

# Exploiting unique features of the gut-brain interface to combat gastrointestinal cancer

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**The gastrointestinal tract comprises a complex ecosystem with extensive opportunities for functional interactions between neoplastic epithelial cells and stromal, immune, neuronal, glial, and other cell types, as well as microorganisms and metabolites within the gut lumen. In this Review, we focus on interactions between gastrointestinal cancers and elements of the central and enteric nervous systems. This previously understudied but rapidly emerging area of investigation has blossomed in recent years, particularly with respect to improved understanding of neural contributions to the development and progression of esophageal, gastric, pancreatic, and colon neoplasia. Cancer neuroscience offers great promise to advance our understanding of how neural-cancer interactions promote alimentary tract neoplasia. The resulting mechanistic insights can be leveraged to identify diagnostic and prognostic biomarkers, and to develop novel therapeutic interventions.**

## Introduction

Advanced cancers of the gastrointestinal (GI) tract are highly resistant to chemotherapy, radiotherapy, and biologicals (1). Even new immunotherapies benefit only a subset of patients with colorectal cancer (CRC) (2). Thus, CRC and gastric cancer remain, respectively, the second and third leading causes of global cancer mortality (3, 4). Although CRC incidence and mortality decreased substantially in the United States over the past 30 years, increasing incidence and mortality in persons younger than 50 years are concerning (5, 6). Moreover, the COVID-19 pandemic adversely impacted cancer screening, thereby upstaging newly diagnosed lesions (7–9). In the United States, the incidence of esophageal and pancreatic ductal adenocarcinomas, both commonly diagnosed at advanced stages, is increasing; esophageal adenocarcinoma and pancreatic ductal adenocarcinoma (PDAC), respectively, cause more than 16,000 and 47,000 deaths yearly (5, 10). Five-year survival rates for advanced esophageal, gastric, pancreatic, and colorectal cancers are all less than 20% (5, 11). Clearly, developing more effective ways to detect and manage these cancers is a high priority — gut-brain interactions impacting GI cancer development and progression provide a largely untapped reservoir of novel diagnostic, prognostic, and therapeutic opportunities.

Abundant evidence implicates the central nervous system (CNS) in GI cancer progression. Chronic behavioral stress is linked to increased cancer risk by mechanisms involving neuroendocrine

signaling (12). In preclinical models, stress-induced adrenergic signaling promotes PDAC progression (13, 14), in part by inducing matrix metalloproteinases (MMPs). MMPs degrade extracellular matrix, facilitating tumor expansion and metastasis (15); these effects are attenuated by  $\beta$ -adrenergic blockade (16). In murine and human studies, pharmacological inhibition of  $\beta$ -adrenergic signaling and chemical denervation of the pancreas improve chemotherapeutic efficacy (17).

Recent attention focused on cancer cell heterogeneity and the role of the tumor microenvironment in modulating tumor growth, invasion, and dissemination. Nonetheless, the spotlight has shone primarily on cancer, stromal, and immune cells (18), with less attention paid to neurons and glial cells (19). While the CNS can modulate disease, the GI tract possesses a unique intrinsic nervous system, the enteric nervous system (ENS), sometimes called the “second brain” or “little brain,” which, alone or in coordination with the CNS, modulates the diverse functions of the gut in health and disease (20). ENS neurons and glial cells are anatomically poised to transmit information multidirectionally to normal and neoplastic GI mucosal cells, stromal cells, immunocytes, and the brain. These complex interactions are further complicated by input from enteroendocrine cells sprinkled throughout the GI tract and by the gut microbiome. GI cancers profit from a landscape uniquely combining neuronal postsynaptic, endocrine, and paracrine signaling with diverse cell-cell contacts and access to key metabolites (Figure 1 and ref. 21).

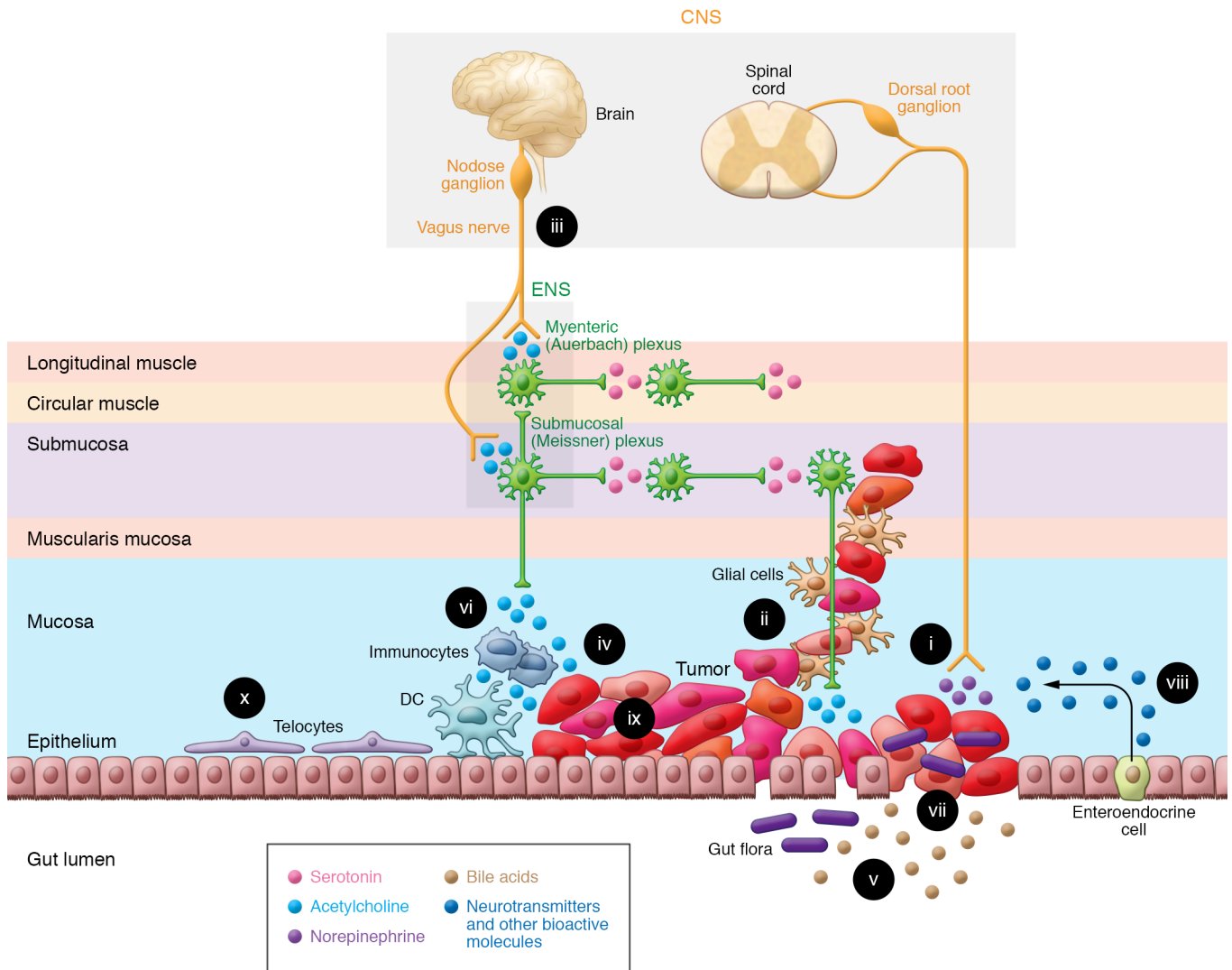
In this Review, we analyze published findings, experimental models, and approaches used to uncover the mechanisms whereby the gut-brain axis modulates GI cancer development and progression. We consider how neoplastic cells advance their survival and progression by hijacking neurotransmitters, growth factors, signaling molecules, and metabolites that normally maintain tissue homeostasis and repair. In so doing, we identify potential therapeutic targets and highlight unresolved questions that can direct

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**Figure 1. The GI neuron-cancer interface.** The ability of the gut-brain axis to modulate GI cancer progression is enhanced by the proximity and multidirectional crosstalk between numerous elements; these complex interactions provide opportunities for therapeutic intervention. (i) Cancer cells release nerve growth factors that promote neuronal tropism toward the tumor, enhancing access to neurotransmitters, metabolites, and the neural scaffold. Advanced cancer stages correlate with increased neural density. (ii) Perineural invasion, associated with worse outcomes, provides a path for tumor spread, access to neurotransmitters, and shielding from immune attack. (iii) Vagal innervation stimulates cancer progression by muscarinic mechanisms and modulates immune function. (iv) Neurotransmitters, like acetylcholine, produced and released by neurons, cancer cells, immunocytes, and possibly gut bacteria stimulate tumor growth, invasion, and dissemination. (v) Fecal bile acids, modified by gut bacteria, modulate immune and cancer cell function by several mechanisms, including activation of cancer cell muscarinic receptors. (vi) Immunocyte function is modulated by neurotransmitters released from the ENS, and cancer, immune, and enteroendocrine cells. (vii) Disruption of the intestinal barrier in the cancer field permits translocation of microorganisms that modulate immune and neural function. (viii) In response to bacterial and neural input, enteroendocrine cells, sprinkled throughout the mucosa, release neurotransmitters and other bioactive molecules. (ix) Cancer cells display intratumor heterogeneity and overexpress receptors for neurotransmitters and bioactive molecules. (x) Subepithelial telocytes are a critical source of pro-proliferative signaling for the intestinal stem cell niche; despite their prominent location, a functional role for telocytes at the neuron-cancer interface remains to be established.

future research. Focusing on cancers of the esophagus, stomach, pancreas, and colon, we leave in-depth analysis of the anatomical gut-brain interface and the role of the gut microbiome to other contributors to this Review series.

### The GI neuron-cancer interface

There is growing interest in understanding the role neurons play in the genesis and growth of non-CNS cancers, particularly with respect to cancers of the GI tract (21, 22). Nonetheless, many inter-

actions between cancers and the nervous system are highly context-dependent. Thus, differences in the innervation of the esophagus, stomach, pancreas, and colon influence crosstalk between neuronal, glial, and cancer cells. The unique proximity of intestinal cancers to the gut microbiome and other fecal contents, and the specialized gut immune system, add layers of complexity. Appreciating the prominence of this tumor microenvironment generated interest in “ecological therapy,” wherein cells that nourish cancer cells are targeted to retard cancer growth (23).

**Table 1. Key features of neuron–cancer cell interactions shared by GI cancers**

Features	GI cancer				References
	Esophageal	Gastric	Pancreatic	Colon	
Neurotrophins expressed by cancer cells	✓	✓	✓	✓	17, 30, 32, 33, 113, 116, 119
Perineural invasion by cancer cells	✓	✓	✓	✓	33, 40–47, 113–120, 130, 131
Vagal innervation affects cancer progression	✓	✓	✓	?	37, 38, 50, 51, 112
Cancers produce and release neurotransmitters	?	✓	?	✓	61–66
Cancers overexpress neurotransmitter receptors	?	✓	✓	✓	50, 60–64, 68, 137
Nerves alter immune function in cancer microenvironment	?	?	✓	✓	22, 38, 84–92
Neurons provide metabolites to cancers	?	?	✓	?	109

✓, feature reported; ?, presence of feature not reported.

Anatomical features facilitate interactions between neurons, immunocytes, gut microorganisms, and other constituents of this complex ecosystem, acting in concert to modulate GI cancer cell proliferation, survival, and invasion (Figure 1). The ganglia of the ENS are concentrated in myenteric (Auerbach) plexuses spanning the entire length of the GI tract, and submucosal (Meissner) plexuses in the small and large intestines (20, 22). Enteric glial cells are positioned in the muscularis propria and mucosa, especially at the base of normal intestinal crypts (24, 25). Beyond providing support for neurons, enteric glial cells, which outnumber neurons, participate actively in a variety of ENS functions, including those vital for neuron maintenance and survival (24). Like neurons, enteric glia express neurotransmitter receptors and transporters, and respond to neurotransmitters, largely by changes in intracellular calcium that modulate cell function (26). Enteric neurons and glia are classified by their roles in regulating cellular architecture, neurotransmitter release, receptor activation, electrophysiological activity, and other functional characteristics; single-cell sequencing may modify classification based on molecular or genetic features (27). Figure 1 illustrates the broad framework of neural–GI cancer interactions in the context of the colon cancer microenvironment — features shared by cancers of the esophagus, stomach, and pancreas (Table 1). Figure 2 zooms in on key interactions between GI cancer cells and the gut neural/glial cell network.

**Nerve growth factors.** Nerve growth factors, or neurotrophins, comprise a highly homologous family of precursor proteins cleaved to active peptides including nerve growth factor (NGF) (28), brain-derived neurotrophic factor (BDNF), glial cell line–derived neurotrophic factor (GDNF), and neurotrophin-3 and neurotrophin-4 (NT-3 and NT-4) (Table 2 and refs. 29, 30). These proteins stimulate nerve development and survival through diverse signaling mechanisms; for example, binding of NGF to tropomyosin tyrosine receptor kinase fusion proteins stimulates receptor homodimerization, autophosphorylation of the tyrosine kinase domain, and activation of PI3K, Ras, phospholipase C (PLC), and other downstream effectors (31).

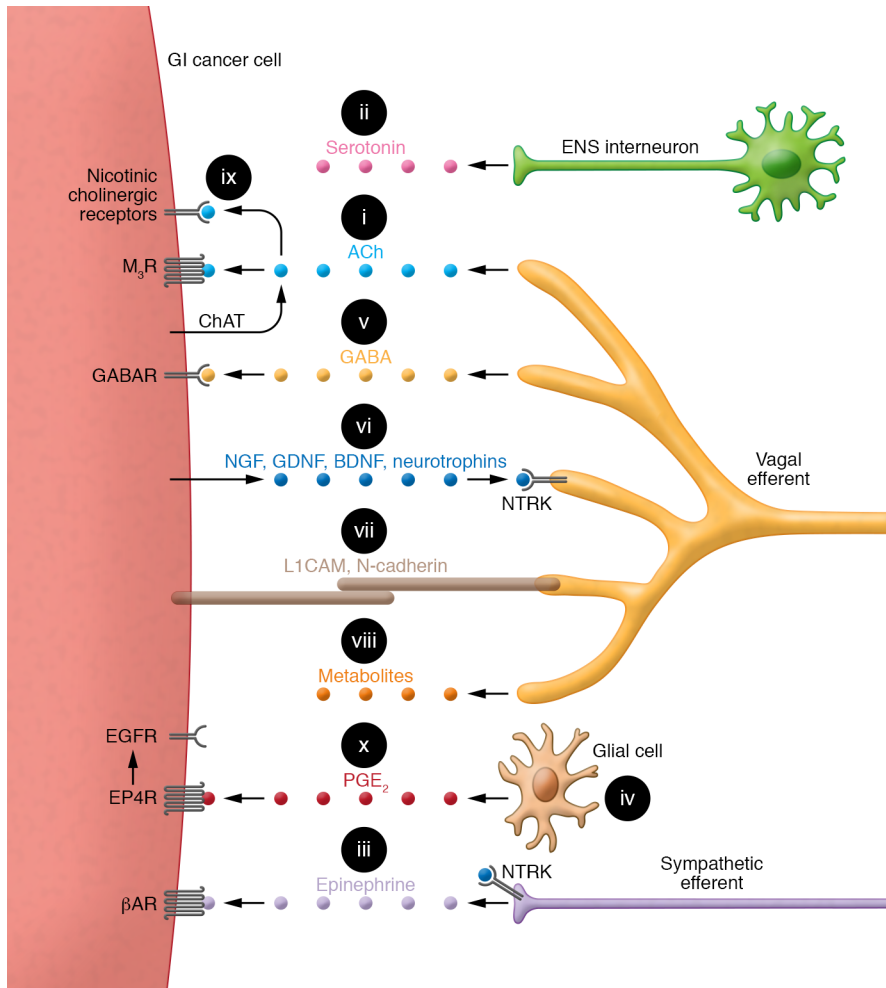
Early-stage cancers release neurotrophins that stimulate local neuronal growth and increased nerve density, features correlated with more aggressive cancers (30, 32). These effects are bidirectional; cancers release neurotrophins that encourage neurogenesis, axonogenesis, and neural migration, while neurons and glial cells release neurotransmitters that stimulate tumor growth and invasion (Figure 2). This was studied extensively in the genesis of PDAC,

where release of neurotrophic growth factors (e.g., NGF) by neoplastic cells and expression of their receptors on neurons correlates with nerve density and tumor aggressiveness (33), a mechanism replicated by NGF overexpression in murine PDAC models (17). In a transgenic mouse PDAC model, surgical denervation of celiac and superior mesenteric ganglia enhanced chemotherapeutic efficacy, supporting the importance of CNS input (17). The complexity and specificity of nerve–cancer interactions is highlighted by murine PDAC models wherein chemical denervation of the pancreas attenuates pancreatic intraepithelial neoplasia and progression (34–36), but surgical vagus nerve transection has opposite actions (37, 38).

**Perineural invasion and neural scaffold.** Although definitions vary, perineural invasion (PNI) is commonly defined as cancer invasion into any of the three layers of the nerve sheath, or cancer surrounding at least 33% of the neural circumference (39, 40); PNI impacts tumor growth, progression, and responses to therapy. PNI advances cancer progression by facilitating biochemical and physical interactions between neural, glial, and neoplastic cells that promote neural and cancer cell proliferation and stimulate cancers to spread along neural planes. Although PNI is associated with worse clinical outcomes for any GI cancer studied (41–44), regional factors such as neural density may selectively augment the importance of PNI for some cancers versus others (40).

The precise molecular mechanisms underlying PNI are uncertain (40), although the release of nerve growth factors and cytokines from cancer and immune cells into the tumor microenvironment is important (45). As illustrated in Figure 1, neurons in the cancer microenvironment can provide a physical scaffold for GI cancer invasion and metastasis (40, 46); cancer cell nests are reported near the myenteric plexus (47). Adherence to and migration along enteric neurons are facilitated by cancer cell expression of key surface molecules (e.g., L1CAM and N-cadherin) (Figure 2 and ref. 46). Notably, retrograde traffic along ENS neurons may include gut bacteria (48), reflecting a perineural cancer scaffold that provides a hub for crosstalk between cancer cells, neurons, glial cells, immunocytes, and translocated microorganisms. Few, if any, experimental models reflect the multitude of interactions between cell types in this complex tumor microenvironment. No treatments currently target PNI.

**Vagal innervation.** Although highly context-dependent, vagal innervation is the most prominent way the CNS regulates GI neoplasia. Surgical interruption of the vagus (vagotomy) appears to



**Figure 2. Key interactions between a generic GI cancer cell and the neural/glial cell network in the tumor microenvironment.** (i) Acetylcholine (ACh) production, mediated by choline acetyltransferase (ChAT), stimulates tumor growth, invasion, and dissemination. Relative quantities of ACh production by cancer cells versus neurons are uncertain. (ii) Serotonin released from ENS interneurons may stimulate colon cancer growth by currently obscure mechanisms. (iii) Epinephrine released from sympathetic neurons stimulates the progression of GI cancer cells overexpressing  $\alpha$ - and  $\beta$ -adrenergic receptors ( $\beta$ AR). (iv) In response to neurotransmitters, glial cells play a major role in modulating and supporting the neuron–cancer cell interface. Glial cells also produce and release tumor growth factors into the tumor microenvironment. (v) GABA stimulates cancer cell proliferation via overexpressed GABA receptors (GABAR). (vi) Nerve growth factors (NGF, GDNF, BDNF, neurotrophins) released from cancer cells interact with neuronal receptors (e.g., NTRK) to promote axonal growth and tropism toward the tumor. (vii) Cancer cells express surface molecules, L1 cell adhesion molecule (L1CAM) and N-cadherin, facilitating adherence and migration along enteric neurons. Homophilic interactions allow L1CAM on cancer cells to adhere to L1CAM expressed on neurons. (viii) Neurons release key metabolites (e.g., serine) into the tumor microenvironment or reprogram cancer cell metabolic pathways. (ix) ACh interaction with nicotinic cholinergic receptors expressed on PDAC stimulates tumor progression. (x) Bidirectional interactions between glial and GI cancer cells involve cancer cell–derived interleukins that stimulate prostaglandin  $E_2$  ( $PGE_2$ ) biosynthesis and paracrine release by enteric glia.  $PGE_2$  stimulates tumor expansion via EP4 receptor–mediated (EP4R-mediated) transactivation of EGFR.

reduce gastric cancer risk (49, 50); gastric neuronal density and cancer stage are correlated (50). In mice, surgical or pharmacological hemivagotomy attenuates proneoplastic Wnt signaling and reduces gastric tumor formation in the denervated stomach (50, 51). Notably, opposite effects are observed in murine PDAC models wherein vagotomy promotes neoplasia (38). As discussed below, this conundrum may be explained by differential expres-

sion of  $M_1$  and  $M_3$  muscarinic receptors ( $M_1R$  and  $M_3R$ ) with conflicting actions on cancer progression. Lastly, a liver-brain-gut neural arc identified by retrograde tracing of hepatic vagal branches may modulate immune responses to CRC (52).

*Neurotransmitters and their receptors on cancer cells.* For decades, traditional neurotransmitters, like acetylcholine (ACh), were considered to derive primarily if not uniquely from neurons. Over the past 15 years, growing interest has focused on non-neuronal neurotransmitter production and release from cancer and immune cells in the tumor microenvironment (53–55) and bacteria in the gut microbiome (56–59). In this regard, most work pertains to effects of ACh on muscarinic receptors expressed by GI cancers (Figure 3 and refs. 60, 61). Human gastric (62, 63) and colon (64) cancer cells express choline acetyltransferase (ChAT) and synthesize and release ACh. Normal pancreatic stellate cells produce ACh (65) and pancreatic cancer cells express choline transporters (66), a surrogate marker of ACh production. Yet, to our knowledge, ACh production by PDAC has not been proved. Cancer cell types that release ACh commonly overexpress  $M_3R$  (64);  $M_3R$  expression correlates with gastric cancer stage and metastasis (62–64). This, and the relatively low concentrations of ACh released by cancer cells, suggest that non-neuronal release of neurotransmitters by cancer, tuft (67), immune, and other cells in the tumor microenvironment modulates cell function by autocrine and paracrine actions. Consistent with these observations,  $M_3R$  deficiency in murine CRC models attenuates neoplasia (68, 69).

$\alpha_{2A}$ -Adrenergic receptor activation in normal gut epithelial cells may stimulate EGFR transactivation and downstream MEK/ERK signaling, which enhances cell migration and wound healing (70, 71). Although adrenergic receptor activation was implicated in PDAC progression, compared with muscarinic neurotransmitters, the role of adrenergic receptor agonists (e.g., epinephrine) in modulating GI cancer growth and progression remains relatively unexplored (72). Gauging the importance of neurotransmitter release from neu-

rons and cancer, immune, enteroendocrine, and other cells in the tumor microenvironment is limited by the challenges of accurate spatial and temporal measurement of very low neurotransmitter concentrations. Moreover, when evaluating neurotransmitter effects in vitro, it is crucial to discriminate physiological from pharmacological (i.e., supraphysiological) neurotransmitter concentrations that may lack disease relevance.



**Table 2. Neurotrophins implicated in GI cancer progression**

Factors	Receptors	GI cancers	Actions	Potential clinical applications	References
NGF	Tropomyosin receptor kinase A (TrkA), p75 neurotrophin receptor (p75NTR)	Esophageal, gastric, pancreatic, and colon cancers	NGF binding to TrkA promotes catecholamine-induced axonogenesis, angiogenesis via VEGF expression, cell proliferation and differentiation via PI3K/Akt and Ras/MAPK, gastric tumorigenesis via ACh/M <sub>3</sub> R/YAP, and PDAC invasion via MAPK-induced overexpression of MMP2. In contrast, NGF binding to p75NTR is proapoptotic.	NGF interaction with p75NTR potentiates antiproliferative effects of 5-FU; by attenuating TrkA signaling, anti-NGF antibodies (e.g., tanezumab) may improve cancer-induced bone pain; Trk inhibitors and anti-NGF antibodies may be effective against gastric adenocarcinomas and PDAC; pan-Trk inhibitors (e.g., larotrectinib, entrectinib) may be effective against cancers expressing Trk variants.	17, 28, 30–33, 105, 109, 113, 116, 125
BDNF	TrkB, p75NTR	Esophageal, gastric, pancreatic, and colon cancers	BDNF binding to TrkB promotes axonogenesis and cancer cell proliferation, differentiation, migration, and invasion, and regulates VEGF/HO-1 expression.	BDNF/TrkB knockdown promotes apoptosis and inhibits CRC growth; BDNF expression is associated with bone metastases; in CRC, BDNF/TrkB signaling contributes to cetuximab resistance; Trk inhibitors may have efficacy against GI cancers.	17, 31, 33, 105, 113, 127
GDNF	RET	Pancreatic and colon cancers	GDNF promotes PDAC invasion via PI3K/Akt- and MEK/ERK-mediated overexpression of MMP9; GDNF drives PDAC chemotaxis in perineural invasion. In contrast, RET appears to be a CRC tumor suppressor.	GDNF/RET-mediated perineural invasion contributes to PDAC-associated pain; RET-selective tyrosine kinase inhibitors (e.g., pralsetinib, selipratinib) for cancers expressing RET variants are in phase I/II clinical trials.	31, 124, 133
NT-3	TrkC, p75NTR	PDAC	NT-3 activation of TrkC, a tumor suppressor, is antiapoptotic; reduced NT-3/TrkC axis activity correlates with CRC progression.	Trk inhibitors may be effective against PDAC.	31, 105, 113, 127
NT-4	TrkB, p75NTR	Pancreatic and colon cancers	NT-4 downregulates cancer cell autophagy via Atg5/MAPK; in CRC, NT-4 promotes epithelial-mesenchymal transition and cell proliferation, migration, and invasion.	NT-4 knockdown promotes autophagy and inhibits CRC growth; Trk inhibitors may be effective against PDAC.	31, 105, 159

ACh, acetylcholine; Atg5, autophagy-associated gene 5; BDNF, brain-derived neurotrophic factor; 5-FU, 5-fluorouracil; GDNF, glial cell line-derived neurotrophic factor; HO-1, heme oxygenase-1; M<sub>3</sub>R, M<sub>3</sub> muscarinic acetylcholine receptor; NGF, nerve growth factor; NT, neurotrophin; YAP, yes-associated protein.

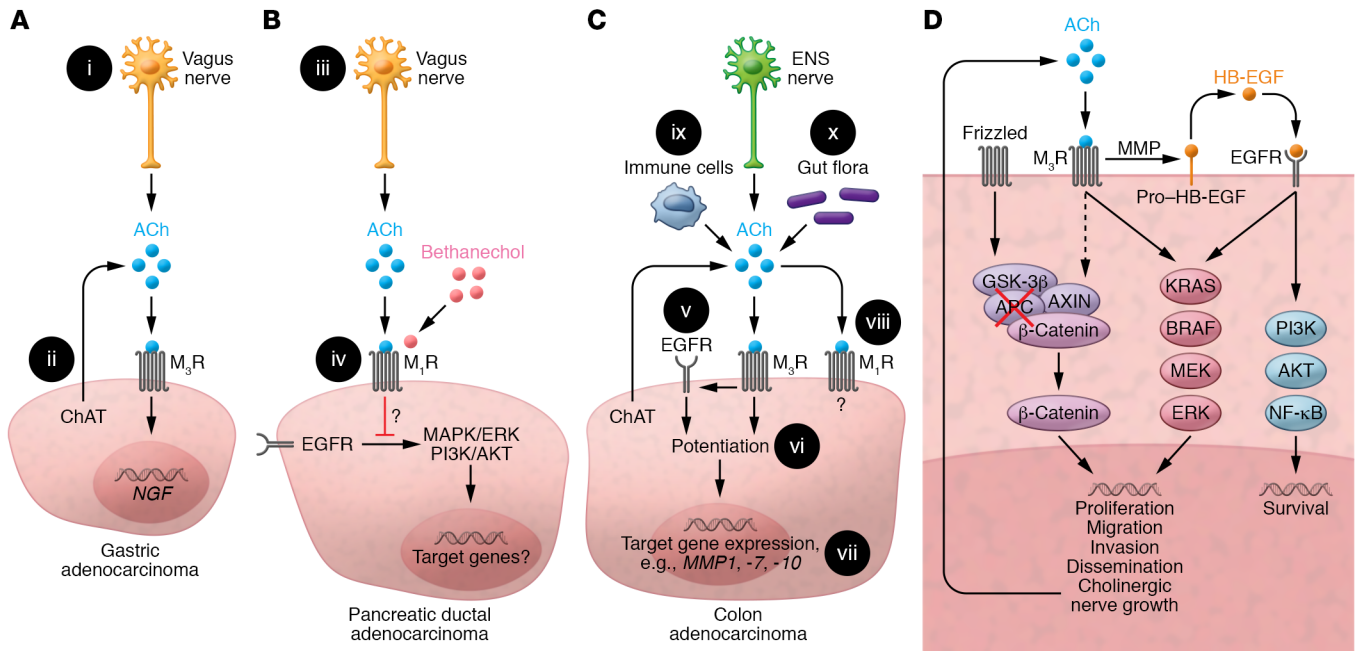
## The GI cancer microenvironment

**Bile acids.** Bile acids (BAs), produced in the liver, excreted into the intestinal lumen, and modified by bacteria in the gut microbiome, are recycled via enterohepatic circulation (73). BAs modulate the function of normal (74) and neoplastic (75) GI epithelial cells by interacting with Takeda G-coupled receptor 5 (TGR5; GPBAR1) and M<sub>3</sub> muscarinic GPCRs (Figure 3 and refs. 75, 76). Long associated with CRC risk, BAs have pleiotropic effects including gut immune modulation (77) and functional interactions with muscarinic receptors (78) overexpressed in CRC (79). These functional interactions mimic those of cholinergic neurotransmitters (e.g., ACh) and, among other actions, result in transactivation of EGFR and signal transduction that stimulates cancer cell proliferation, survival, and invasion (80, 81). Interestingly, chenodeoxycholic acid also inhibits the pro-oncogenic effects of *Bacteroides fragilis* toxin (82).

**Gut immunocytes.** By modulating lymphatic traffic, egress from lymph nodes, and T cell activation, CNS adrenergic nerve fibers suppress immune activity in highly innervated GI organs like the stomach and pancreas (83–85). This may limit immune surveillance and checkpoint inhibitor efficacy (86, 87); surgical or chemical denervation may improve the efficacy of immunotherapy (83, 87). Neurons in the ENS also regulate the activity of enteric immunocytes (88–90) that synthesize and release non-neuronal ACh in the cancer microenvironment (Figure 3 and refs. 91, 92). Macrophages in the endoneurium release cytokines that facilitate PNI by attracting tumor cells to neurons (45). A recently identified neural arc connecting the brain and gut via the liver may modulate immune responses to GI cancers by a mechanism involving ACh neurotransmission (52).

**Gut microbiome.** Intestinal barrier disruption encourages transmural infiltration of bacteria and fungi comprising the gut microbiome (93). Cancers arising from GI epithelial cells at the host–gut microbiome interface break the single-layer barrier formed by tight junctions between normal epithelial cells (Figure 1 and refs. 94, 95). Dysbiosis resulting from “barrier-breaking” effects of cancer can activate multiple signaling systems (96, 97). For example, NF-κB and STAT3 pathways regulate the function of regional immune cells and neurons (98). Tumors hijack these developmental, wound healing, and antiinflammatory signaling programs to foster their own progression. Some bacterial metabolites, e.g., ACh and BAs, are GPCR agonists that can alter both neuron and cancer cell function (Figure 3 and refs. 99, 100). Mucosal microbial biofilms from humans with CRC are carcinogenic in murine models (101), and some bacterial products (e.g., *B. fragilis* toxin) contribute to barrier-breaking effects of cancers (102).

**Enteroendocrine cells.** Sprinkled throughout the epithelial lining of the GI tract (Figure 1), enteroendocrine cells under neuronal, hormonal, and paracrine control synthesize and release a variety of neurotransmitters. Paracrine signaling by enteroendocrine cell-derived serotonin modulates the activity of neurons, immunocytes, and cancer cells (103, 104). Whether serotonin release from enteroendocrine cells or neurons in the ENS advances or retards GI cancer progression remains uncertain; this is likely context- and concentration-dependent (Figure 2 and ref. 105). Similar to pulmonary epithelial neuroendocrine cells that produce and release ACh, which stimulates small cell lung cancer progression (106), neuroendocrine tumors such as pheochromocytomas may release neurotransmitters and growth factors that enhance GI cancer progression (107, 108).



**Figure 3. Muscarinic receptor activation in GI cancer.** (A) Gastric adenocarcinoma. (i) ACh release from vagal efferents activates  $M_3$  muscarinic receptors ( $M_3R$ ); vagotomy attenuates neoplasia. (ii) Cancer cells express ChAT, key for non-neuronal ACh synthesis; resulting ACh levels and their autocrine and paracrine impact on tumor progression remain uncertain.  $M_3R$  activation induces nerve growth factor (NGF) expression. (B) PDAC. (iii) Treating mice with bethanechol, a non-subtype-selective muscarinic receptor agonist, activates muscarinic receptors. (iv)  $M_1R$  activation attenuates PDAC progression by undefined mechanisms involving repressed EGFR signaling. (C) CRC. (v)  $M_3R$  signaling transactivates EGFR; this is mediated by MMP7-mediated release of HB-EGF, an EGFR ligand. (vi) Concurrent activation of  $M_3R$  and EGFR potentiates target gene expression. (vii)  $M_3R$  activation selectively induces *MMP1*, *MMP7*, and *MMP10* expression. *MMP1* and *MMP7* facilitate cell invasion. *MMP7* also catalyzes the release of EGFR ligands (e.g., HB-EGF). (viii)  $M_1R$  expression and activation attenuate colon cancer progression by unknown mechanisms. (ix) Immuncytes and (x) gut flora provide additional sources of non-neuronal ACh. (D) Post- $M_3R$  signaling alters gene expression and cancer cell function by impacting various signaling pathways. APC and/or  $\beta$ -catenin gene mutations free  $\beta$ -catenin from proteasomal destruction, promoting transcription of  $\beta$ -catenin target genes.  $M_3R$  activation transactivates EGFR and augments  $\beta$ -catenin signaling. Resulting changes in downstream gene transcription stimulate cancer cell proliferation, survival, migration, invasion, and dissemination. Notably, induction of neurotrophin expression can promote neural growth and tropism, a feedback loop providing additional access to ACh and other neurotransmitters.

**Metabolites.** Cancer cells have high metabolic requirements and limited blood supply. Intriguing work suggests neuronal axons can supply scarce amino acids and nutrients to GI cancer cells in this nutrient-depleted tumor microenvironment (109, 110). Banh and colleagues found that serine deprivation impaired PDAC growth but permitted the selective translation and secretion of NGF to increase neural density and provide PDAC cells with access to axon-derived serine (109). Rabben et al. found that gastric cancers are glutamine-dependent; vagotomy induced a metabolic switch from glutaminolysis to oxidative phosphorylation and glycolysis (Warburg effect) (110). These studies uncover the provision of neuron-derived metabolites and neuron-induced metabolic reprogramming of GI cancer cells as potential therapeutic targets (Figure 2).

### Interactions of specific GI cancers with gut neural networks

Organ-specific features of GI cancer–nervous system interactions are reported for the esophagus, stomach, pancreas, and colon. In most cases, distinguishing between generic and truly organ-specific features awaits further clarification.

**Esophageal cancer.** Esophageal cancers are composed of squamous cell carcinomas and adenocarcinomas; the impact of the gut-brain axis on esophageal cancer is evidenced by the surge in adenocarcinomas, once uncommon and now the most fre-

quent form of esophageal cancer in developed nations (111). Gastroesophageal reflux, due primarily to poorly understood defects in CNS (vagal parasympathetic and spinal sympathetic) and ENS control of lower esophageal sphincter pressure and esophageal motility, predisposes to preneoplastic Barrett’s epithelium and esophageal adenocarcinoma (112). Thus, although the impact of neurons and glia on esophageal cancer progression is less clear than their impact on progression of other GI cancers (Table 1), there is compelling evidence that the nervous system plays a central role in the development of esophageal adenocarcinomas.

Among other mechanisms, neural innervation promotes esophageal tumor progression via neurotrophins and their receptors (Table 2) (45, 113–115). Nerve bundles and neuropeptide-immunoreactive nerve fibers expressing neurotrophic receptor kinase 1 (NTRK1, also called TrkA), an NGF-binding receptor, are commonly observed in esophageal cancers that overexpress NGF (113, 116); an esophageal cancer subtype expresses high levels of Trk-T1 neurotrophin receptor mRNA (114). Low-affinity p75 neurotrophin receptors (p75NTR), expressed in the stem cell population of normal esophageal epithelial cells, were detected in approximately half of 187 esophageal squamous cell carcinomas (113). RNAi knockdown of p75NTR expression in esophageal squamous cancer cells inhibited proliferation and induced apoptosis (117). Notably, NTRK gene fusions involving

**Table 3. Advantages and limitations of experimental models to assess GI nerve-cancer interaction**

Categories	Examples	Advantages	Limitations	References
<b>In vitro</b>				
GI cancer, glial, and neuronal cell cultures	Human and murine GI cancer cell lines; cancer cell coculture with glial and neuronal cells	Reductionist models permit selective parsing and precise manipulation of molecular and genetic pathways; coculture permits studies of cell-cell interactions	Small repertoire of commonly used cell types; use of multiple cell lines may not validate the importance of findings; multiple passages may damage DNA; coculture excludes physical connections and other factors at the GI cancer-neural interface; using supraphysiological concentrations of reagents (e.g., neurotransmitters) limits disease relevance	46, 64, 80, 81, 163–165, 174
<b>Ex vivo</b>				
Organoid models	Growth factor–induced expansion of murine and human epithelial and cancer stem cells	Multicellular composition of murine or human GI epithelial and cancer cells that retain many in vivo functions	GI epithelial and cancer organoid models lack neural and immune elements as well as a diverse gut microbiome; absence or loss-of-function mutations of key genes may retard or prevent organoid development	17, 34, 37, 181–184
<b>In vivo</b>				
Xenograft models	Injection of human or murine GI cancer cells in immunodeficient mice	Compared with cutaneous cell injection, orthotopic xenograft models may mimic human disease progression	Conventional human cancer cell xenografts in immunodeficient mice rarely mimic human disease progression and commonly replicate information already obtained from cell culture studies; major species differences in innate immunity	124, 157, 184
Carcinogen-induced tumor models	AOM/DSS-induced colon tumors	AOM, alone or combined with DSS, selectively induces colon neoplasia	Failure to mimic human disease (e.g., lack of metastasis in AOM/DSS models); “off-target” effects of vagotomy and other interventions may have unexpected, unappreciated effects	17, 37, 68
Genetically engineered animal models	Apc gene mutations in mice and pigs with colon neoplasia; Kras mutations in KC and KPC mice with PDAC	No carcinogen needed; physiological molecule levels in microenvironment; autochthonous tumors preserve neuron and immune cell interface	Only a few genes can be manipulated per model, with long latency to cancer development; limited generalizability due to heterogeneous genetic variants in spontaneous cancers; genetic drift may occur between generations; pig models are not practical; most <i>Apc<sup>Min/+</sup></i> models primarily develop adenomas in the small intestine, not colon, and females have confounding mammary and gynecological tumors	17, 33–37, 69, 165–167, 190, 191
Human tissue samples	FFPE and freshly frozen tissues	Allows comparison of in vivo expression of key molecules and cell-cell connections; can select specific cells for investigation with laser capture microscopy	Limited ability to manipulate test subjects; even generous margins (≥10 cm) to obtain “normal” tissue may not exclude cancer field effects; lability of RNA may confound analysis	46, 79, 146

AOM, azoxymethane; DSS, dextran sodium sulfate; FFPE, formalin-fixed paraffin-embedded; KC, (LSL-*Kras*<sup>+/LSL-G12D</sup> *Pdx1*-Cre); KPC, (LSL-*Kras*<sup>+/LSL-G12D</sup> LSL-*Trp53*<sup>+/R172H</sup> *Pdx1*-Cre).

NTRK1, NTRK2, or NTRK3 detected in a subset of esophageal, pancreatic, and colon cancers are targets for two FDA-approved TRK inhibitors, entrectinib and larotrectinib (118).

PNI in approximately half of esophageal squamous carcinomas identifies a clinical subset with a worse prognosis (43) and reduced survival (119). Meta-analysis identified PNI as a biomarker for advanced esophageal and esophagogastric junction cancers (120). PNI correlates with advanced TNM stage, poor cell differentiation (120), shorter disease-free survival, and increased rates of local recurrence (121), factors associated with overexpression of NGF (116).

**Gastric cancer.** The proximal two-thirds of the stomach is endowed with extensive vagal innervation (122) that regulates secretion of gastric acid and pepsinogen by cholinergic mechanisms (123). Epidemiological observations suggest a link between cholinergic innervation and gastric neoplasia; neuronal density correlates with more advanced stages of gastric cancer (50), and vagotomy for peptic ulcer disease may reduce long-term cancer risk (49). Robust evidence for this association was provided by elegant murine studies showing reduced gastric neoplasia following surgical or pharmacological denervation along with improved responses to chemotherapy and prolonged survival (50), findings confirmed by others (51). In

these murine models, vagotomy attenuated nuclear translocation of  $\beta$ -catenin and expression of several Wnt/ $\beta$ -catenin target genes, including *Ccnd1*, *Axin2*, *Myc*, *Lgr5*, and *Cd44* (50), providing a plausible mechanism underlying the benefits of denervation.

As for other GI cancers (68, 69), *M<sub>3</sub>R* deficiency or inhibition suggests a prominent role for this receptor subtype (50, 51). Gastric cancer cells express ChAT, synthesize and release ACh, and overexpress *M<sub>3</sub>R* (Figure 2); *M<sub>3</sub>R* expression correlates with gastric cancer stage and lymph node metastasis (62, 63). *M<sub>3</sub>R* activation by autocrine release of ACh stimulates cell proliferation by an *M<sub>3</sub>R*/EGFR/ERK-dependent mechanism (63); *M<sub>3</sub>R* knock-down suppressed growth and promoted apoptosis of human gastric cancer cell xenografts (62). In murine gastric epithelial cells, ACh release from Dclk1-positive tuft cells and neurons induced NGF expression by a YAP-mediated mechanism (ACh/NGF/*M<sub>3</sub>R*/YAP axis), which promotes neuron proliferation and cancer progression (Figure 3A and ref. 32).

**Pancreatic ductal adenocarcinoma.** Neurotrophins are overexpressed by PDACs and intrapancreatic cancer neurons; adding neurotrophins and coculture of PDAC and neural cells stimulates PDAC cell proliferation (Table 2 and ref. 124). Exogenous NGF dose-dependently increases MMP2 expression and enhances

pancreatic cancer cell invasion by activating ERK signaling (125). Pancreatic cancers overexpress GDNF, which may have chemokinetic effects on tumor cells and upregulates MMP9 expression and activity. MMP9, a gelatinase (type IV collagenase), facilitates cancer cell invasion and metastasis (15, 126). BDNF and NT-3 also stimulate PDAC invasion into the basement membrane (127, 128).

Following retroperitoneal nerve dissections that revealed neural involvement in PDAC (129), strong evidence accumulated linking neural input to cancer progression. Neural invasion, almost uniformly present in PDAC, shortens survival (44, 130–133). Neuronal support and mutations in axon guidance genes are also implicated in PDAC progression (134). Rare RET mutations in PDAC are associated with GDNF-dependent tumor invasion (Table 2 and ref. 135).

Neurotransmitters like ACh, adrenergic agonists,  $\gamma$ -aminobutyric acid (GABA), and glutamate, released from neuron and glial cell networks infiltrating PDAC, play important roles in tumor growth and dissemination (136). Cholinergic signaling via muscarinic receptors directly and indirectly suppresses pancreatic tumorigenesis and cancer stemness (37). In genetically engineered mice, subdiaphragmatic vagotomy accelerated, and a muscarinic agonist, bethanechol, suppressed, PDAC development; bethanechol, which improved survival by an  $M_1$ R-dependent mechanism (Figure 3B and ref. 37), is in early clinical trials. Likewise, in a murine PDAC model, subdiaphragmatic vagotomy promoted tumor growth and reduced survival, but not in mice deficient in TNF- $\alpha$  (38). In line with non-neuronal ACh production (54), ACh produced by human and rat pancreatic stellate cells may modulate pancreatic exocrine secretion and neoplasia (65). The specific downstream target genes for muscarinic receptor signaling in PDAC remain uncertain (Figure 3B).

$\beta$ -Adrenergic signaling mediates the accelerated PDAC growth and invasion observed with chronic stress (16). In an orthotopic mouse model of PDAC, in vivo optical imaging revealed that stress-induced neural activation increased tumor growth and metastasis. These effects were reproduced by pharmacological activation of  $\beta$ -adrenergic signaling and reversed by  $\beta$ -blockade, which also extended animal survival (13). Compared with controls, PDAC-bearing mice exposed to chronic stress had larger tumors and shortened lifespans, effects attenuated by a  $\beta$ -blocker (13).  $\beta$ -Blockade in PDAC is being evaluated in clinical trials.

Other neurotransmitters and receptors are implicated in PDAC progression. For example, GABA, which primarily inhibits CNS neuronal excitability, unexpectedly stimulates PDAC cell proliferation (105). These actions are most likely mediated via overexpressed GABA receptor  $\pi$  subunits that signal by elevating intracellular calcium and activating MAPK/ERK signaling (137). In accord with increased PDAC risk in tobacco users, nicotine-derived nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, a potent mutagen, carcinogen, and nicotinic ACh receptor agonist, induced PDAC in hamsters (138). Aggressive pancreatic cancers overexpress NMDA glutamate receptors that stimulate pro-growth signaling pathways when activated by glutamatergic nerves (139, 140).

**Colorectal cancer.** Neural influences are evident early in CRC development — colon cancer stem cells (CCSCs) proliferate in an environment with a denser enteric glial cell network than in normal colon, particularly at the tumor invasive front (25). In precancerous

lesions and CRC, enteric glial networks exhibit structural abnormalities (141, 142); S-100 $\beta$  and glial fibrillary acidic protein immunostaining reveal denser and more branched networks and glial cells intimately associated with both CRC cells and adjacent neurons (Figures 1 and 2 and ref. 25). Bidirectional interactions between glial and CRC cells involve CRC-derived IL-1 $\alpha/\beta$  stimulation of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) biosynthesis and paracrine release by enteric glia (Figure 2). PGE<sub>2</sub> stimulates CCSC growth and expansion via EP4 receptor-mediated transactivation of EGFR (25, 143–146). Activation of enteric glia by IL-1 may promote tumorigenesis by effects on immunocytes similar to those in inflammatory bowel diseases that increase CRC risk (147). In human CRC cell lines, immunodeficient mice, and primary human CRC cells, enteric glia stimulate an increase in the number and size of CCSC-derived tumors (25), identifying enteric glia as potential therapeutic targets.

Neurons also facilitate tumor development by serving as physical scaffolds for CRC cell migration and metastasis (Figure 1 and refs. 40, 46). ENS neurons are uniquely unmyelinated; the lack of perineurium and endoneurium sheath layers presents an unimpeded interface with tumors (46, 148) and glial cells, which, through paracrine signaling, enable CCSC activation (25). Enteric neurons and EpCAM-positive CRC cells are closely associated, particularly at the tumor invasive front, facilitating physical interactions between the two cell types (Figure 2 and ref. 46).

Synergistic interactions between bacterial species are associated with CRC initiation and progression (149); in mouse models, microorganisms (e.g., *B. fragilis*, *E. coli*, and *Fusobacterium nucleatum*; refs. 150–152) release factors, including *B. fragilis*-derived BFT toxin (101, 102), *F. nucleatum*-derived FadA and Fap2 adhesins, and NF- $\kappa$ B, that enhance CRC progression (153–155). It is likely that these factors modulate ENS activity; NF- $\kappa$ B, for example, plays a key role in regulating CNS inflammation (156). These multidirectional interactions between the gut microbiome, CRC cells, and enteric neurons are likely to promote cancer progression; stronger evidence awaits better experimental models (153). Because differences in immune, epithelial, and neural cell functions and microbial diversity in one region of the colon may impact cancer development and progression at other sites, purely reductionist approaches to explore links between the microbiome, ENS, and neoplasia may be misleading (157, 158).

The ability of CRC cells to adhere to enteric neurons and migrate to new anatomic locations is facilitated by cell surface molecules (e.g., L1CAM and N-cadherin) (Figure 2 and ref. 46) and can be modulated by neurotrophins (e.g., NT-4) (Table 2 and ref. 159). When cocultured with primary enteric neurons, enteric glia, and mesenchymal cells, CRC cells from established lines and primary CRC cells colocalize with enteric neurons (46). Moreover, in contrast to nontransformed intestinal epithelial cells and mesenchymal cells, CRC cells migrate across longer distances to reach enteric neurons and adhere to them with greater force (46). Successful invasion of CRC cells through the high-resistance neural sheath layers of collagen and basement membrane identifies hardier cells and confers survival and proliferative advantages (40). Besides the prognostic value of PNI, single-cell gene profiling may identify expression patterns predictive of PNI and cancer, neuronal, and glial genes that provide therapeutic opportunities (40–42, 160, 161).



As in gastric cancer and PDAC, cholinergic muscarinic receptors in CRC are the most prominent neurotransmitter targets. Treatment with a non-subtype-selective muscarinic agonist, bethanechol, promotes murine colon neoplasia (162). Of five muscarinic receptor subtypes,  $M_1R$  and  $M_3R$  activity most prominently modulates colon cancer progression (Figure 3C).  $M_3R$  overexpression in primary CRC predicts metastases, and in murine models of sporadic and genetic CRC, global  $M_3R$  deficiency robustly attenuates intestinal neoplasia (68, 69, 79).  $M_3R$  activation selectively induces the expression of *MMP1*, *MMP7*, and *MMP10*, which facilitates CRC invasion and spread (163). Blocking expression and activation of *MMP1* in vitro abolishes ACh-induced colon cancer cell invasion into endothelial cell monolayers (164). Selective BAs (e.g., deoxycholytaurine) can activate  $M_3R$  (78, 80), providing a mechanism whereby increased fecal BA levels augment murine colon neoplasia (165, 166). The mechanisms underlying the actions of  $M_3R$  in GI cancer are summarized in Figure 3D.

In contrast to  $M_3R$ , the role of  $M_1R$  in GI cancer remains obscure. As in animal models of PDAC (37), in azoxymethane-treated mice,  $M_1R$  deficiency modestly augmented colon neoplasia and, notably, negated the beneficial effects of  $M_3R$  deficiency (167). A therapeutic strategy directed at muscarinic receptor signaling will likely require targeting of  $M_1R$  and  $M_3R$  simultaneously.

## Impediments to studying gut-brain interactions in GI cancer

Capturing the intricacies of gut-brain interactions experimentally is challenging. While reductionist experimental systems such as cell coculture are valuable approaches to parse cross-directional cell signaling, they fail to capture the complex milieu and interactions between cells in living organisms. These models may not accurately reflect relevant concentrations of neurotransmitters and other biologically active molecules, diffusion limits in the extracellular space, and other parameters important to distinguish physiological from pharmacological effects (Table 3). The human GI tract features a particularly complex and dynamic ecosystem that may not be reproduced even by *in vivo* mouse and other animal models, which are also confounded by species differences (Figure 1).

Technical limitations and insufficient attention to quality control — e.g., confirming the specificity of antibodies, particularly those directed at GPCRs (168); authenticating cell lines and transgenic mice; optimizing tissue fixation, preservation, and autofluorescence; and ensuring high-quality mRNA measurement (169) — further impact data quality, interpretation, and translational value. An additional challenge is replicating *in vitro*, *ex vivo*, and *in vivo* the physical forces cancer cells exert for PNI and migration along a neural scaffold (Figure 1 and refs. 170, 171). Collectively, these limitations contribute to the poor track record of experimental models in predicting therapeutic success of novel interventions in clinical trials, and to the paucity of treatments directed at the gut-brain axis (172, 173).

*In vitro* models. The majority of information regarding the effects of neurotransmitters on oncogenic cell signaling and function derives from *in vitro* cell models. These use a relatively small repertoire of human cancer cell lines, many established decades ago. Extensive passaging is likely to have altered their genetic makeup and key biological features (174). Use of primary GI cancer cells may address these concerns but is limited by the contin-

uous need to replenish tissue samples and the innate heterogeneity of cancers (174–176). Coculturing primary GI cancer and ENS cells provides useful information but does not fully capture complex *in vivo* cell interactions (46). Biomedical journals commonly require investigators to replicate findings in multiple cancer cell lines, but adherence to this guidance is not uniform; as recently as 2018, an otherwise exemplary study employed only one established human colon cancer cell line without providing a rationale for cell line selection, although key findings were replicated in primary human colon cancer cells (46). Even using multiple cell lines does not assure scientific validity or reproducibility in more complex systems, particularly given the lack of cross-directional input from the neural and glial components of the tumor microenvironment and substantial intratumoral and neural network diversity (177). To some extent, the use of single-cell RNA sequencing may address the latter limitation (178), but changes in gene expression, which must be confirmed by quantitative PCR, are not necessarily mirrored by commensurate changes in protein expression.

*Ex vivo* models. Conventional organoid models developed from GI cancer stem cells can provide useful information regarding the factors promoting growth, invasion, and metastasis, but, among other limitations, organoids lack neural and immune elements (179–182). Even novel 3D organoids-on-a-chip, which permit the growth of mini-intestines on scaffolds that mimic basement membranes, fail to incorporate neural elements (179, 183). These limitations may be overcome by tissue engineering to develop scaffold-guided organoid morphogenesis from tissue stem cells that more faithfully mimic *in vivo* biology (183). In addition to furthering investigation into the role of neurons in GI neoplasia, development of increasingly accurate patient-derived organoid models may pave the way for advances in precision medicine by predicting the efficacy of novel therapies directed at the gut-brain axis (183).

*In vivo* models. Xenografts developed from human cancer cells injected into the skin of immunodeficient mice are common “*in vivo*” models. More cynically, these models represent only a change in culture medium from *in vitro* solutions to live organisms; xenograft experiments almost uniformly mirror *in vitro* findings without offering novel mechanistic insights, providing only an incremental advance and limited validation of *in vitro* findings. Orthotopic xenografts may more faithfully replicate human cancer progression and metastasis, e.g., human colon cancer cells implanted in the mouse sigmoid colon (157, 184). Patient-derived xenografts (PDXs) can provide real-time information to develop cancer-specific treatment (176).

*In vivo* models commonly fail to account for the impact of human immune and neural cells on GI cancer progression, even when PDX models employ “humanized” mice. Variability in gut microbiota can also confound outcomes; causal inferences based solely on murine studies should be avoided (185, 186). Investigators using human surgical tissues commonly use adjacent uninvolved tissue as control, but even the use of broad margins, 10 cm or more from the cancer, may be confounded by macroscopically indistinguishable cancer “field effects” affecting “control” cells (187). Innervation maps forming the basis for understanding nerve-tumor interactions derive largely from studies of noncancerous tissue, whereas GI cancers may restructure and rewire neuronal networks (30). Off-target effects of surgical manipulations, e.g., vagotomy, may alter the GI cancer-neuron interface in unexpected ways (38, 50, 51).

Genetically engineered mice and pigs (188–190) are limited by fundamental species differences in physiology and pathophysiology (191). For example, the mouse and human immune systems have very different major histocompatibility genes. Humanized genetically engineered mouse models have long latency periods and fail to recapitulate late-stage human disease, the most difficult clinical management problem. Even combining advanced techniques and models fails to mimic faithfully the complexity of the human GI tumor microenvironment.

## Conclusions and perspectives

Despite impressive progress, therapeutic interventions directed at the GI cancer gut-brain axis are currently limited to targeting neurotrophin, muscarinic, and  $\beta$ -adrenergic receptors. To advance the field, a more comprehensive understanding of the GI neuronal-glia-cancer cell interface is needed, akin to that for the gut microbiome (154, 192) and immune system (18, 193). Specific areas ripe for exploration include (a) determining how precancerous changes in the GI tumor secretome alter enteric glial networks and facilitate tumorigenesis; (b) using single-cell RNA-Seq and spatial transcriptomics to develop a more complete inventory of the cells, genes, and proteins comprising the tumor-neuron adhesion complex and molecular guidance factors, and better understanding how their expression alters cancer progression; (c) using similar methods to learn how immune cells, e.g., tumor-associated macrophages, mediate interactions between the ENS and GI cancer cells; (d) elucidating how GI cancers attract neurons and other constituents of the ENS and vice versa; (e) exploring whether molecules like L1CAM, whose expression correlates with PNI in PDAC (194), are viable therapeutic targets; (f) investigating whether PNI provides a mechanism for GI cancer cells to evade immune detection and treatment; (g) cataloging axon-derived metabolites that enhance cancer cell survival and growth (109, 195); and (h) improving experimental models to more faithfully capture the extraordinary complexity of the GI tumor microenvironment and the integration of neural and glial networks (196).

Exciting research opportunities in GI cancer neuroscience will result from leveraging advances in tissue preparation, clearing, and higher-resolution optical imaging to resolve CNS and ENS circuitry; computational biology for single-cell mRNA sequencing and metabolomics; optogenetics using light to monitor and control the activity of individual neurons and biochemical pathways modified by gene editing; 3D electrophysiological recording; and artificial intelligence (18, 197). Integrating newly discovered cell types and signaling pathways will yield novel mechanistic insights and therapeutic targets. For example, subepithelial telocytes, which provide pro-proliferative signals to stem cells throughout the small intestine and colon (198, 199), may contribute to crosstalk between colon cancer stem cells and components of the tumor microenvironment (Figure 1 and ref. 200). Filling key gaps in knowledge has great potential to advance our understanding of the role the gut-brain axis plays in GI cancer progression and empower us to leverage this information to improve therapeutic outcomes. Because of shared pathways and mechanisms (Figure 2 and Figure 3D), novel therapeutics targeting the gut-brain connection for one GI cancer will likely be applicable to others.

## Author contributions

AS, GX, and JPR conceived, wrote, reviewed, edited, and approved the submitted manuscript.

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