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Nervous system control of intestinal host defense in *C. elegans*

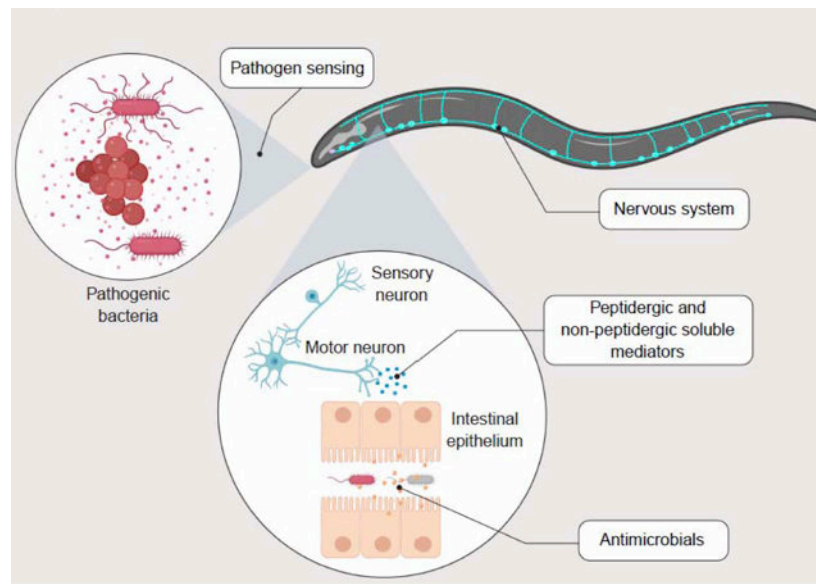
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Abstract

Interplay between the nervous and immune systems is critical for homeostasis, and its dysfunction underlies pathologies like multiple sclerosis, autism, leukemia, and inflammation. The nematode *C. elegans* provides an opportunity to define evolutionarily conserved mechanisms of regulation of host innate immunity and inflammation in a genetically tractable whole-animal system. In the past few years, the *C. elegans* nervous system has emerged as an integral part of host defense against pathogens, acting through diverse mechanisms to repress or induce protective transcriptional responses to infection in distal tissues. In this review, we discuss current knowledge of the mechanisms through which the *C. elegans* nervous system controls the expression of host defense genes in the intestinal epithelium. Although still incomplete, the insights derived from such work have broad implications for neural regulation of epithelial function at mucosal barriers in higher organisms in health and disease.

Graphical Abstract



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Conflict of interest statement

The authors declare no conflicts of interest.

Introduction

Free-living nematodes like *Caenorhabditis elegans* ingest microbes as a source of nutrition. Their habitats harbor diverse communities of microorganisms, some of which have evolved virulence potential, or the capability to fight back against predation[1]. This poses an evolutionary conundrum to nematodes, not unlike that posed to humans in relation to their microbiota: how to detect and protect against pathogenic microbes, while foraging for nonpathogenic or commensal nutritional sources? Hence the evolution in nematodes of sophisticated molecular sensing and defense mechanisms, the majority of which are shared with mammals, and thus must have existed in their last common ancestor[2,3]. This article reviews the most recent advances in our understanding of the critical role that the nervous system plays in detecting pathogenic microbes and in inducing the expression of antimicrobial host defense genes in the intestine in the model nematode *C. elegans*. The insights derived from this work have implications for our understanding of the evolution of host defense strategies in animals, and can shed light on yet undiscovered mechanisms of neuro-immune regulation in higher organisms.

C. elegans host defense

Invertebrates possess only innate mechanisms of host defense, which may include pattern recognition receptors such as the Toll-like receptors (TLR), downstream signaling pathways, and transcription factors like NF- κ B that induce the expression of antimicrobial peptides upon infection[4]. In addition, patrolling phagocytes in their “blood” contribute to pathogen clearance. In these animals, innate host defense results from interactions between endocrine signals and cellular defenses to enhance survival of infection.

Not so in *C. elegans*. These nematodes have lost genes encoding NF- κ B and several key components of known TLR pathways[3]. Furthermore, although *C. elegans* does possess 3 pairs of phagocyte-like coelomocytes, these do not patrol and do not appear to ingest bacteria[5]. One might be tempted to think that *C. elegans* represents a more primordial host defense system in relation to, say, arthropods like *Drosophila*. However, its phylogenetic position in relation to more basal metazoans like cnidariae, which do carry genes for TLR pathways and patrolling phagocytes, clearly favors the hypothesis that nematodes lost TLR pathways and phagocytes in the course of evolution[3], presumably in favor of alternative mechanisms of defense that increased their fitness. What might these mechanisms of defense be?

Research conducted over the last 15 years, following the pioneering work of Ausubel and Ewbank[6,7], has demonstrated that infections with pathogenic bacteria, fungi, and viruses elicit pathogen-specific programs of gene expression in many tissues throughout the nematode body[8]. In the specific case of ingested pathogenic bacteria, *C. elegans* rely on their simplified intestinal epithelium to fight infections[3]. Different bacterial infections elicit distinct transcriptional programs in these cells, which include genes that may encode antimicrobial peptides and enzymes, and which enhance host survival of infection[9,10]. Although such activities have been formally demonstrated in only a handful of cases, the majority of these genes have human correlates, if not direct homologs[11,12].

The induction of these putative or demonstrated antimicrobials has been used with remarkable success to identify upstream signaling pathways that regulate their expression. In a relatively short time, forward and reverse genetics approaches have facilitated the discovery and characterization of multiple evolutionarily-conserved signaling pathways, which are engaged in pathogen-specific manners to produce the organismal defense response. Thus, pathways mediated by p38 MAPK[13,14], β -catenin[15], hypoxia-inducible factor[16], heat shock factor[17], transcription factor EB (TFEB)[18], the unfolded protein response[19], ZIP-type transcription factors[20,21], GATA transcription factors[22], NRF2 and the oxidative stress response[23,24], and insulin signaling[25,26], among others, play important roles under different infection scenarios (reviewed in[27]). Despite all this important knowledge, which helped identify new pathways in mammalian systems[18,28], the question remained: how does *C. elegans* detect infection to trigger host response pathways?

Neural regulation of host defense gene expression

Recent evidence points to the nervous system in general, and the chemosensory system in particular, as key for pathogen sensing in *C. elegans*[29]. *C. elegans* is one of the most popular model organisms to study the nervous system, because several features make the *C. elegans* nervous system an appealing model for higher organisms. Compared to the human brain, which contains about 100 billion (10^{11}) neurons[30], the *C. elegans* nervous system comprises just 302 neurons[31]. These neurons are connected through about 5,000 synapses and a few hundred gap junctions, which have been comprehensively mapped[31].

At the molecular level, the *C. elegans* nervous system shares many features with its mammalian counterparts because its genome encodes many pathways that are conserved in mammals[32]. For example, *C. elegans* produce most neurotransmitters that are present in mammals (with the exception of glycine and histamine)[32,33]. Moreover, the *C. elegans* nervous system expresses neurotransmitter receptors, ion-channels, and GPCRs with mammalian counterparts[34]. Thus, *C. elegans* is poised to facilitate fundamental understanding of neural regulation of innate immunity.

Insulin and insulin receptor

The first study to demonstrate a key role of the nervous system in *C. elegans* host defense found that mutants defective in neurotransmitter secretion were resistant to *P. aeruginosa*[35]. Concordantly, compared to wild type such mutants exhibited increased expression of genes encoding antimicrobial factors (e.g. saposin *spp-1*, antimicrobial peptide *abf-2*, and lysozyme *lys-7*). On the other hand, mutants with enhanced neuronal secretion were more susceptible to *P. aeruginosa* than wild type, which correlated with decreased expression of antimicrobial peptides and other host defense genes in the intestinal epithelium[35]. Thus, the nervous system appeared to function in communication with the intestinal epithelium to repress host defense.

The molecular mechanism was shown to involve the insulin pathway. DAF-16, homologous to mammalian transcription factor FOXO3, functions downstream of insulin receptor DAF-2. DAF-2 represses DAF-16 to prevent the expression of a broad range of longevity

and stress defense genes[26]. Under stress conditions, DAF-2 activity diminishes, causing DAF-16 activation by nuclear translocation and expression of stress resistance genes. In the mentioned study, the pathogen resistance phenotype of neurotransmitter defective mutants was suppressed by mutation of *daf-16*, implicating the insulin pathway. Moreover, such neurotransmitter defective mutants exhibited constitutive nuclear translocation of DAF-16 (FOXO3) in the intestinal cells in the absence of infection. Thus, the nervous system functions to repress DAF-16, presumably by activating DAF-2 via an agonistic ligand.

Consistent with this idea, the insulin gene *ins-7*, which encodes a putative DAF-2 ligand[36], was upregulated in uninfected neurotransmission-defective mutants and in infected wild type animals. Moreover, *ins-7* mutants exhibited increased nuclear translocation of DAF-16 (FOXO3) and enhanced survival during infection, similar to neurotransmitter defective mutants. Furthermore, these phenotypes were reversed by neuronal rescue with wild type *ins-7*. These results implied that neurotransmission was a key mechanism of control of *ins-7* expression and downstream DAF-16 (FOXO3) activity via DAF-2[35••]. While INS-7 induction during infection appears to be counterproductive for the nematode, whether this is an example of the pathogen “hacking” the host response, or if INS-7 confers an undescribed advantage to the host in a more ecologically relevant scenario, is not clear.

In mammals, insulin signaling plays multiple roles in immunity, including both pro- and anti-inflammatory functions[37]. For example, hyperglycemia, a metabolic disorder presenting excessive blood glucose, causes increased production of pro-inflammatory mediators and cytokines[38]. Insulin, which acts to reduce blood glucose, opposes this effect[38]. Furthermore, insulin directly modulates immune cells such as human monocytes to enhance immunocompetence[38]. Thus, the repression of host defense genes by INS-7 and the anti-inflammatory function of insulin in mammals may be shared evolutionarily ancient functions of insulin.

Neuropeptides and NPR-1

The G-protein coupled receptor (GPCR) family, which includes olfactory and bacterial molecule receptors, is expanded in the *C. elegans* genome compared with vertebrates[34]. Because GPCRs are known to function as receptors for molecules of bacterial origin (e.g. mammalian formylated peptide receptors FPR1 and FPR2), mutants lacking individual GPCR genes were screened for defective survival of *P. aeruginosa* infection. This screen identified *npr-1* {homologous to mammalian neuropeptide Y (NPY) receptor (NPYR)} to be required for defense[39].

npr-1 had been previously implicated in avoidance of oxygen and attraction to food[40,41]. Using multiple approaches, the study demonstrated that *npr-1* mutants exhibited defective induction of host defense genes, such as C-type lectin *clec-85* and lysozymes *lys-2* and *lys-8*, which are controlled by the p38 MAPK pathway[13,26]. Moreover, the promotion of defense gene expression by *npr-1* required genes encoding known signaling components downstream of NPR-1 (NPYR), including guanylate cyclase *gcy-35* and cGMP-gated ion channel subunits *tax-2* and *tax-4*[42]. Although the mechanism of NPR-1 activation during infection was not identified, this study found that *npr-1* function in AQR, PQR, and URX sensory neurons was sufficient for protection against infection[39]. The proposed model was

that NPR-1 (NPYR) inhibits the activity of sensory neurons AQR, PQR, and URX, while GCY-35, TAX-2, and TAX-4 are required for their activation (Figure 1). Whether infection reduced the secretion of a peptide agonist for NPR-1 (NPYR) was not known, nor how this pathway controlled p38 MAPK activity to enact gene induction during infection. In addition, subsequent work showed that NPR-1 is involved in aversive learning to avoid infection with *P. aeruginosa* after initial exposure[43]. Thus, the emerging consensus is that NPR-1 (NPYR) signaling contributes to host defense via control of both gene expression and behavioral immunity.

Mammalian NPY and NPYRs have been shown to play a role in the immune response and inflammation[44]. Sympathetic neurons that innervate lymphoid organs produce NPY[45,46]. NPY1 and NPY2 receptors are expressed in immune cells, such as macrophages, neutrophils, granulocytes, and lymphocytes[44], and NPY has been shown to promote both pro- and anti-inflammatory activities in these cells[47,48]. NPY may also function directly in gastrointestinal immunity, since NPY-positive nerve fibers in the mouse ileal lamina propria are found in close proximity with gut immune cells, including IgA+ lymphocytes[49]. Further, there is evidence that NPY exerts a proinflammatory function in the mouse intestine[47,50]. Thus, NPR-1 (NPYR) may regulate an important, evolutionarily ancient pathway for host defense in nematodes and mammals.

Octopamine and OCTR-1

The same genetic screen that identified *npr-1* revealed that mutations in *octr-1* resulted in enhanced resistance of *P. aeruginosa* infection[51]. *octr-1* encodes a GPCR specific for octopamine, which is an invertebrate neurotransmitter that is closely related to mammalian noradrenaline[52]. Consistent with their resistance phenotype, *octr-1* mutants exhibited increased expression of a specific set of host defense genes, which are thought to participate in a noncanonical unfolded protein response (UPR)[51]. Using a similar neuron-specific rescue approach as for NPR-1, OCTR-1 was found to function in ASH and ASI sensory neurons. These findings led to the proposal that OCTR-1 functions in ASH and ASI neurons to suppress noncanonical UPR genes in uninfected animals, which are required for host defense during bacterial infection (Figure 1). However, the cellular source of octopamine in uninfected animals and the mechanism of control of the UPR genes remained unclear.

More recently, *tbh-1* mutants unable to synthesize octopamine were found to be resistant to *P. aeruginosa* and to express higher levels of host defense genes compared with wild type, consistent with the previous findings using *octr-1* mutants[53••]. Furthermore, nematodes lacking RIC octopaminergic neurons phenocopied *tbh-1* mutants in infection assays. Conversely, exogenous octopamine supplementation was sufficient to repress host defense genes and cause hypersusceptibility to infection, indicating that octopamine is necessary and sufficient to repress host defense against *P. aeruginosa*[53••]. *In vivo* neuronal activity, recorded using genetically-encoded Ca^{2+} sensitive fluorophore GCAMP6, revealed that RIC activity diminished during *P. aeruginosa* infection in comparison with uninfected animals[53••]. Thus, octopamine released from RIC neurons may function as a repressor of unwanted host defense gene expression in uninfected animals, acting via OCTR-1 expressed in postsynaptic ASH neurons. During infection, an unknown mechanism may reduce RIC

activity, enhancing ASH neuron activity and increasing expression of specific host defense genes that enhance survival (Figure 1).

C. elegans OCTR-1 is considered to be an ortholog of human $\alpha 2A$ adrenergic receptor for norepinephrine[53••]. Norepinephrine regulates innate immune responses in mammals and other vertebrates, suggesting that the role of octopamine and/or norepinephrine in immunomodulation is evolutionarily conserved[54]. A recent study showed that elimination of noradrenergic neurons using 6-hydroxydopamine improved survival of mice during *Klebsiella pneumoniae* peritonitis[55]. Two complementary mechanisms of action were revealed: sympathetic-derived norepinephrine directly reduced production of monocyte chemoattractant protein-1 (MCP-1) by peritoneal macrophages, and splenic-nerve-derived norepinephrine prevented egress of splenic monocytes[55]. Elimination of noradrenergic neurons thus abrogated these two mechanisms, enhancing the ability of the innate immune system to clear the infection.

Dopamine and DOP receptors

Enteropathogenic *E. coli* (EPEC) kills *C. elegans* via secreted toxins, which were found to be indole and other derivatives of tryptophan[56,57]. Brief exposure to virulent or avirulent EPEC significantly improved the survival of *C. elegans* to subsequent infection with the pathogen, a phenomenon then termed “conditioning”[57]. Conditioned animals expressed higher levels of several host defense genes in the intestine, which candidate analysis showed to depend on the insulin and p38 MAPK pathways. Tissue-specific rescue experiments revealed that the insulin receptor DAF-2 and FOXO3 homolog DAF-16 functioned in the nervous system for conditioning[57].

Because serotonin and dopamine had been previously implicated in food sensing[58,59], these two neurotransmitters were examined as potential upstream signals in the neural control of conditioning. Mutants defective in synthesis of dopamine, but not serotonin, exhibited defective conditioning, implicating dopamine in the conditioning of *C. elegans* for EPEC infection[57]. Furthermore, mutants defective in dopamine synthesis, reuptake, or signaling through D2-like dopamine receptor DOP-3 were also defective in conditioning. The proposed model posited that exposure to EPEC triggered dopamine release, which drove DAF-16 (FOXO3) and p38 MAPK activation to increase the expression of antimicrobial peptides in conditioned animals[57]. However, the mechanism of EPEC detection to induce dopamine release, and the mechanism connecting dopaminergic signaling to the insulin and p38 pathways were not elucidated.

In contrast, a recent study showed that dopamine signaling through D1-like DOP-4 receptor resulted in the inhibition of p38 MAPK, downregulation of host defense genes, and increased susceptibility during *P. aeruginosa* infection[60••]. In this study, the cellular source of dopamine was the CEP mechanosensory neurons, and required active DOP-4 in downstream chemosensory ASG neurons. Thus, the neural circuits and biological role of dopaminergic signaling may depend on the specific pathogenic insult.

Additionally, dopamine signaling has been implicated in the modulation of behavioral plasticity, such as during food sensing, in *C. elegans*[61,62]. *C. elegans* possess eight pairs

of dopaminergic neurons, with sensory projections that are directly exposed to the external environment[63]. Their anatomical position and exposure to the environment makes dopaminergic sensory neurons prime candidates for pathogen sensing by the nervous system. However, whether the sensory endings of dopaminergic neurons directly detect pathogenic bacteria remains unknown.

Multiple immune cells in mammals express dopamine receptors, and dopamine is known to modulate their functions. For example, human monocytes and macrophages express all five subtypes of dopamine receptors [64] and dopamine alters their cytokine production upon stimulation[65]. In resting macrophages, dopamine caused increased production of IL-6 and CCL2[65]. After LPS stimulation, dopamine caused increased production of IL-6, CCL2, CXCL8, and IL-10, and decreased production of TNF- α [65]. Several studies have shown that neurological disorders that arise from dysregulation of dopaminergic system in humans, such as Parkinson's disease and schizophrenia, correlate with abnormal immune function[66]. Thus, dopamine appears to be another shared and evolutionarily ancient signaling molecule in host defense.

Acetylcholine and muscarinic receptors

A reverse genetics approach was used to identify GPCRs that are required for the induction of C-type lectin gene *clec-60* (used as a reporter of the broader host response) during *S. aureus* infection[67••]. Simultaneous silencing of genes *gar-2* and *gar-3*, which encode muscarinic acetylcholine receptors, prevented the induction of *clec-60* and other *S. aureus*-induced genes. Consistent with the notion that muscarinic signaling was important for the host response, the same study defined a potential signaling pathway downstream of GAR-2 and GAR-3, comprised of EGL-30 (G α_q), PLC-1 (phospholipase C ϵ), diacylglycerol, and EGL-8 (protein kinase C β) that was necessary and sufficient for induction of *clec-60* and other host defense genes(Figure 1)[67••]. Moreover, infection with *S. aureus* increased endogenous acetylcholine release, and acetylcholine analog arecoline was sufficient to trigger this pathway in the absence of infection. Because *gar-2* and *gar-3* were found to function in the intestine, and because acetylcholine is produced only in the nervous system in *C. elegans*[68,69•], these results suggested the existence of a novel neuro-intestinal axis.

Downstream of muscarinic signaling, the same study defined a canonical *Wingless*-integration site (Wnt) signaling pathway, which was activated by acetylcholine via the transcriptional induction of Wnt gene *cwn-2* and its putative receptor *mig-1* (Frizzled) (Figure 1)[67••]. Activation of this Wnt pathway was necessary and sufficient to drive the expression of host defense genes like *clec-60*[67••]. However, the mechanism of *S. aureus* detection by the nervous system to trigger acetylcholine release remained unknown.

Nonetheless, a similar muscarinic-Wnt signaling axis was recently implicated in the development of gastric cancer in mice and humans[70•], suggesting that this mechanism is evolutionarily conserved. Moreover, murine nociceptive sensory neurons can directly sense *S. aureus* infection. For example, two recent studies showed that *S. aureus* α -hemolysin and N-formylated peptides, released during infection, cause Ca²⁺ influx in nociceptor neurons, thereby activating them[71••,72]. Activation of nociceptive neurons by *S. aureus* α -hemolysin led to the release of neuropeptide CGRP in a dose-dependent manner.

Furthermore, microarray analysis showed high expression levels of CGRP receptor in innate immune cells, such as neutrophils and macrophages, in addition to nociceptive neurons themselves, and deletion of CGRP receptor prevented the inhibition of pro-inflammatory signaling caused by α -hemolysin[72]. These studies demonstrated how a pore-forming toxin can trigger release of neuropeptides from neurons, which can act on innate immune cells, thus modulating the inflammatory response[72].

Although nociceptor neurons can be cholinergic, there is no known direct connection of intestinal epithelial cells with cholinergic nerve fibers in mammals. However, acetylcholine has been shown to induce secretion of antimicrobials in the mammalian intestinal epithelium. For example, activation of muscarinic acetylcholine receptors in small intestine isolated from mice increased secretion of antimicrobial defensins from Paneth cells and enhanced crypt antimicrobial activity *ex vivo*[73–75]. Conversely, muscarinic inhibitor atropine abolished antimicrobial secretion by Paneth cells[74]. Moreover, acetylcholine induced the expression of antimicrobial peptides in mouse intestinal epithelial cells[76•]. Recently, a study showed that muscarinic signaling is essential for clearing *Salmonella* infection in mice[77•]. Thus, muscarinic signaling to the gut epithelium appears to be an evolutionary ancient form of regulation of intestinal host defense.

Conclusion

From the studies described above and others, it is increasingly clear that different neuronal circuits and neurotransmitters have distinct relevance to *C. elegans* host defense under different infection scenarios. What is lacking is an integrated understanding of how these distinct circuits and neurotransmitter systems are collectively regulated during any particular pathogenic encounter. It is important to understand how they become activated or inhibited at the whole nervous system level by any particular pathogen, for a clear view of the various pathogen-sensing functions of the nervous system in the context of the whole organism.

Similarly, it is not clear exactly what signals are being sensed to detect the presence of pathogen. Most likely some of these involve soluble chemical compounds or macromolecules that sensory neurons can detect in the context of the thin watery films in which *C. elegans* live. Recent studies suggest that secondary metabolites produced by *P. aeruginosa* modulate behavior through one such mechanism of detection[78]. However, other mechanisms of pathogen detection may also operate, such as detection of translational repression by bacterial toxins[21] or morphological alterations to the intestine caused by bacterial colonization of the lumen[79••,80••]. In many cases, potential detected signals have been identified, but the mechanisms of detection remain unclear.

Finally, the exact mechanisms of neuron-epithelium communication to regulate epithelial gene expression are not known. In mammals, recent work has demonstrated direct synapsing of neurons unto epithelial enteroendocrine cells of the intestine[81••] and extensive neural innervation of the mucosa to the immediate vicinity of the intestinal epithelium[81••]. Unlike mammals, direct synapsing of neurons on epithelial cells has not been observed in *C. elegans*; however, in the tiny nematode body several neurons are in anatomical positions that

would be consistent with distal chemical communication, as has been demonstrated in mammals. Most mediators of such communication in nematodes have not been identified.

Given these considerations, much further work is required to completely understand neuronal control of intestinal epithelial gene expression even in a simple organism like *C. elegans*. The next few years will likely witness great progress in addressing these outstanding questions, with great potential implications for the mammalian microbiota-gut-brain axis[82].

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Highlights

- *C. elegans* responds to pathogens by transcriptional induction of evolutionarily-conserved host defense genes
- Neuron-intestine communication is essential for host defense gene induction in several infection paradigms
- Conserved neurotransmitter pathways can function as negative or positive regulators of host defense gene expression
- Further dissection of the mechanisms of neuro-epithelial communication is needed for full understanding of organismal responses to infection

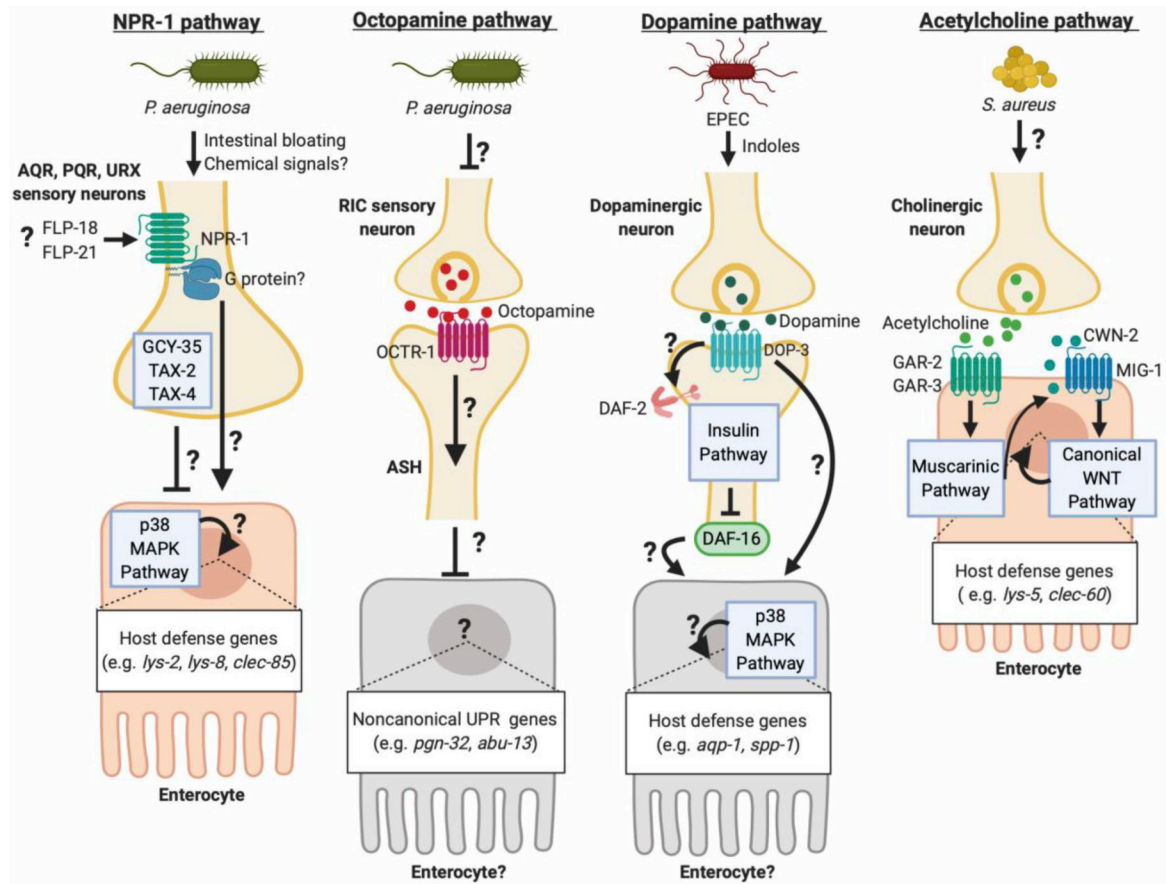


Figure 1. Multiple neuronal pathways regulate intestinal host defense in *C. elegans*. NPR-1 (NPYR) positively controls the expression of host defense genes during *P. aeruginosa* infection. In parallel, GCY-35, TAX-2, and TAX-4 negatively regulate their induction. FLP-18 and FLP-21 neuropeptides are upregulated during infection, and act through NPR-1 (NPYR) for behavioral avoidance of infection, but they have not been shown to affect defense gene expression[79]. Octopamine, released from RIC sensory neurons, acts through receptor OCTR-1 in ASH neurons to inhibit expression, presumably in the intestinal epithelium, of non-canonical UPR genes that promote host defense. This may be a mechanism of preventing inappropriate activation in noninfected animals. Dopamine, released during infection, acts on DOP-3 receptor on neurons to drive “conditioning” to EPEC. This was dependent on both insulin signaling and p38 MAPK, presumably via induction of host defense genes in the intestinal epithelium. Acetylcholine, released during *S. aureus* infection, drives muscarinic signaling in the intestinal epithelium and induces the expression of Wnt homolog CWN-2 in the same tissue. CWN-2 activates canonical Wnt signaling through MIG-1 (Frizzled), resulting in the intestinal epithelial expression of host defense genes. Created with BioRender.