Carmn* may play similar roles in IFAN development and neuroplasticity, this would require additional testing to determine the regulatory targets of the long noncoding RNA. Although not a scRNA-seq study, Muller et al[30] identified cocaine- and amphetamine-regulated transcript (encoded by gene *Cartpt*) as a novel IFAN marker as well. However, in scRNA-seq studies *Cartpt* is expressed by several other neuronal clusters[20,23] and therefore would need to be used in combination with other markers for IFAN identification.

Enteric Glia

Between 1 and 7 subtypes of enteric glia are classified on the basis of expression patterns. Of note, the 2 studies that found only 1 glial subtype sequenced biopsy tissue from IBD patients and thus only captured mucosal glia.[31,32] In mice the number of glial subtypes may differ throughout development or by gut region, because 7 subtypes were identified in postnatal day (PN) PN21 mouse small intestine,[24] whereas adult mouse colon only has 2–4 subtypes.[20,23,33] Alternatively, this is due to the differences in resolution because these datasets contain varied numbers of captured glial cells. One glial subtype in PN21 mice is classified as a progenitor cell because of topoisomerase *Top2a* expression.[24] This is not a defining marker identified in adult enteric glia and suggests this glial subtype plays a larger role in the juvenile development period than adulthood. Expression of the vesicular monoamine transporter *Slc18a2* and GDNFα receptors further supports developmental convergence of glial subtypes, because these mark 2–3 subtypes at PN21 and only 1 subtype each in adulthood.[20,24] Several other markers of enteric glial subtypes are identified only in a single study. These include differential expression of *Foxd3* and *Aldh1a3* in PN21 subtypes[24] and neurotensin receptor *Ntsr1* in adult subtypes.[20] In humans, 1 glial subtype expressed *P2Y12R, NRXN1,* and *XKR4,*[20] which could also be potential markers. Although differential expression of these markers could reflect developmental and species differences, this heterogeneity may also be due to the dynamic and reactive expression patterns known of enteric glia in varying environments.[15,16] Regardless, the paucity of glial discussion in ENS scRNA-seq studies cannot resolve this, and glial heterogeneity warrants further investigation. Some of these studies did in fact create glial expression datasets,[20,23,24] and reanalysis or meta-analysis of these data with a glial focus would likely help resolve these differences and identify additional subtype expression patterns.

Region-, Species-, and Sex-Dependent Expression

Region-Dependent Expression

The number of neuronal subtypes is mostly conserved across gut regions[20,21]; however, the proportion of neuronal types and subtypes varies. Catecholaminergic neurons are more highly concentrated in the duodenum than ileum, whereas *Sst*$^+$ and *Cck*$^+$ neuronal subtypes are more prevalent in the ileum.[22] The ileum contains more sensory neurons than the colon, and the colon contains more secretomotor/vasodilator neurons. This difference likely reflects the colon's need to regulate fluid absorption and secretion.[20] Subtype-specific genes also vary between regions of the small and large intestine. *Chat*$^+$/*Nos*$^+$ descending interneurons, *Gad2*$^+$ interneurons,[21–23] and/or

Gad2$^+$ secretomotor/vasodilator neurons[20] are more prevalent in the small intestine. Genes including *Unc5d*, *Col25a1,*[34] *Htr2b,*[20,30] and *Htr3a*[20] are all highly enriched in the colon and are putative markers for subtypes of ascending interneurons, inhibitory motor neurons,[21] excitatory motor neurons,[21,22] and IPANs/inhibitory motor neurons,[23] respectively.

Regional expression patterns also reflect gut physiology. Genes involved in signaling with enteroendocrine cells such as *Cckar,*[21] *Tacr3, Npy,* and the glucagon receptor *Gcgr* are highest in duodenal neurons.[30] Meanwhile, distal gut segments are enriched for the glutamate receptor,[20] *Sst, Cartpt, Penk,* and *Grp*.[30] Glutamate is mostly absorbed in terminal ileum,[20] whereas these other distally enriched genes are important in colonic motility.[30] Transcription factor *Pou3f3* (*Brn1*) is also higher in the colon than small intestine.[21,23,30,34] This gene is important in central nervous system development,[23] so perhaps it plays a role in colonic ENS development as well. Finally, *Ahr* is highly expressed in colonic neurons,[23] and neuronal *Ahr* integrates microbial cues with colonic motility.[34] For other genes with regional variation their expression may be functionally relevant but is currently unclear. Duodenal neurons enrich for growth factors such as *Fst1* and *Wif1,* whereas distal neurons enrich for *Agrp,*[30] *Ano5, Pde1c, Panrt2*,[34] *Pantr1,* and *Zfhx3*.[23]

Neurotransmitter ligand/receptor expression differs across the colon as well and highlights colonic region-dependent signaling priorities. Somatostatin (*Sst*) signaling may be more prominent in proximal colon. Meanwhile, several pathways are distally enriched, including serotonin (*Htr3a* and *Htr3b*), glutamate (*Gria3* and *Grid1*), ACh (*Chrna7* and *Chrm1*), chromogranin B (*Chgb*), enkephalin (*Penk*), norepinephrine (NE), secretogranin II (*Scg2*), and *Vip*.[20] However, *Htr3a* and *Htr3b* are higher in the duodenum than ileum,[30] suggesting additional roles for these receptors proximally. Particular neuronal subtypes also demonstrate regional colonic distribution. *Calca*$^+$*/Nog*$^+$*/Nmu*$^+$ sensory neurons are more highly prevalent in the proximal colon, whereas *Lgr5*$^+$ inhibitory motor neurons are more common distally.[20]

Enteric glial gene expression also varies between gut locations, including between colonic mucosa and muscularis externa in humans. Mucosal glia more highly expressed ferritin genes (*FTH1* and *FLT*), heat shock protein *CRYAB*, and galectin-1 (*LGALS1*), whereas myenteric glia expressed genes involved in cell adhesion such as *NRXN1* and *CADM2*.[20] These are likely also reflective of known gut physiology. Ferritin helps regulate iron absorption in the mucosa,[35] whereas CRYAB modulates mucosal inflammation and barrier integrity,[36] suggesting mucosal glia participate in these functions. This is not a surprising role for enteric glia because it is important in related peripheral glia such as Schwann cells.[37]

Species-Dependent Expression

Historically enteric neurons from smaller mammals are considered smaller, simpler, and easier to classify than those from larger species such as humans. Perhaps this is in part due to different proportions of neuronal cell types, because these types display varying cell body size and complexity.[4] ScRNA-seq research supports this phenomenon because the proportions of neuronal types differ between species. Both excitatory and inhibitory motor neurons are enriched in humans, whereas all the other types (sensory neurons, interneurons, and secretomotor/vasodilator neurons) are less abundant. However, single cell collection methods have variable efficacy in capturing rare cells or cells with differing morphologies,[38] so to what extent these findings are due to technical limitations is unknown. ScRNA-seq research highlights both conserved functions and complex molecular differences. For instance, development of the ENS is highly conserved. Parallel scRNA-seq of mouse and human neural crest cells identified similar progression of gene expression patterns between both species, suggesting conserved mechanisms of neural fate determination.[39] Specifically, ligand-receptor interactions important for neuronal development are highly conserved between mice and humans.[25] However, hedgehog signaling is subtly different between species, promoting both neuronal and glial differentiation in mice but only neuronal differentiation in humans.[39]

The number of neuronal subtypes based on neurochemical coding is also relatively conserved between species,[20,22] and some co-markers are shared. *Chat*$^+$ neurons in mice and humans express *Galntl6, Tshz2, Alk, Bnc2*,[20] *Rbfox1, Pbx3,* and *Tbx2.*[23] *Nos*$^+$ neurons in both species express *Dgkb*[20] and *Tbx3.*[23] Putative interneurons express *Grm7,* and sensory neurons express *Cbln2*.[20] Interestingly, secretomotor/vasodilator neurons from both species share markers with other neuron types and therefore may require co-expression patterns to identify. These neurons express *Vip, Kcnd2, Etv1,* and *Scgn*.[20]

However, murine and human enteric neuronal expression patterns are more different than similar and may reflect divergent molecular signaling mechanisms. May-Zhang et al[21] estimate that only 40% of neuron-specific genes are conserved between mice and humans, with variations in subtype- and location-dependent expression. This is interesting considering mouse and human gene expressions are considered more similar than different within brain regions.[40] These findings may also be influenced by technical differences. Nonetheless, these suggest differential regulation of feeding and energy within the ENS through the melanocortin, leptin, and serotonin pathways. Human neurons highly express the melanocortin receptor *MC1R,* whereas mouse neurons express its antagonist *Agrp*. Similarly, human neurons highly express leptin receptor *LEPR* and serotonin synthesis enzyme *TPH2.* Murine *Lepr* expression was not detected in scRNA-seq studies, whereas *Tph2* was undetected or detected in only a very small proportion (0.2%) of enteric neurons.[20,21]

Differences in gene expression between mice and humans further complicate discovery of subtype markers as well. Human neurons sampled and sequenced by Drokhlyansky et al[20] did not express *CHAT,* and the authors used expression of the choline transporter *SLC5A7* to mark these neurons instead. They hypothesize the lack of *CHAT* expression is due to their specific methods,[20] which is likely the case because other studies do not report this same

concern in scRNA-seq human data.[23] It is important to note that the strategy of using *Slc5a7* to mark cholinergic neurons would not likely be appropriate in mice because *Slc5a7* may also be expressed by nitrergic neurons[24]; however, *Slc18a3* as used by Morarach et al[22] where *Chat* detection was low could be another alternative. Cell-specific markers for IPANs are unclear and therefore make species comparisons somewhat premature. May-Zhang et al[21] suggest that *Klhl1* may be a species-specific marker of murine IPANs, labeling an entirely different subset of neurons in humans classified as *CALB1*$^+$/*NXPH2*$^+$ Dogiel type III neurons of the small intestine. However, *Klhl1* is expressed by non-IPAN mouse neuronal subtypes as well,[21,22] and *KLHL1*$^+$ human neurons may reflect unidentified neuronal subtype(s) conserved between mice and humans. *Nmu* expression likely reflects true IPANs based on its clear and conserved expression by murine and human IPANs,[20] expression in putative murine IPAN clusters across datasets,[20–24] and is also morphologically verified.[22]

Enteric glial subtypes appear somewhat conserved between humans and mice, where 3 clusters were identified in each species by Drokhlyansky et al.[20] These clusters may correspond to one other but are not explicitly compared. However, human glia demonstrates higher complexity because patient-specific subtypes also clustered, likely reflecting the impact of human genetic variability and disease status on gene expression. Enteric glial expression involving ENS development is mostly conserved between mice and zebrafish, but canonical marker expression differs. McCallum et al[41] found that although some canonical markers such as *Sox10* and *Plp1b* are expressed in zebrafish enteric glia, *Gfap* and *S100b* are not. Additional developmental genes such as *Sox2* and *Foxd3* are conserved between species, further validating the zebrafish as a reasonable organism to study mammalian ENS development.

Sex-Dependent Expression

Many of the established concepts regarding ENS neurochemical coding and physiology relied on data from studies that either did not consider sex as a variable or aimed to remove it as a variable. Current omics studies investigating sex differences also remain relatively limited. However, scRNA-seq studies that did assess sex differences did not observe overt sex-related differences in clustering of enteric neuron subtypes, regardless of age or species.[20,22,23] Although ENS cell clustering is similar between sexes, there are still differentially expressed genes within all or specific clusters.[20,21] Although most of these genes are X- and Y-chromosome related, some are not. May-Zhang et al[21] observed that *SLC6A14* and *MUC5B* are enriched in female human neurons, whereas *Cntnap5a* is higher in putative IPANs (cluster 5), and *Sst* is higher in excitatory motor neurons innervating longitudinal muscle (cluster 0) in female mice. Similarly, robust sex differences have not been observed between glial cell subtypes. It is currently unclear whether these data reflect a true lack of sex differences or are too underpowered to detect subtle sex differences. This would be an important area to address in future studies.

Omics Contribution to Understanding Enteric Nervous System Dysfunction and Disease

High-throughput omics data highlight ENS expression patterns and how they are altered in abnormal states. Here we discuss ENS gene expression in the context of dysmotility, development, communication with immune cells, and dysbiosis. Finally, we link known genetic disease markers with ENS expression. Highlights of these findings are summarized in Table 3.

Dysmotility

Genome-wide association studies and related genetic studies have identified mutations associated with dysmotility in humans. How these mutations contribute to disease risk through gene expression is often unclear. Omics data suggest some of these mutations affect expression of genes involved in cell cycling and differentiation in the ENS. For instance, mutations in DNA repair gene *RAD21* are associated with chronic intestinal pseudo-obstruction. This mutation lowers expression of neuronal differentiation factor *Runx1,* which subsequently reduces enteric neuron numbers and slows intestinal transit in zebrafish.[25,42] Transcription factors *Dlx1* and *Dlx2* are also important for bowel motility, where *Dlx1/2* mutants have decreased *Vip* and increased *Penk* and *Plp1* expression,[43] suggesting Dlx1/2 signaling modulates neuronal subtype populations and peripheral glia. *Sox6* also helps drive neuronal subtype differentiation, and absence of *Sox6*-driven dopaminergic neurons contributes to gastroparesis in mice.[25] This may relate to symptoms of gastroparesis in Parkinson's disease patients, but the connection to diagnosable human disease requires further investigation.

Not surprisingly, omics data also support that altered neurotransmitter and neuromodulator signaling in the ENS contribute to dysmotility. A mutation in subunit gene *GABRG1* of the excitatory ion channel GABA-A is associated with irritable bowel syndrome (IBS) and decreased *GABRG1* expression in IBS patients.[44] Expression of serotonin receptor *HTR2B* decreases in obstructed defecation patients.[45] *Htr2b* is primarily expressed by excitatory motor neuron subtypes[21,22] and in the distal gut.[20,30] These data suggest decreased prevalence or activity of excitatory motor neurons contributes to dysmotility, whereas others suggest roles for inhibitory neurons. GDNF signaling is typically highlighted in neuronal development[46,47] because loss of this signaling during development leads to colonic aganglionosis in mice.[48] However, scRNA-seq highlighted a potential role for GDNF in acute dysmotility during adulthood as well, albeit further testing is warranted to see whether this has functional relevance. Wright et al[23] discovered that in adult mice GDNF receptor α (*Gfra1*) is preferentially expressed by nitrergic neurons and glia and confirmed that GDNF preferentially exerts its effects through *Gfra1*. Furthermore, GDNF signaling enhanced colonic contractility. For other neuropeptides involved in dysmotility the neuron populations affected are unclear. Secretoneurin is involved in gastrointestinal motility[49,50] and expressed by the

majority of interganglionic enteric neurons.[51] Its precursor protein secretogranin II increases in these neurons in response to early life stress[49] and therefore may impact stress-dependent dysmotility.

Development

Genetic and omics studies investigating neuronal development are often in the context of Hirschsprung disease (HSCR). These studies could encompass their own review, and here we focus instead on increased resolution of the enteric neuronal development timeline and where differential expression of developmental genes may disrupt this.

Many important transcription factors and signaling regulators for ENS development were discovered in early omics studies. These include now canonical developmental markers such as *Sox6, Pbx3, Dlx1, Dlx2, Ascl1, Phox2b,* and *Elavl4.*[25,43,50,52,53] *Dlx2* is decreased in aganglionic mouse bowel,[52] and its expression is enriched in neuronal cells compared with non-neuronal cells in murine embryonic gut,[25] further validating *Dlx2* as an important neuronal-specific regulator in development. Interestingly, *Dlx2* is enriched in non-neuronal cells in the zebrafish embryo gut,[50] suggesting interspecies differences in the role of *Dlx2*. However, many other canonical ENS development genes are conserved between mice and zebrafish, including *Phox2b, Elavl3,* and *Elavl4.*[41]

Integrating findings from prior omics and newer scRNA-seq studies expands on prior omics work identifying integral genes in ENS development by further resolving cellular subtypes and time points where gene expression differs. In addition, these studies are performed in both humans and mice and may shed some light on interspecies variability in ENS development. In mice at E12.5, the ENS clusters into glial progenitors, neuronal progenitors, and mixed groups.[54] Recent scRNA-seq studies in humans suggest that neural crest progenitors are present by embryonic week (EW) EW6.5 and have already created the basic architecture of the submucosal and myenteric plexuses by EW8.[55,56] However, correlating the timeline of ENS development between mice and humans is complex and likely contains discrepancies. For instance, Cao et al[57] could map scRNA-seq human enteric glial clusters to murine clusters but could not replicate this in enteric neurons, suggesting

Table 3. Genes and Proteins Involved in ENS Dysfunction and Disease Suggested by Omics

Dysfunction/disease topic	Major finding(s) by omics	Genes/proteins of interest	References
Dysmotility	Genes that affect the number of enteric neurons and modulate neuronal subtype populations	*RAD21, Runx1, Dlx1, Dlx2, Sox6*	26,42,43
	Genes/proteins that affect neuronal excitability/function	*GABRG1, HTR2B, Gfra1,* Secretoneurin	21–23,44,45,49,50
Development	Genes important for ENS development	*Sox6, Pbx3, Dlx1, Dlx2, Ascl1, Phox2b, Elavl4*	26,43,50,52,53
	Genes involved in neuronal differentiation/subtype fate determination	*Sox6, Ascl1, SEMA3A, Etv1, Bnc2*	22,26,55,58
	Genes expressed by glial progenitor clusters	*COL20A1, TFAP2B, GFRA3, ARTN, RXRG*	55,56
Neuroimmune communication	Genes/proteins for ligands or receptors in neuron-immune cell communication	*CX3CL1, CX3CR1,* β2-AR, Arg1, *Oprm1, Cnr2*, IL-12, IL-18	20,60,61,65
	Identified IPAN as neuronal subtype that communicates with ILC2s to modulate immune response	*Nmu*	20–24
	Genes involved in glial-immune communication	*Cxcl10, S100b*	33,71,72
Dysbiosis	Genes/proteins in ENS-gut microbiome communication that modify ENS function	*Ahr,* NGF (host) chaperonin 60, SCFAs (microbes)	34,73–76
	Genes in ENS-gut microbiome communication that affect cell survival/cell death	*Cartpt*, IL18	30,65
	Host genes affected by microbiome composition	*MCT2, GRID2IP*	82
Gastrointestinal disease markers	Known/novel markers for Hirschsprung disease (HSCR) expressed in enteric neurons	*RET, PHOX2B, GFRA1, ECE1,* ARF4, KIF5B, RAB8A	20,83
	Known markers for Parkinson's disease expressed in enteric neurons	*DLG2, SNCA, SCN3, Lrrk2*	20
	Known markers for autism spectrum disorder expressed in enteric neurons and/or enteric glia	*GABRB3, DSCAM, NLGN3, NRXN1, ANK2*	20,85
	Known/novel markers for inflammatory bowel disease (IBD) expressed in enteric neurons and/or enteric glia	*Ptger4, LSAMP, BACH2, NONHSAG044354*	20,70,87,89
	Novel markers for irritable bowel syndrome (IBS)	elastase 3a, cathepsin L, proteasome alpha subunit-4	90

ENS, enteric nervous system; ILC2s, group 2 innate lymphoid cells; IPAN, intrinsic primary afferent neuron.

differences in neuronal cluster development timelines. Regardless of timeline differences, canonical markers of functional neuronal types can be traced across development in both mice and humans.[25,53,58,59] In humans excitatory neurons emerge first, followed by inhibitory neurons at EW14. *TAC1*$^+$ and *VIP*$^+$ neurons continue to differentiate until EW16. Electrical excitability also begins to form at this point with the expression of voltage-gated sodium channel *SCN3A*.[58] In mice *Vip* is apparent by E15.5,[53] and dopaminergic neurons appear later at E18.[25,26] Specific transcription factors help regulate these fates, where in mice *Sox6* and *Ascl1* help drive dopaminergic differentiation,[25] and in humans *SEMA3A* may regulate *TAC1*$^+$/*VIP*$^+$ neuron development.[58]

ScRNA-seq identified a novel early binary split in neuronal development with putative genetic markers that are conserved between humans and mice. In mice this split mapped to the timeline E15.5 to E18, whereas in humans this corresponded to EW6 to EW11. At the binary split one group expresses *Etv1/ETV1* and contains inhibitory motor neurons and select sensory neurons/interneurons, whereas another group expresses *Bnc2/BNC2* and contains excitatory motor neurons and additional sensory neurons/interneurons.[22,55] Although corroboration between 2 scRNA-seq studies is promising, the exact roles of these genes require further study. Mutant phenotypes focusing on these genes could confirm their importance. Regardless, these scRNA-seq studies identified a novel archetype in neuronal development that may be conserved between species. The developmental fate of these binary split clusters was further investigated in mice, where the gene expression patterns of these initial 2 clusters remain into adulthood as *Nos1*$^+$*Npy*$^+$ inhibitory motor neurons and *Ndufa4l2*$^+$ excitatory motor neurons, respectively. Meanwhile, other neuronal types and subtypes down-regulate these markers to diversify into the other characterized enteric neuronal subtypes.[22] The role of these early clusters in adulthood is currently unclear, but because of their shared expression patterns with early life progenitors, these cells may shed additional light on the debated phenomenon of adult neurogenesis.

Human scRNA-seq studies also discuss development of enteric glia. Glial progenitors are detectable at early time points, with 5 glial progenitor clusters identified by one study at EW7–8[56] and 1 progenitor and 1 maintained glial cluster at EW6–11 by another study.[55] In addition to the maintained cluster, 3 additional glial clusters were detected at EW12–17.[55] Interestingly, this maintained cluster appears shared between both datasets and co-expressed *MAL, FGL2, GFRA3,* and *RXRG.*[56] However, one study suggests this cluster represents non-enteric glia originating in the sacrum or trunk due to *TFAP2B* expression,[55] whereas the other study suggests this represents lymphoid associated glia due to expression of immune markers *FGL2, MAL,* and *TGFBR3*.[56] Whether either or both of these classifications are correct requires further investigation.

Neuroimmune Communication

Neuroimmune communication within the gut was initially suggested by innervation surrounding Peyer's patches and immune cells in the lamina propria and immunostaining for neurotransmitter receptors on these immune cells.[3,4] Omics data suggest molecular mechanisms of interaction between specific neuronal types or subtypes and immune cells. For instance, secretomotor/vasodilator neurons may communicate with monocytes via chemokine *CX3CL1* to *CX3CR1*.[20] Adrenergic neurons communicate with muscularis macrophages through β2 adrenergic receptors during bacterial infection and increase expression of protective and wound-healing genes such as Fizz1 (*Retnla*) and *Il10* in these cells.[60] Muscularis macrophages in turn communicate with enteric neurons using arginase 1 to protect them from NLRP6-inflammasome activation and cell death.[61] Together these highlight protective signaling mechanisms in enteric neuroimmune communication.

Neurons may also communicate with immune cells through opioid and cannabinoid receptors, but it is unclear whether these signals would ameliorate or exacerbate inflammation because the impact of opioid and cannabinoid signaling on gut inflammation is complex.[62–64] Neurons could use enkephalins to signal opioid receptor mu 1 (*Oprm1*) on T cells and Dagla to signal cannabinoid receptor 2 (*Cnr2*) on B cells. Inhibitory motor neurons also produce interleukins (ILs) IL12 and IL18, which may interact with T cells.[20] However, these are transcriptional data and require protein-level mechanistic studies to validate and determine the role of these specified communications. In addition, neuronal IL18 regulates antimicrobial activity in goblet cells[65] and therefore may play a similar role in T cells.

ScRNA-seq data have also added resolution to previously known neuroimmune communications. For instance, non-omics data support neuromedin U produced by intestinal neurons communicates with group 2 innate lymphoid cells through their neuromedin U receptor 1 (NMUR1).[66,67] Although these neuromedin U–producing neurons were postulated to be cholinergic with mucosal projections, their functional type was yet unknown. ScRNA-seq clusters confirmed that IPANs express *Nmu* as a specific marker[20–24] and therefore clearly identifies IPANs as the cells communicating with group 2 innate lymphoid cells to modulate immune response. This is a rewarding example of novel scRNA-seq subtypes complementing non-omics studies to understand biomolecular function.

Specific mechanisms of communication between enteric glia and immune cells were recently reported in non-omics work.[68,69] Although one recent omics study did highlight a putative mechanism of glial-immune interaction in mice, other findings mainly highlight general immune response. Progatzky et al[33] identified up-regulation of enteric glial *Cxcl10* as an important mediator of interferon gamma signaling and ultimately inflammatory and granulomatous response to helminth infection. Other studies support glial-immune communication in chemical models of inflammation. In dinitrobenzene sulfonic acid (DNBS) colitis enteric glia up-regulate genes in immune-related pathways including cytokine activity and antigen processing and presentation.[70] Glia treated with lipopolysaccharide + interferon gamma also up-regulate several proinflammatory cytokines, chemokines, and interleukins in cell culture[71] and

rat small intestine.[72] Interestingly, glial *S100b* decreased in both models. Typically S100b release increases inducible nitric oxide synthase expression and nitric oxide production,[71,72] so perhaps this is a compensatory/protective mechanism.

Dysbiosis

Omics research primarily uses 16s rRNA sequencing to correlate altered microbiome diversity with gastrointestinal disease, but here we will focus on host ENS changes. Not surprisingly, the microbiome alters enteric neuronal gene expression in the ileum and colon but not proximal intestinal regions.[30,34] Many genes are regulated by colonic microbes and affect ENS function. For instance, the microbiome impacts colonic motility by up-regulating *Ahr* expression on enteric neurons.[34] Commensal bacteria release extracellular vesicles containing heat shock system proteins such as chaperonin 60, which increase both colonic motor complex amplitude and IPAN activity,[73] suggesting roles for this communication in both motor and afferent intrinsic pathways. Microbial dysbiosis also correlates with afferent signaling in visceral hypersensitivity. Specifically, taxa that produce short-chain fatty acids increase in multiple inflammatory disease models.[74–76] Intrinsic enteric neurons are not considered directly involved in pain transduction pathways,[4] but communication between the ENS and extrinsic sensory neurons can modulate pain perception.[70] Short-chain fatty acids increase expression of enteric glial fibrillary acid protein and nerve growth factor, where nerve growth factor contributes to visceral hypersensitivity.[77] Taken together these data suggest that the gut microbiome and its biomolecular mediators have effects on many aspects of known ENS function.

Microbial dysbiosis likely impacts motor neuron development. Mice that receive antibiotics at PN10 had increased colonic motility corresponding with increased cholinergic neurons and decreased nitrergic neurons, whereas antibiotic-treated 6-week-old or adult mice had the opposite results.[78–80] Although these findings suggest age-dependent relationships between enteric neurons and microbiota, these groups also received different antibiotics, and this may also explain these results. Commensal microbiota also regulate the survival of specific IFANs by preventing inflammasome-dependent cell death.[30] These IFANs express the marker cocaine- and amphetamine-regulated transcript (*Cartpt*) and help regulate blood glucose levels through communication with the liver and pancreas. Conversely, enteric neurons prevent infection by pathogenic bacteria. Neuronal IL18 promotes goblet cell production of antimicrobial peptides and subsequently prevents invasion of the pathogenic species *Salmonella typhimurium*.[65] Together these data highlight signaling mechanisms between enteric neurons and gut bacteria that help regulate homeostasis and prevent infection.

Dysbiosis is also associated with disease pathogenesis and/or disease markers, particularly in IBS. Although taxonomic changes of the microbiome in IBS are subtle, these bacteria alter host serum metabolites,[81] likely reflecting altered bacterial metabolites as well. This metabolic disturbance contributes to enteric neuron dysfunction and dysmotility in IBS. For instance, mice that receive fecal transplants from IBS with diarrhea patients recapitulate decreased colonic transit times despite little change in microbial composition.[81] In addition, short-chain fatty acids can regulate colonic motility through the monocarboxylate transporter 2, where mutations in the gene for monocarboxylate transporter 2 ligand delphilin (*GRID2IP*) confer IBS disease risk.[82] This similarly suggests disturbances in microbial metabolites may be key in IBS dysmotility, although functional studies are required to validate this connection.

ENS Expression of Gastrointestinal Disease Markers

Many previously identified disease markers and risk genes are enriched in enteric neurons compared with other colonic cell types. Note that our previous sections on pathologies may also include genes that could be considered disease markers. However, in those contexts we investigated how omics data highlighted potential functions of these genes in pathogenesis. Here we discuss genes that omics studies specifically identify as putative markers of specific medical diagnoses. Drokhlyansky et al[20] highlighted the genes *RET, PHOX2B, GFRA1,* and *ECE1* as markers of HSCR that are enriched in neurons compared with non-neuronal cells.[20,22,23] The inclusion of both *RET* and *GFRA1* is interesting because both are considered receptors for GDNF, but *Gfra1* signaling was discussed earlier as a means of dysmotility in adult mice,[23] and *RET* is the canonical receptor for GDNF implicated in HSCR.[48] Indeed murine haploinsufficiency of either GDNF or RET mimics intestinal aganglionosis/hypoganglionosis seen in HSCR. This supports the role for GDNF-RET signaling in HSCR, and thus the role of *GFRA1* in this context is still unclear. Perhaps this highlights complex signaling patterns across development through adulthood that require further investigation, where milder or altered perturbations of the same genes lead to different diseases that present at different ages. This complexity is further suggested by findings in obstructed defecation patients where expression of HSCR-related genes (including *RET, PHOX2B,* and *GFRA1*) are down-regulated.[45] However, the functional relevance of this differential expression is unknown. In addition to these HSCR genes involved in neuronal development,[50,52,54] a single recent proteomics study in HSCR patients suggests new markers of disease ARF4, KIF5B, and RAB8A.[83] Decreased expression of these proteins in colons of HSCR patients was also validated with Western blot and immunostaining. These genes are involved in cellular trafficking functions and theorized to be important for neuronal processes development. Validation of these targets could highlight specificities in the pathogenesis of HSCR in addition to serving as novel disease markers.

Meanwhile, ENS expression of Parkinson's disease risk genes suggests neurodegenerative processes may preferentially affect certain neuronal subtypes. Parkinson's

disease risk genes *DLG2, SNCA*, and *SCN3* are enriched across most neuron subtypes in humans, but murine *Lrrk2* is more highly expressed in inhibitory motor neuron and secretomotor/vasodilator neuron subtypes. *Lrrk2* expression in enteric neurons also increases with age.[20] LRRK2 dysfunction in the brain contributes to neuroinflammation and subsequent neuronal death in late-onset Parkinson's disease.[84] Similar mechanisms may occur in the gastrointestinal tract and preferentially target certain neuronal subtypes to produce symptoms. However, whether these are species differences or whether LRRK2 functions similarly in the ENS is unclear.

The effects of autism spectrum disorder risk genes in the ENS may also reflect central nervous system pathology. Enteric neurons enrich for genes expressed in the central nervous system such as GABA receptor *GABRB3* and adhesion molecules *DSCAM* and neuroligin-3 (*NLGN3*).[20,85] The effects of this autism spectrum disorder *NLGN3* mutant in the brain recapitulate in the ENS, where enteric neurons have increased GABA-A sensitivity and subsequently shortened intestinal transit time.[85] Autism spectrum disorder may also involve enteric glial pathology because glia enrich for risk genes *NRXN1* and *ANK2* compared with other intestinal cell types,[20] but the effect of this on gastrointestinal dysfunction in autism spectrum disorder is unknown. *Ank2* is also enriched in a specific glial subtype in mice,[20] and perhaps this glial subtype contributes to disease.

Glia are also implicated in inflammatory bowel disease (IBD). Mutations in the prostaglandin receptor EP4 (*PTGER4*) confer risk in IBD,[86–88] and *Ptger4* is expressed in enteric glia.[70] Enteric glial *Ptger4* decreases with DNBS colitis but increases if the tachykinin receptor NK2R is blocked, indicating that communication between $NK2R^{+}$ enteric neurons and/or extrinsic afferents and enteric glia may play a role. A mutation in *LSAMP* is associated with IBD risk in African Americans.[87] *Lsamp* is also expressed in murine enteric glia and decreases in DNBS colitis.[20,70] Furthermore, *Lsamp* is preferentially enriched in certain glial subtypes.[20] Taken together these data suggest certain enteric glial subtypes may play a role in autism spectrum disorder and IBD, but follow-up investigation is required to validate this.

Novel disease biomarkers are also suggested by omics research and may serve to further classify and identify patients. A mutation in long noncoding RNA NONHSAG044354 is associated with IBD risk and may regulate *BACH2* expression in the transverse colon.[89] *BACH2* is enriched in enteric neurons and most highly expressed in excitatory motor neuron subtypes.[20] Perhaps *BACH2* expression in these neurons is altered by long noncoding RNAs in certain IBD patients, and differential expression of either NONHSAG044354 or BACH2 may confer disease risk. Omics research suggests that IBS patients could be diagnosed by co-expression of biomarkers, because expression of elastase 3a, cathepsin L, and proteasome alpha subunit-4 effectively distinguishes IBS supernatants from healthy controls.[90] Furthermore, elastase 3a from IBS patients activates enteric neurons, suggesting this may not only be a potential biomarker of IBS but also involved in enteric neuronal dysfunction.

Conclusions

ScRNA-seq identifies novel putative markers of ENS cell subtypes, some of which are replicated between studies and suggest distinct neuronal and glial populations (Figure 2 and Table 2, and similarly assessed by Wright et al[23]). With the recent generation of many promising ENS omics datasets it may be surprising that many details of ENS cellular heterogeneity are inconsistent or still unresolved. Although some of this is due to the difficulty of accessing and reporting on the data generated, some of these discrepancies are due to experimental differences between datasets. For instance, differing numbers of cells collected in scRNA-seq studies affect the resolution of clusters and differentially expressed genes. In addition, these samples span across different locations of the gut, ages, species, and cellular material sequenced (ie, isolated nuclei vs whole cells).[20–24] Regardless, these data still expand our understanding of ENS cellular heterogeneity and highlight further complexity in enteric neuron and glial classification. Meanwhile, other omics methods supplement these findings by elucidating ENS cell type- and subtype-specific roles in physiology and disease. Together these findings demonstrate the ability of omics data to identify novel molecules and pathways in the ENS.

Current omics methods can generate data in a relatively unbiased manner. The combination of this potential for novelty with the ever-increasing power and sensitivity of omics technology makes omics an ideal methodology for exploring complex and multi-modal questions. These characteristics also make omics data exponential hypothesis-generating tools that will promote scientific advancement for years beyond their creation. This is demonstrated by the sheer number of downloads, citations, and re-analyses of recent omics publications.[91]

However, some of these same characteristics also present current challenges in omics methods. Because of the sheer amount of data created in any given experiment, it can be difficult to synergize and make overall sense of collective findings. This is especially the case when accessing data through publications as opposed to datasets. In some cases it may be better to access the data directly, but this can prove challenging without bioinformatics expertise. Fortunately, some dataset creators have also supplied companion websites to access their data, and this helps mitigate this concern. Some notable examples include Zeisel et al[24] (http://mousebrain.org/), Progatzky et al[33] (https://biologic.crick.ac.uk/ENS/EGCinflammation), Fawkner-Corbett et al[56] (https://simmonslab.shinyapps.io/FetalAtlasDataPortal/), Elmentaite et al[55] (https://www.gutcellatlas.org/), and Cao et al[57] (https://descartes.brotmanbaty.org/). There are additional databases that streamline access to multiple omics studies, such as the Single Cell Portal by the Broad Institute.[92] Although these are all important and promising works that increase the accessibility of omics results, an ideal database would collectively represent all works in a singular, consistent space and allow relatively easier comparison between

datasets. By implementing this the omics community would ideally also provide standardized means of accessing, searching, and representing omics data. We realize this is a difficult task but hope that as omics data continue to grow, means of improving accessibility will continue to improve in tandem. These could also include new meta-analyses of previously published datasets.

Another challenge in omics is that it easily generates comparative data but requires specific experimental design to provide causative data. This historically made understanding expressional changes in the ENS challenging because it meant correlating tissue-level differential expression with genes known to have ENS expression without knowing whether this gene expression change occurred in ENS cells or whether ENS cells even expressed said gene in the first place. Thankfully newer omics technologies allow ENS-specific sequencing, where techniques such as scRNA-seq clearly demonstrate their ability to identify enteric neurons and glia out of many other cell types. As these modalities continue to grow, future research may sequence live cells in situ[93] and/or integrate single cell techniques for multiple biomolecules together to provide a better mechanistic picture.[1] Future research may also resolve whether some ENS cell clusters are distinct cell populations or temporal expression patterns responding to the current microenvironment. Because of the rate that omics technology improves and ENS omics research is recently published, it is only a matter of time until our understanding of ENS genetic architecture delves deeper once again.

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