Immunology and Inflammation
Neuroscience

Neuronal NPR-15 controls the interplay between molecular and behavioral immune responses through the amphid sensory neuron-intestinal axis in *C. elegans*

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Abstract

The survival of hosts during infections relies on their ability to mount effective molecular and behavioral immune responses. Despite extensive research on these defense strategies in various species, including the model organism *Caenorhabditis elegans*, the neural mechanisms underlying their interplay remain poorly understood. Previous studies have highlighted the role of neural G protein-coupled receptors (GPCRs) in regulating both immunity and pathogen avoidance, which is particularly dependent on aerotaxis. To address this knowledge gap, we conducted a screen of mutants in neuropeptide receptor family genes. We found that loss-of-function mutations in *npr-15* activated immunity while suppressing pathogen avoidance behavior. Through further analysis, NPR-15 was found to regulate immunity by modulating the activity of key transcription factors, namely GATA/ELT-2 and TFEB/HLH-30. Surprisingly, the lack of pathogen avoidance of *npr-15* mutant animals was not influenced by oxygen levels. Moreover, our studies revealed that the amphid sensory neuron ASJ is involved in mediating the immune and behavioral responses orchestrated by NPR-15. Additionally, NPR-15 was found to regulate avoidance behavior via the TRPM gene, GON-2, which may sense the intestinal distension caused by bacterial colonization to elicit pathogen avoidance. Overall, this study expands our knowledge of how neuronal GPCRs can simultaneously modulate immunity and behavior. Given the conserved nature of the GPCR and immune signaling pathways studied, the findings contribute to a broader understanding of host defense strategies and mechanism underlining the interplay between immunity and behavior across species.

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eLife assessment

This **important** work by Aballay et al. advances our understanding of how G protein-coupled receptors (GPCRs) regulate immunity and pathogen avoidance. The authors provide **convincing** evidence for the GPCR NPR-15 to mediate immunity by altering the activity of several key transcription factors. Nevertheless, both reviewers have raised questions to be addressed, at which time this work will be of broad interest to immunologists specifically, and biologists in general.

Introduction

Hosts employ multiple defense mechanisms to combat infections, including molecular immune defenses (1, 2, 3) and behavioral defense responses to invading pathogens (4, 5, 6). These strategies are conserved across species (5, 6, 7, 8), but their relationship and mechanistic interplay are not yet fully elucidated. While the immunological defense response is effective, it is metabolically costly and may lead to inflammatory damage (9, 10, 11, 12). On the other hand, the avoidance behavioral response serves as a crucial first line of defense, enabling hosts to prevent or minimize contact with pathogens (13, 14, 15). Although both immune and behavioral responses to pathogen infection are well documented in *Caenorhabditis elegans* (16, 17, 18, 19, 20), the relationship between these survival strategies remains poorly understood.

C. elegans is a valuable model organism for studying the genetic mechanisms that control host immune and behavioral responses to pathogens (21, 22). Although *C. elegans* lacks adaptive immunity, part of its innate immune response comprises evolutionarily conserved pathways and immune effectors (22). *C. elegans* has been widely used in research studies to investigate these pathways due to its well-defined nervous system and genetic tractability, making it an ideal model organism to explore the evolutionarily conserved pathways that are critical to immunity and avoidance behavior (23, 24, 25). Notably, intestinal changes triggered by bacterial pathogen colonization activate the DAF-7/TGF-β pathway and the G-protein coupled receptor (GPCR) NPR-1 pathway, which also regulates aerotaxis behavior (15, 16, 19, 26).

To uncover mechanisms that control immune and behavioral responses to invading pathogens independently of aerotaxis, we focused on studying mutants in *npr* genes that have not been previously linked to either host strategy against pathogen infection. Our investigation revealed that loss-of-function mutations in the NPR-15 encoding genes enhanced pathogen resistance when infected by Gram-negative and Gram-positive bacterial pathogens. Intriguingly, *npr-15* mutants exhibited a lack of pathogen avoidance behavior that was found to be independent of oxygen sensation. These findings point towards the involvement of a novel mechanism in the regulation of immune response and avoidance behavior. Further analysis unveiled that the resistance to pathogen infection in *npr-15* mutants is mediated by the transcription factors GATA/ELT-2 and TFEB/HLH-30. These evolutionarily conserved transcription factors play vital roles in regulating immunity in *C. elegans* (27, 28, 29, 30, 31). Additionally, we discovered that NPR-15 controls avoidance behavior through the intestinal-expressed transient receptor potential melastatin (TRPM) ion channel, GON-2, which has recently been demonstrated to modulate avoidance behavior in Gram gram-positive bacteria (32). Moreover, our results indicate that the amphid sensory neuron, ASJ, plays a crucial role in the interplay between immune response and avoidance behavior. These findings provide valuable insights into the intricate interplay between the nervous system, immunity, and avoidance behavior, which have implications for both *C. elegans* biology and broader host-pathogen interactions across species.

Results

NPR-15 loss-of-function enhances pathogen resistance and inhibits avoidance behavior independently of aerotaxis

Out of 34 mutants in *npr* genes that were not previously linked to the control immunity (Table S1A), only animals lacking NPR-15 [*npr-15(tm12539)* and *npr-15(ok1626)* null animals] exhibited enhanced survival against *Pseudomonas aeruginosa*-mediated killing compared to wild-type (WT) animals (**Figure 1A**, S1A and Table S1B). Furthermore, we found that the *npr-15(tm12539)* animals exhibited less visible bacterial colonization and significantly reduced colony-forming units compared to WT animals (**Figure 1B-C**). The enhanced resistance to pathogen of *npr-15(ok1626)* animals appears to be universal, as the mutants were also found to be resistant to additional human pathogens, including Gram-negative *Salmonella enterica* strain 1344 and Gram-positive *Staphylococcus aureus* strain NCTCB325 and *E. faecalis* strain OG1RF (**Figure 1D-F**), suggesting that NPR-15 suppresses defense against bacterial pathogens in general. When exposed to live *E. coli*, the primary food source of *C. elegans* in the laboratory, *npr-15(tm12539)* animals exhibited increased lifespan compared to WT animals (**Figure 1G**). However, there were no significant differences in longevity between *npr-15(tm12539)* and WT animals when they were exposed to lawns of *E. coli* that were rendered non-proliferating by ultraviolet light (UV) treatment (33) (**Figure 1H**). Expression of *npr-15* under the control of its own promoter rescued the enhanced survival of *npr-15(tm12539)* animals to different bacterial pathogens (**Figures 1A-G**), indicating that the functional loss of NPR-15 enhanced the animal's survival against live bacteria.

Considering that *C. elegans* exhibits avoidance behavior when encountering certain pathogenic bacteria (15, 17, 18, 19), we examined the lawn occupancy of *npr-15(tm12539)* and WT animals on the partial lawn of *S. aureus* cultured in the center of an agar plate (**Figure 1I**). Unexpectedly, we found that *npr-15(tm12539)* exhibited significantly reduced pathogen avoidance when exposed to *S. aureus* (**Figure 1J**). To investigate whether aerotaxis played a role in the lack of avoidance of *S. aureus* exhibited by *npr-15(tm12539)*, we studied lawn occupancy in the presence of 8% oxygen. As shown in Figure S1B, exposure to 8% oxygen to *npr-15(tm12539)* animals did not rescue the lack of avoidance to *S. aureus*, although it did rescue the lack of avoidance of *npr-1* mutants. Moreover, the survival of *npr-15(tm12539)* animals on full-lawn assays, where agar plates were completely covered by pathogenic bacteria to eliminate the possibility of pathogen avoidance, was significantly higher than that of WT animals (Figure S1C-D). These findings suggest that NPR-15 suppresses pathogen resistance and enhances avoidance behavior in response to pathogen infection, independently of oxygen concentrations.

Because *C. elegans* pharyngeal pumping directly affects bacterial intake (16, 19, 34, 35), we asked whether the resistance to infections and reduced pathogen avoidance behavior in *npr-15(tm12539)* animals could be attributed to a decrease in pathogen intake. We found that *npr-15(tm12539)* animals exhibited pumping rates comparable to that of WT animals (Figure S1E), indicating that the dose of pathogens is similar in both cases. Moreover, it has recently been demonstrated that bacterial accumulation in animals defective in the defecation motor program (DMP) causes intestinal distension that elicits a robust immune response (19) and modulates pathogen avoidance (19, 26, 32, 36, 37). However, we found that the defecation cycle of *npr-15(tm12539)* animals is indistinguishable from that of WT animals (Figure S1F). Taken together, our results show NPR-15 loss-of-function enhances pathogen resistance and inhibits avoidance behavior, suggesting NPR-15 modulates the interplay between molecular and behavioral immunity.

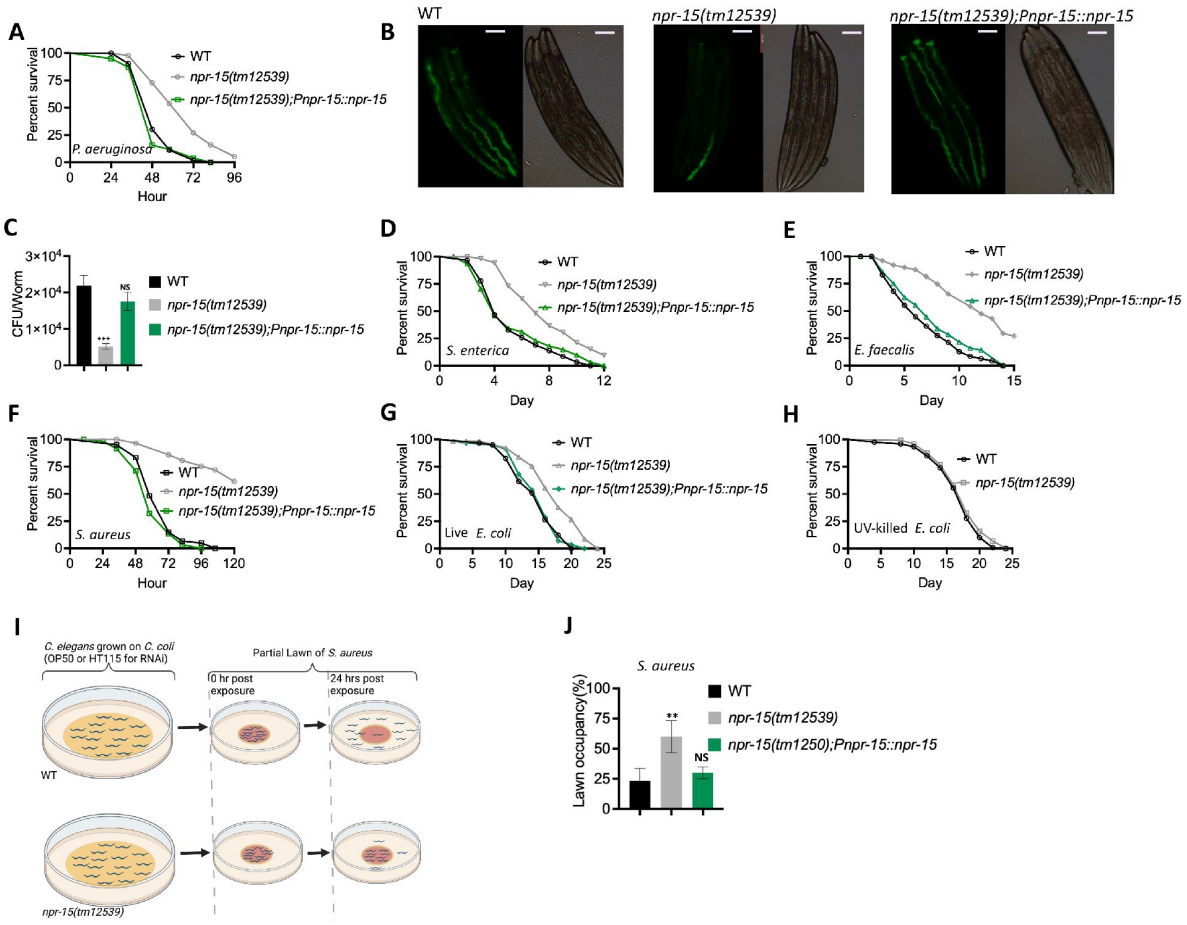

Figure 1.

NPR-15 loss-of-function enhanced pathogen resistance and inhibit avoidance behavior.

A. Wild type (WT), *npr-15(tm12539),* and *npr-15(tm12539);Pnp-15::npr-15* animals were exposed to *P. aeruginosa* partial lawn and scored for survival. WT vs *npr-15(tm12539)*, P < 0.0001; *npr-15(tm12539);Pnp-15::npr-15*, P=NS.

B. Colonization of WT, *npr-15(tm12539),* and *npr-15(tm12539);Pnp-15::npr-15* animals by *P. aeruginosa*-GFP after 24 hours at 25°C. Scale bar, 200 µm

C. Colony-forming units per animal [WT, *npr-15(tm12539), npr-15(tm12539);Pnp-15::npr-15*] grown on *P. aeruginosa* –GFP for 24 hours at 25°C. Bars represent means while error bars indicate SD of three independent experiments; ***p < 0.001.

D. WT, *npr-15(tm12539),* and *npr-15(tm12539);Pnp-15::npr-15* animals were exposed to *S. enterica* partial lawn and scored for survival. WT vs *npr-15(tm12539)*, P < 0.0001; *npr-15(tm12539);Pnp-15::npr-15*, P = NS.

E. WT, *npr-15(tm12539),* and *npr-15(tm12539);Pnp-15::npr-15* animals were exposed to *E. faecalis* partial lawn and scored for survival. WT vs *npr-15(tm12539)*, P < 0.0001; *npr-15(tm12539);Pnp-15::npr-15*, P = NS.

F. WT, *npr-15(tm12539),* and *npr-15(tm12539);Pnp-15::npr-15* animals were exposed to *S. aureus* partial lawn and scored for survival. WT vs *npr-15(tm12539)*, P < 0.0001; *npr-15(tm12539);Pnp-15::npr-15*, P = NS.

G. WT, *npr-15(tm12539),* and *npr-15(tm12539);Pnp-15::npr-15* animals were exposed to live *E. coli* and scored for survival. WT vs *npr-15(tm12539)*, P < 0.0001; *npr-15(tm12539);Pnp-15::npr-15*, P = NS.

H. WT and *npr-15(tm12539)* animals were exposed to UV-killed *E. coli* and scored for survival. WT vs *npr-15(tm12539)*, P=NS.

I. Schematics of avoidance behavior assay on *S. aureus*.

J. Lawn occupancy of WT *C. elegans* and *npr-15(tm12539)* animals on a partial lawn of *S. aureus* at 24 h. Bars represent means while error bars indicate SD; **p < 0.001, and NS = not significant.

The loss of NPR-15 leads to the upregulated immune and neuropeptide genes

To understand the immune mechanisms controlled by NPR-15 in defense against pathogen exposure, we conducted transcriptomic analyses to identify dysregulated genes in *npr-15(tm12539)* compared to WT animals (**Figure 2A** and Table S2). To identify gene groups that were controlled by NPR-15, we performed an unbiased gene enrichment analysis using a Wormbase enrichment analysis tool (*https://wormbase.org/tools/enrichment/tea/tea.cgi*) that is specific for *C. elegans* gene data analyses (38). The study revealed 10 ontology clusters with high enrichment scores of vital biological functions for upregulated and downregulated genes in *npr-15(tm12539)* (**Figure 2B**, S2). Overall, the gene expression data showed significant upregulation of immune/defense response and neuropeptide signaling pathway genes (**Figure 2B**). Genes associated with synaptic signaling, ligand-gated channel activity, lipid metabolism, and response to biotic stimuli were also upregulated in *npr-15(tm12539)* animals.

To further explore potential immune pathways involved in pathogen resistance in NPR-15-deficient animals, we performed a gene enrichment analysis using the immune gene subset with the Worm Exp tool (*https://wormexp.zoologie.uni-kiel.de/wormexp/*) (39). This tool integrates all published expression datasets for *C. elegans* to analyze and generate pathways. Our analysis revealed a high enrichment of pathways crucial for *C. elegans* defense against bacterial infections, including the ELT-2, HLH-30, PMK-1, and DAF-2/DAF-16 insulin pathways (**Figure 2C** and Table S3) (28, 30, 31, 40, 41, 42, 43, 44, 45). Notably, we observed that several of the PMK-1, HLH-30, and DAF-16-dependent genes were also controlled by ELT-2 (**Figure 2D**), suggesting a potentially major role of ELT-2-dependent genes in the enhanced resistance to the pathogen phenotype of *npr-15(tm12539)* animals. To validate these findings, we performed Quantitative PCR (qPCR) and confirmed the upregulation of ELT-2-dependent immune genes such as *dct-17*, C29F3.7, T19D12.4, C32H11.4, C34H4.1 and *clec-86* (**Figure 2E**). We also validated HLH-30-dependent genes T21F4.1, *argn-1*, F53A9.8, and *pnp-1* (**Figure 2F**). These transcriptomic and gene analyses indicate that NPR-15 primarily regulates *C. elegans* defense against bacterial infections by inhibiting immune genes, several of which are controlled by ELT-2, HLH-30, and PMK-1.

NPR-15 controls immunity in an ELT-2/HLH-30-dependent manner via sensory neuron, ASJ

To investigate whether the enhanced resistance to pathogen infection of *npr-15(tm12539)* animals is due to the upregulation of immune genes, we examined the role of RNAi-mediated suppression of the immune pathways shown in **Figure 2C**. We inactivated *elt-2*, *pmk-1, hlh-30,* and *daf-16* in WT and *npr-15(tm12539)* animals and exposed them to *S. aureus*. We found that *elt-2* RNAi completely suppressed the enhanced resistance to *S. aureus* infection in *npr-15(tm12539)* animals (**Figure 3A**). Partial suppression of pathogen resistance in *npr-15(tm12539)* animals was observed with *hlh-30* RNAi (**Figure 3B**). Furthermore, when both *hlh-30* and *elt-2* were inactivated in *npr-15(tm12539)* animals and were exposed to *S. aureus*, their susceptibility to infection was comparable to that of *elt-2* RNAi animals (**Figure 3C**). However, *pmk-1*, *daf-16* RNAi failed to suppress the pathogen resistance of *npr-15(tm12539)* (**Figure 3D-E**). As a control, we performed *skn-1* RNAi, which had no effect on the pathogen resistance of *npr-15(tm12539)* (**Figure 3F**). To confirm these findings, we crossed mutants in the aforementioned immune regulators to *npr-15(tm12539)* animals and exposed them to *S. aureus* (Figure S3A-D). These results indicate that NPR-15 suppresses ELT-2 and HLH-30-dependent molecular immunity.

Because NPR-15 is expressed in six sensory neuronal cells (ASG, ASI, ASJ, ASE, AFD, and AWC) as well as an interneuron, AVK (**Figure 4A**) (46), we studied whether the NPR-15-expressing cells could control the defense response against pathogen infection. Firstly, we examined the neuronal connectome and communication between the NPR-15-expressing cell and found well-established

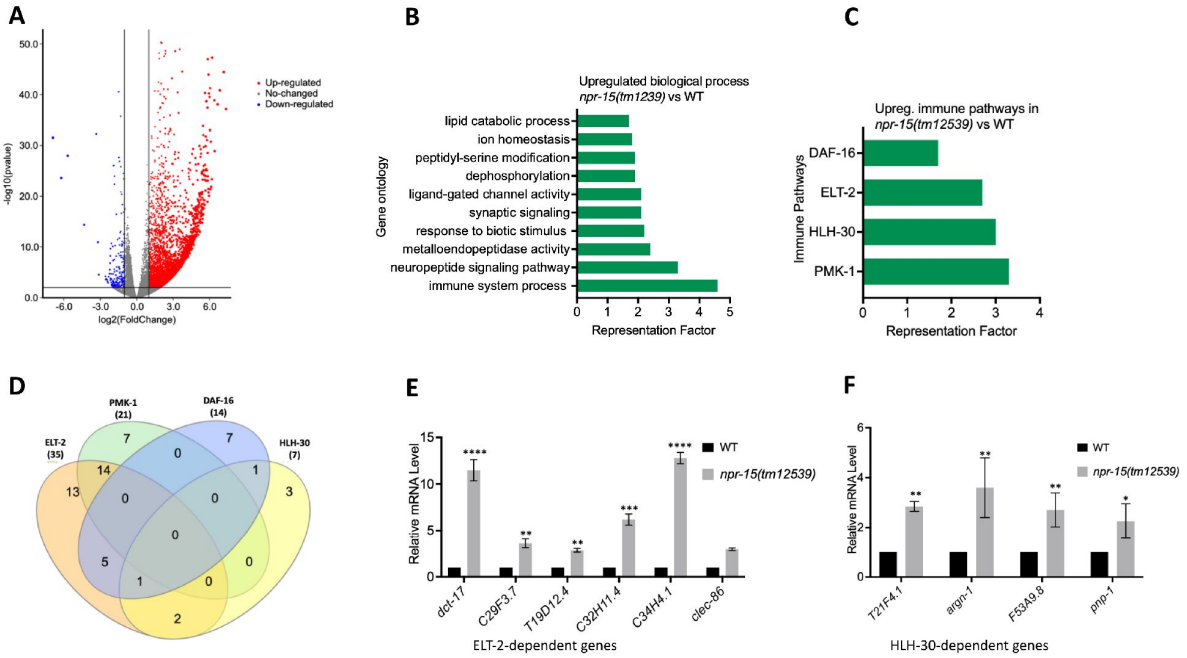

Figure 2.

NPR-15 inhibits the expression of immune and aversion-related genes/pathways.

A. Volcano plot of upregulated and downregulated genes in *npr-15(tm12539)* vs WT animals. Red and blue dots represent significant upregulated and downregulated genes respectively, while the grey dots represent not significant genes.

B. Gene ontology analysis of upregulated genes in *npr-15(tm12539)* vs WT animals. The cutoff is based on the filtering thresholds of P < 0.05 and arranged according to the representation factor.

C. Representation factors of immune pathways for the upregulated immune genes in *npr-15(tm12539)* vs WT animals.

D. Venn diagram showing the upregulated immune genes in each pathway in *npr-15(tm12539)* vs WT animals.

E. qRT-PCR analysis of ELT-2-depenent immune gene expression in WT and *npr-15(tm12539)* animals. Bars represent means while error bars indicate SD of three independent experiments; *p < 0.05, **p < 0.001 and ***p < 0.0001.

F. qRT-PCR analysis of HLH-30-depenent immune gene expression in WT and *npr-15(tm12539)* animals. Bars represent means while error bars indicate SD of three independent experiments; *p < 0.05, **p < 0.001 and ***p < 0.0001.

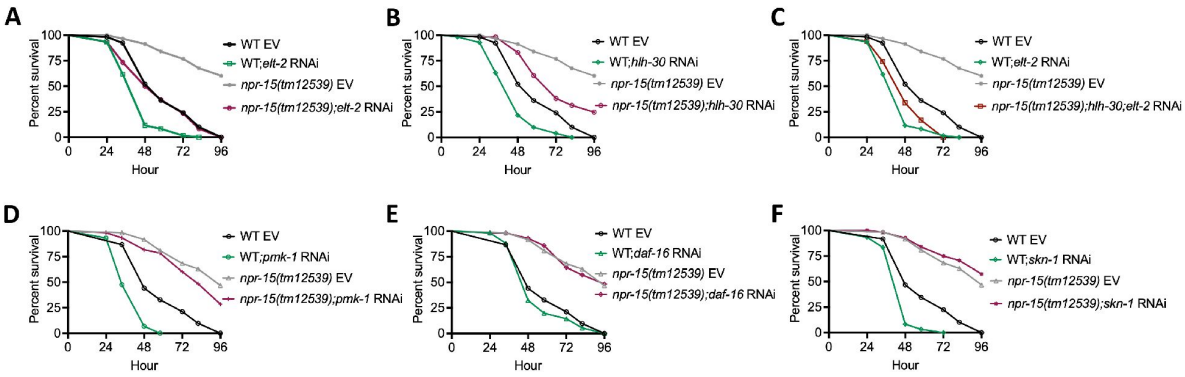

Figure 3

NPR-15 loss-of-function enhances immunity via ELT-2 and HLH-30 when exposed to *S. aureus*.

A. WT and *npr-15(tm12539)* animals fed with *elt-2* RNAi were exposed to *S. aureus* full lawn and scored for survival. EV, empty vector RNAi control. WT EV vs. *npr-15(tm12539)* EV, P < 0.0001; *elt-2* RNAi, P <0.0001; *npr-15(tm12539);elt-2* RNAi, P=NS. *elt-2* RNAi vs *npr-15(tm12539); elt-2* RNAi, P<0.001.

B. WT and *npr-15(tm12539)* animals fed with *hlh-30* RNAi were exposed to *S. aureus* full lawn and scored for survival. EV, empty vector RNAi control. WT EV vs. *npr-15(tm12539)* EV, P < 0.0001; *hlh-30* RNAi, P <0.05; *npr-15(tm12539);hlh-30* RNAi, P<0.05. *hlh-30* RNAi vs *npr-15(tm12539); hlh-30* RNAi, P<0.0001.

C. WT and *npr-15(tm12539)* animals fed with *hlh-30* and *elt-2* RNAi were exposed to *S. aureus* full lawn and scored for survival. EV, empty vector RNAi control. WT EV vs. *npr-15(tm12539)* EV, P < 0.0001; *npr-15(tm12539);hlh-30;elt-2* RNAi, P <0.001. WT;*elt-2* RNAi vs *npr-15(tm12539);hlh-30;elt-2* RNAi, P=NS.

D. WT and *npr-15(tm12539)* animals fed with *pmk-1* RNAi and animals were exposed to *S. aureus* full lawn and scored for survival. EV, empty vector RNAi control. WT EV vs. *npr-15(tm12539)* EV, P < 0.0001; *pmk-1* RNAi, P <0.0001; *npr-15(tm12539);pmk-1* RNAi, P< 0.0001. *pmk-1* RNAi vs *npr-15(tm12539);pmk-1* RNAi, P<0.0001.

E. WT and *npr-15(tm12539)* animals fed with *daf-16* RNAi were exposed to *S. aureus* full lawn and scored for survival. EV, empty vector RNAi control. WT EV vs. *npr-15(tm12539)* EV, P < 0.0001; *daf-16* RNAi, P =NS; *npr-15(tm12539);daf-16* RNAi, P<0.0001. *daf-16* RNAi vs *npr-15(tm12539);daf-16* RNAi, P<0.0001.

F. WT and *npr-15(tm12539)* animals fed with *skn-1* RNAi were exposed to *S. aureus* full lawn and scored for survival. EV, empty vector RNAi control. WT EV vs. *npr-15(tm12539)* EV, P < 0.0001;*skn-1* RNAi, P =NS; *npr-15(tm12539);skn-1* RNAi, P<0.0001. *skn-1* RNAi vs *npr-15(tm12539);skn-1* RNAi, P<0.0001.

synaptic connections between all the sensory neurons (**Figure 4A**). Next, we asked if NPR-15 could be acting in a neuron-intrinsic manner to control immune response. To address this question, we specifically inactivated *npr*-15 in neurons and the intestine using tissue-specific RNAi strains and assessed the survival of the animals after *S. aureus* infection. Unlike intestine-specific RNAi (strain MGH171) (Figure S4A), neural-specific RNAi (strain TU3401) of NPR-15 resulted in a pathogen resistance phenotype similar to that of *npr-15(tm12539)* animals (Figure S4B). This finding was further supported by rescuing NPR-15 under the control of a pan-neuronal promoter and exposing the animals to *S. aureus*. The results demonstrated that pan-neuronal promoter-driven expression of NPR-15 rescued the enhanced survival phenotype of *npr-15(tm12539)* animals (**Figure 4B**). These results suggest that NPR-15 suppresses immunity through the nervous system. We next studied the specific NPR-15-expressing neuronal cells (ASG, ASI, ASJ, ASE, AFD, AWC) that could control the defense response against pathogen infection. To identify the neuronal cells responsible for the NPR-15-mediated immune control, we crossed strains lacking the neurons with *npr-15(tm12539)* animals and studied their effect on defense against pathogen infection (**Figure 4C-E** and S4C-E). We found that ASJ(-) animals exhibited resistance to pathogen-mediated killing similar to that of *npr-15(tm12539)* animals (**Figure 4C**). Although the ASG(-) and ASE(-) neuron-ablated strains demonstrated pathogen resistance phenotype, there is a significant difference compared to that of *npr-15(tm12539)* animals (**Figure 4D-E**). Hence, we further confirmed the role of NPR-15/ASJ neurons in suppressing immunity by rescuing NPR-15 on ASJ unique promoter and performing survival experiments with the rescued strain. Results showed that the ASJ-specific rescue of NPR-15 successfully blocked the enhanced survival of *npr-15(tm12539)* animals (**Figure 4F**). We also quantified the expression of immune genes and found that they were upregulated in ASJ(-) animals (**Figure 4G**). Collectively, these findings suggest that NPR-15 controls immunity in a manner similar to that of ASJ neurons.

The lack of avoidance behavior by NPR-15 loss-of-function is independent of immunity and neuropeptide genes

Having established the upregulation of immune genes in *npr-15(tm12539)* animals compared to WT animals (**Figure 2B-F** and Table S3), we determine whether the reduced pathogen avoidance of *npr-15(tm12539)* animals could be attributed to the upregulation of immune pathways. To investigate this, we employed RNA interference to suppress immune transcription factors/regulators and evaluated their impact on pathogen avoidance behavior in both WT and *npr-15(tm12539)* animals. Our results indicate that none of the tested immune regulators (*elt-2, pmk-1, daf-16,* and *hlh-30*) were able to suppress the lack of pathogen avoidance behavior observed in response to *S. aureus* (Figure S5A-D). Furthermore, we inactivated immune genes that are not controlled by the immune regulators tested above, but none of them were able to suppress the lack of avoidance behavior in the *npr-15(tm12539*) animals (Table S5). These findings indicate that the avoidance behavior observed in *npr-15(tm12539)* is independent of the upregulated immune genes.

Additionally, previous studies have shown that neuropeptides expressed in the intestine can modulate avoidance behavior (47), and we found that neuropeptides are among the most highly upregulated genes in *npr-15(tm12539)* animals (**Figure 2B** and Table S4). To elucidate the neuronal-intestinal axis through which NPR-15 regulates avoidance behavior and explore the mechanism by which immunity and avoidance interplay via this axis, we inactivated the upregulated intestinal-expressed neuropeptides in *npr-15(tm12539)* animals. Unfortunately, our experiments revealed that none of the inactivated intestinal-expressed neuropeptides were able to suppress the lack of avoidance behavior of *npr-15(tm12539)* animals in response to *S. aureus* (Figure S5E and Table S5). Therefore, it can be concluded that the absence of avoidance behavior by loss of NPR-15 function is independent of both immune and intestinal neuropeptide signaling pathways.

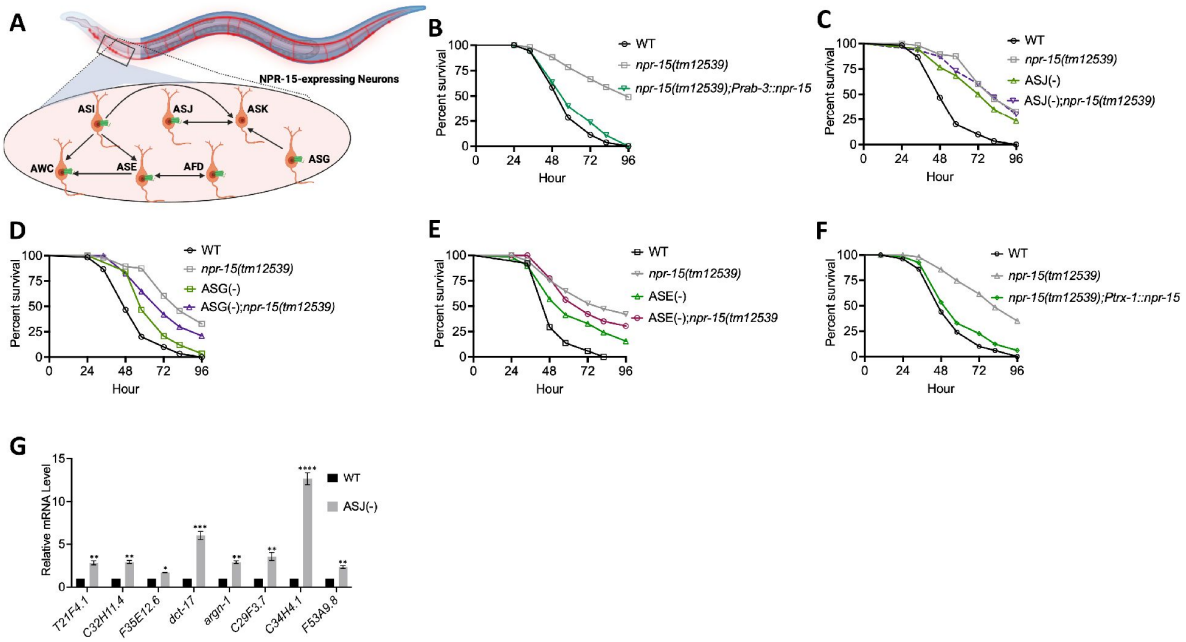

Figure 4.

NPR-15 suppresses molecular immune response via sensory neurons, ASJ.

A. Neuronal connectome of NPR-15-expressing neurons.

B. WT, *npr-15(tm12539),* and *npr-15(tm12539);Prab-3::npr-15* animals were exposed to *S. aureus* full lawn and scored for survival. WT vs *npr-15(tm12539)*, P < 0.0001; *npr-15(tm12539);Prab-3::npr-15*, P=NS.

C. WT, *npr-15(tm12539),* ASJ(-), and ASJ(-);*npr-15(tm12539)* animals were exposed to *S. aureus* full lawn and scored for survival. WT vs *npr-15(tm12539)*, P < 0.0001; ASJ(-), P < 0.001; ASJ(-);*npr-15(tm12539)*, P < 0.0001. ASJ(-) vs. ASJ(-);*npr-15(tm12539)*, P =NS.

D. WT, *npr-15(tm12539),* ASG(-), and ASG(-);*npr-15(tm12539)* animals were exposed to *S. aureus* full lawn and scored for survival. WT vs *npr-15(tm12539)*, P < 0.0001; ASG(-), P < 0.001; ASG(-);*npr-15(tm12539)*, P < 0.0001. ASG(-) vs. ASG(-);*npr-15(tm12539)*, P < 0.05.

E. WT, *npr-15(tm12539),* ASE(-), and ASE(-);*npr-15(tm12539)* animals were exposed to *S. aureus* full lawn and scored for survival. WT vs *npr-15(tm12539)*, P < 0.0001; ASE(-), P < 0.001; ASE(-);*npr-15(tm12539)*, P < 0.0001. ASE(-) vs. ASG(-);*npr-15(tm12539)*, P < 0.05.

F. WT, *npr-15(tm12539), npr-15(tm12539);Ptrx-1::npr-15* animals were exposed to *S. aureus* full lawn and scored for survival. WT vs *npr-15(tm12539);Ptrx-1::npr-15*, P = NS.

G. qRT-PCR analysis of ELT-2 and HLH-30-depenent immune gene expression in WT and ASJ(-) animals. Bars represent means while error bars indicate SD of three independent experiments; *p < 0.05, **p < 0.001 and ***p < 0.0001.

NPR-15 controls pathogen avoidance via sensory neuron, ASJ, and intestinal TRPM channel, GON-2

Having demonstrated that NPR-15 controls immune response via sensory neurons, ASJ (**Figure 4C**, 4F-G), we sought to identify the neurons involved in the lack of avoidance behavior to *S. aureus* observed in *npr-15(tm12539)* animals. First, we investigated the avoidance behavior of a pan-neuronal rescued strain of NPR-15, which successfully rescued the suppressed pathogen avoidance of *npr-15(tm12539)* animal (**Figure 5A**). Next, we asked which of the NPR-15-expressing-neuronal cells could control pathogen avoidance. To answer this question, we evaluated the pathogen avoidance of the different neuron-ablated strains that were crossed with *npr-15(tm12539)* animals. Consistently with our previous findings, we found that only ASJ(-) exhibited reduced pathogen avoidance similar to that of *npr-15(tm12539)* animals (**Figure 5B**). This observation was further confirmed by rescuing NPR-15 under the control of an ASJ unique promoter (**Figure 5C**). The pathogen avoidance of other neuronal-ablated strains was comparable to that of WT animals (**Figure 5D-G**). These results suggest that the loss of NPR-15 function suppresses behavioral immunity via sensory neurons, specifically ASJ.

In a recent study, it was shown that transient receptor potential melastatin (TRPM) ion channels, specifically GON-2 and GTL-2, are associated with a lack of pathogen avoidance (32). To investigate further, we inactivated *gon-2* and *gtl-2* in *npr-15(tm12539)* animals and WT animals. Our findings showed that only *gon-2* null animals, but not *gtl-2*, exhibited pathogen avoidance behavior similar to that of *npr-15(tm12539)* animals (**Figure 5H-I**). This suggests that both NPR-15 and GON-2 may function in a shared pathway to regulated pathogen avoidance behavior. As it has been previously demonstrated that GON-2 modulates avoidance behavior through the intestine (32), we confirmed the role of intestinal-expressed GON-2 in pathogen avoidance by inactivating *gon-2* in an RNAi intestine-specific strain (MGH171), as well as in an RNAi neuron-specific strain TU3401) that was used as a control. These animals were exposed to *S. aureus* to study their lawn occupancy. Our results showed that the inactivation of *gon-2* in MGH171 and MGH171;*npr-15(tm12539)* (**Figure 5J**), but not in TU3401 (Figure S6), exhibited comparable avoidance behaviors to that of *npr-15(tm12539)* animals. These results suggest that NPR-15 acts through the intestinal TRPM channel GON-2 to control pathogen avoidance behavior. Since we have previously shown that only ASJ(-) animals among other neuron-ablated strains exhibit a similar avoidance behavior to *npr-15(tm12539)* animals (**Figure 5B**, 5D-G), we investigated whether NPR-15 control of avoidance behavior towards *S. aureus* in a GON-2-dependent manner involves ASJ. We used RNAi to inactivate *gon-2* in ASJ(-), ASJ(-);*npr-15(tm152799)*, *npr-15(tm12539)*, and WT animals before exposing them to *S. aureus* to assay their lawn occupancy. Our results showed that the avoidance behavior of ASJ(-);*gon-2* is comparable to that of ASJ(-), ASJ(-);*npr-15(tm12539)*, and *gon-2* null animals when exposed to *S. aureus* (**Figure 5K**). These results suggest that NPR-15 controls *S. aureus*, avoidance in a GON-2-dependent manner via the ASJ neuron. In summary, the neuronal GPCR/NPR-15 plays a dual role: suppressing the immune response and enhancing avoidance behavior towards *S. aureus* through sensory neurons, specifically ASJ. The control of immunity against the pathogen *S. aureus* is dependent on the ELT-2 and HLH-30 transcription factors, while the control of avoidance behavior is GON-2-dependent and mediated through the intestine (**Figure 6**).

Discussion

GPCRs play a crucial role in neuronal and non-neuronal tissues in shaping both immune and avoidance behavioral responses toward invading pathogens (48). In this study, we investigated the dual functionality of GPCR/NPR-15 in regulating molecular innate immunity and pathogen avoidance behavior independently of aerotaxis. Additionally, we aimed to understand the mechanism by which the nervous system controls the interplay between these crucial survival strategies in response to pathogenic threats. Our findings indicate that NPR-15 suppresses immune

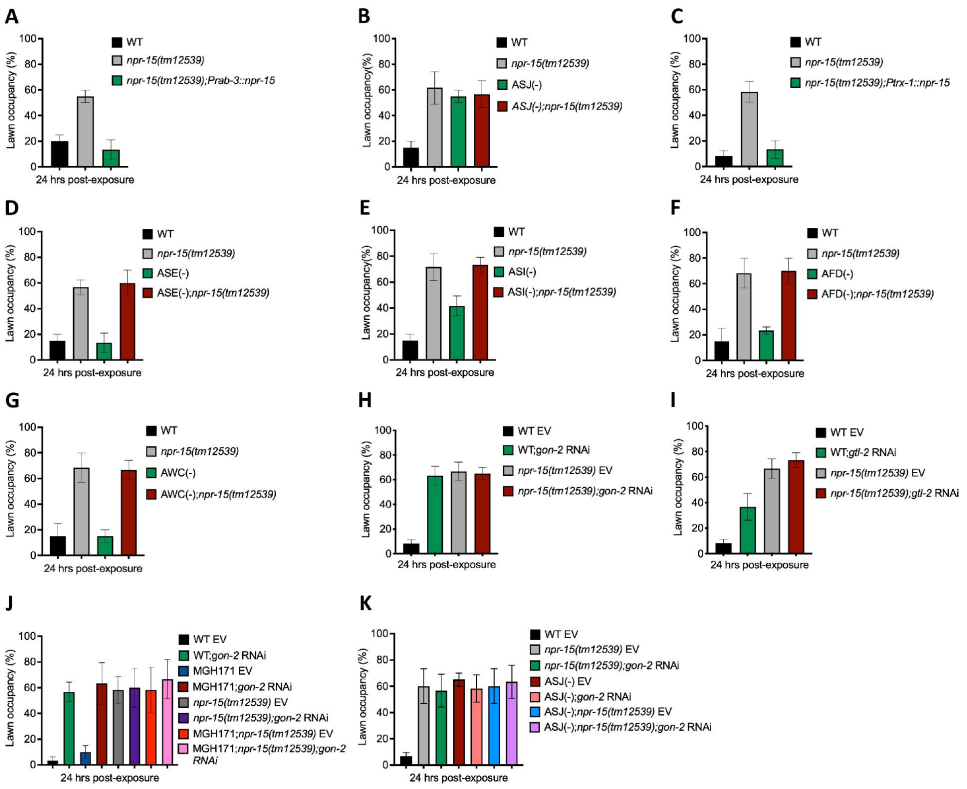

Figure 5

NPR-15 loss-of-function inhibits pathogen avoidance behavior in an ASJ-dependent manner.

A. WT, *npr-15(tm12539),* and *npr-15(tm12539);Prab-3::npr-15* animals were exposed to partial lawn of *S. aureus* and scored for lawn occupancy. WT vs *npr-15(tm12539)*, P < 0.0001; *npr-15(tm12539);Prab-3::npr-15*, P=NS.

B. WT, *npr-15(tm12539),* ASJ(-), and ASJ(-);*npr-15(tm12539)* animals were exposed to partial lawn of *S. aureus* and scored for lawn occupancy. WT vs *npr-15(tm12539)*, P < 0.0001; ASJ(-), P < 0.001; ASJ(-);*npr-15(tm12539)*, P < 0.0001. ASJ(-) vs. ASJ(-);*npr-15(tm12539)*, P =NS.

C. WT, *npr-15(tm12539),* and *npr-15(tm12539);Ptrx-1::npr-15* animals were exposed to partial lawn of *S. aureus* and scored for lawn occupancy. WT vs *npr-15(tm12539)*, P < 0.0001; *npr-15(tm12539);Ptrx-1::npr-15*, P=NS.

D. WT, *npr-15(tm12539),* ASE(-), and ASE(-);*npr-15(tm12539)* animals were exposed to partial lawn of *S. aureus* and scored for lawn occupancy. WT vs *npr-15(tm12539)*, P < 0.0001; ASE(-), P < 0.001; ASE(-);*npr-15(tm12539)*, P < 0.05. ASE(-) vs. ASE(-);*npr-15(tm12539)*, P < 0.0001.

E. WT, *npr-15(tm12539),* ASI(-), and ASI(-);*npr-15(tm12539)* animals were exposed to partial lawn of *S. aureus* and scored for lawn occupancy. WT vs *npr-15(tm12539)*, P < 0.001; ASI(-), P < 0.001; ASI(-);*npr-15(tm12539)*, P < 0.0001. ASI(-) vs. ASI(-);*npr-15(tm12539)*, P < 0.001.

F. WT, *npr-15(tm12539),* and AFD(-) animals were exposed to partial lawn of *S. aureus* and scored for lawn occupancy. WT vs *npr-15(tm12539)*, P < 0.001; AFD(-), P =NS.

G. WT, *npr-15(tm12539),* and AWC(-) animals were exposed to partial lawn of *S. aureus* and scored for lawn occupancy. WT vs *npr-15(tm12539)*, P < 0.001; AWC(-), P =NS.

H. WT and *npr-15(tm12539)* fed with *gon-2* RNAi and were exposed to the partial lawn of *S. aureus* and scored for lawn occupancy. EV, empty vector RNAi control. WT EV vs WT;*gon-2*, P<0.0001; *npr-15(tm12539);gon-2*, P<0.0001. WT;*gon-2* vs *npr-15(tm12539);gon-2*, P=NS.

I. WT and *npr-15(tm12539)* fed with *gtl-2* RNAi and were exposed to the partial lawn of *S. aureus* and scored for lawn occupancy. EV, empty vector RNAi control. WT EV vs WT;*gtl-2*, P<0.001; *npr-15(tm12539);gtl-2*, P<0.0001. WT;*gtl-2* vs *npr-15(tm12539);gtl-2*, P<0.001.

J. WT, *npr-15(tm12539),* RNAi intestine-specific strain MGH171, MGH171; *npr-15(tm12539)* fed with *gon-2* RNAi and were exposed to the partial lawn of *S. aureus* and scored for lawn occupancy. EV, empty vector RNAi control. MGH171 EV vs MGH171;*gon-2*, P<0.0001. MHG171;*gon-2* vs *npr-15(tm12539);gon-2*, P=NS.

K. WT, *npr-15(tm12539),* ASJ(-), ASJ(-);*npr-15(tm12539)* fed with *gon-2* RNAi and were exposed to the partial lawn of *S. aureus* and scored for lawn occupancy. EV, empty vector RNAi control. WT;*gon-2* RNAi vs ASJ(-);*gon-2*, P=NS.

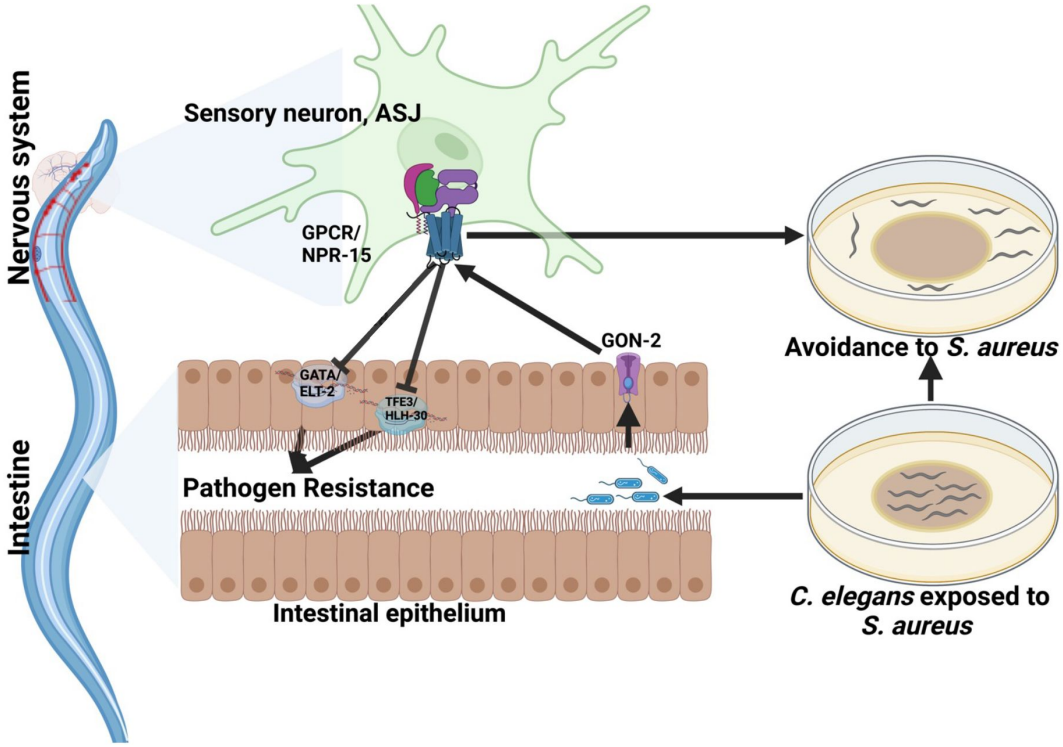

Figure 6

GPCR/NPR-15 suppressed immune response and avoidance behavior via sensory neurons, ASJ. The immune response control is dependent on ELT-2 and HLH-30 transcription factors. While NPR-15 controls avoidance behavior to *S. aureus* via intestinal-expressed TRPM channel, GON-2.

response against different pathogen infections by inhibiting the activity of the GATA/ELT-2 and HLH-30 immune transcription regulators. Moreover, we found that the control of the pathogen avoidance behavior by NPR-15 is independent of aerotaxis and dependent on intestinal GON-2. Furthermore, we demonstrated that NPR-15 controls the immunity-behavioral response via an amphid sensory neuron, ASJ, in response to *S. aureus* infection. These results provide novel insights into the role of neuronal GPCR signaling in the intricate regulation of immune activation and avoidance behavioral responses evolutionarily conserved across metazoans (5, 13, 14).

We explored the mechanistic interconnections and interplay between immunity and avoidance behavior during pathogen infection, focusing on the role of GPCR/NPR-15 via the sensory neuron ASJ. We demonstrated that NPR-15 suppresses immune response by decreasing the expression of immune genes controlled by GATA/ELT-2 and HLH-30, which are key regulators of epithelial immune responses in *C. elegans* against both Gram-negative and Gram-positive bacteria (27, 28, 29, 31). Various GPCRs have been implicated in immune response control. For instance, the octopamine receptor OCTR-1 (20), olfactory learning receptor OLRN-1 (49), D1-like dopamine receptor DOP-4 (50), and neuropeptide receptor family NPR-1, NPR-9, and NPR-8 (16, 35, 51) have been shown to regulate immune responses via the p38/PMK-1 MAPK pathway. In contrast to these GPCRs, our study highlights the role of NPR-15 in maintaining homeostasis during defense against bacterial infections by suppressing excessive immunity through the inactivation of ELT-2 and HLH-30 transcription factors. Interestingly, while NPR-15 prevents immune activation, it is necessary for eliciting avoidance behavior toward *S. aureus*. Previous research has identified NPR-1 (16) and PCDR-1 (52) as GPCRs involved in regulating pathogen avoidance behavioral responses. Unlike NPR-15 mutations, mutations in NPR-1 lead to increased susceptibility to pathogens infection (16), and aerotaxis plays a critical role in the behavioral response of *npr-1* mutants (16, 53). On the other hand, PCDR-1 appears to play a minimal role in regulating immune and behavioral responses to pathogens (52).

ASJ neurons are known to regulate different biological functions such as lifespan (54), dauer activities (55, 56), head-directed light avoidance (57), and food search (58). We show here that ASJ neurons play a role in the control of immune and behavioral responses against pathogen infection through GPCR/NPR-15. Several other aforementioned GPCRs are known to control immunity via neuronal cells in *C. elegans*. NPR-1 functions in AQR, PQR, and URX sensory neurons to control the p38/PMK-1 pathway (16). OCTR-1 activity in ASH and ASI neurons suppresses the innate immunity in the p38/PMK-1 pathway and unfolded protein response (UPR) pathways (20, 59). Furthermore, OLRN-1 is required for the AWC olfactory neurons to activate immunity (60), while DOP-4, a D1-like dopamine receptor, functions in ASG neurons to modulate the p38/PMK-1 pathway (50). NPR-9, a human gastrin-releasing peptide receptor ortholog, regulates innate immunity through AIB interneurons (51), and NPR-8 modulates innate immunity during infections via AWB, ASJ, and AWC neurons (35). These studies collectively emphasize the critical role of neuronal GPCRs in the immune response to pathogen infection in *C. elegans*.

Conclusion

Our research uncovers the dual regulatory role of NPR-15, an evolutionarily conserved neuronal GPCR, in both immunity and avoidance behavior, independent of aerotaxis, mediated by amphid sensory neurons. The host relies on behavioral responses to minimize or totally avoid pathogen exposure, effectively preventing the activation of the immune pathways and production of immune effector molecules, which can be metabolically costly. Moreover, the sustained and prolonged activation of the molecular immune system can have detrimental effects on the host.

Understanding the organismal control of molecular and behavioral immune responses to pathogens can provide valuable insights into universal mechanisms used across species to maintain homeostasis during infections.

Materials and Methods

Bacterial strains

The bacterial strains used in this study are *Escherichia coli* OP50, *E. coli* HT115(DE3) (61), *Pseudomonas aeruginosa* PA14, *P. aeruginosa* PA14-GFP (62, 63), *Salmonella enterica* Serovar Typhimurium 1344 (64), and *Staphylococcus aureus* strain NCTCB325 (65). Gram-negative bacteria were grown in Luria-Bertani (LB) broth. *Staphylococcus aureus* strain NCTCB325 was grown in Tryptic Soy Agar prepared with nalidixic acid. All bacteria were grown at 37°C.

C. elegans Strains and Growth Conditions

Hermaphrodite *C. elegans* (var. Bristol) Wild-type (WT) was used as control unless otherwise indicated. *C. elegans* strains RB1429 *npr-15(ok1626)*, CX14394 *npr-5(ok1583)*, RB1836 *npr-14(ok2375)*, RB1289 *npr-18(ok1388)*, RB1405 *npr-22(ok1598)*, VC2526 *npr-35(ok3258)*, DA609 *npr-1(ad609)*, IM222 *npr-1(ur89)*, CX4148 *npr-1(ky13)*, CZ9957 *gtl-2(n2618)*, EJ1158 *gon-2(q388)*, EJ26 *gon-2(q362)*, LH202 *gtl-2(tm1463)*, JIN1375 *hlh-30(tm1978)*, CF1038 *daf-16(mu86)*, KU25 *pmk-1(km25)*, RB1668 *C02H7.2(ok2068)*, RB1632 *T02E9.1(ok2008)*, VC2421 *R106.2(ok3192)*, PS8315 *npr-29(sy1270)*, RB1365 *npr-16(ok1541)*, RB1958 *T07D4.1(ok2575)*, PS8484 *npr-31(sy1360)*, RB1429 *npr-15(ok1626)*, RB1325 *C53C7.1(ok1442)*, RB1938 *Y116A8B.5(ok2541)*, PS8177 *npr-23(sy1203)*, PS8317 *npr-33(sy1272)*, PS8450 *npr-27(sy1315)*, PS8444 *npr-21(sy1309)*, PS8442 *npr-26(sy1307)*, CX14394 *npr-5(ok1583)*, RB1836 *W05B5.2(ok2375)*, RB1405 *Y59H11AL.1(ok1598)*, MGH171 *alxIs9* [*vha-6p::sid-1::SL2::GFP*], TU3401 *uIs69* [*pCFJ90 (myo-2p::mCherry) + unc-119p::sid-1*], DA609 *npr-1(ad609)*, DA508 *npr-1(n1353)*, GR2245 *skn-1(mg570)*, were obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN). We also obtained FX22538 *npr-15(tm12539)*, FX21950 *npr-3(tm1583)*, FX01583 *npr-3(tm11950)*, FX01782 *npr-4(tm1782)*, FX01497 *npr-6(tm1497)*, FX01498 *npr-12(tm1498)* and FX01504 *npr-13(tm1504)*, FX01665 *npr-34(tm1665)*, FX31809 *npr-30(tm6617)*, FX21079 *npr-28(tm1155)*, FX22538 *npr-15(tm12539)*, FX21950 *npr-3(tm11950)*, FX01583 *npr-3(tm1583)*, FX01782 *npr-4(tm1782)*, FX01497 *npr-6(tm1497)*, FX01498 *npr-12(tm1498)*, FX01504 *npr-13(tm1504)*, FX03170 *npr-17(tm3170)*, FX03225 *npr-17(tm3225)*, FX03170 *npr-17(tm3170)*, FX03225 *npr-17(tm3225)*, FX31312 *npr-22(tm8953)* from National Bioresource Project (NBRP), Japan. ASE(-);*npr-15(tm12539)*, ASI(-);*npr-15(tm12539)*, ASJ(-);*npr-15(tm12539)*, ASG(-);*npr-15(tm12539)*, *daf-16(mu86); npr-15(tm12539)*, *pmk-1(km25);npr-15(tm12539)*, *hlh-30(tm1978);npr-15(tm12539)*, *skn-1(mg570);npr-15(tm12539)* and ASG(-):*npr-15(tm12539)* were obtained by standard genetic crosses. Rescued strain *npr-15(tm12539);Pnpr-15::npr-15*, neuronal rescued strain *npr-15(tm12539);Prab-3::npr-15* and *npr-15* rescued in ASJ neuron-specific, *npr-15(tm12539);Ptrx-1::npr-15*were generated as described below. The ASG(-) ablated Ex[Ptax-2::CZ::ced-3(p17)::unc-54 3'UTR + Plim-6::ced-3(p15)-NZ::unc-54 3'UTR, pRF4] was generated from our previous project (66). The strains were crossed with the WT laboratory N2. All strains were grown at 20°C on nematode growth medium (NGM) plates seeded with *E. coli* OP50 as the food source (67) unless otherwise indicated. The recipe for the control NGM plates is: 3 g/l NaCl, 3 g/l peptone, 20 g/l agar, 5 µg/ml cholesterol, 1 mM MgSO4, 1 mM CaCl2, and 25 mM potassium phosphate buffer (pH 6.0). The NGM plates were without antibiotics except indicated.

RNA Interference (RNAi)

Knockdown of targeted genes was obtained using RNAi by feeding the animal with *E. coli* strain HT115(DE3) expressing double-stranded RNA (dsRNA) homologous to a target gene (68, 69). RNAi was carried out as described previously (Sun et al., 2011; Singh and Aballay, 2017). Briefly, *E. coli* with the appropriate vectors were grown in LB broth containing ampicillin (100 µg/mL) and

tetracycline (12.5 µg/mL) at 37°C overnight and plated onto NGM plates containing 100 µg/mL ampicillin and 3 mM isopropyl β-D-thiogalactoside (IPTG) (RNAi plates). RNAi-expressing bacteria were grown at 37°C for 12-14 hours. Gravid adults were transferred to RNAi-expressing bacterial lawns and allowed to lay eggs for 2-3 hours. The gravid adults were removed, and the eggs were allowed to develop at 20°C to young adults. This was repeated for another generation (except for ELT-2 RNAi) before the animals were used in the experiments. The RNAi clones were from the Ahringer RNAi library.

C. elegans Survival Assay on Bacterial Pathogens

P. aeruginosa and *S. enterica* were incubated in LB medium. *S. aureus* was incubated in a TSA medium with nalidixic acid (20 µg/mL). The incubations were done at 37°C with gentle shaking for 12 hours. *P. aeruginosa* and *S. enterica* were grown on a modified NGM agar medium of 0.35% peptone and TSA respectively. For partial lawn assays, 20 µl of the overnight bacterial cultures were seeded at the center of the relevant agar plates without spreading. For full lawn experiments, 20 µl of the bacterial culture was seeded and spread all over the surface of the agar plate. No antibiotic was used for *P. aeruginosa* and *S. enterica*, while nalidixic acid (20 µg/mL) was used for the TSA plates for *S. aureus*. The seeded plates were allowed to grow for 12 at 37°C. The plates were left at room temperature for at least 1 hr before the infection experiments. 20 synchronized young adult animals were transferred to the plates for infection, three technical replicate plates were set up for each condition (n = 60 animals) and the experiments were performed in triplicate. The plates were then incubated at 25°C. Scoring was performed every 12 hr for *P. aeruginosa* and *S. aureus*, and 24 hr for *S. enterica*. Animals were scored as dead if the animals did not respond to touch by a worm pick or lack of pharyngeal pumping. Live animals were transferred to fresh pathogen lawns each day. All *C. elegans* killing assays were performed three times independently.

Bacterial lawn avoidance assay

Bacterial lawn avoidance assays were performed by 20 mL of *P. aeruginosa* PA14 and *S. aureus* NCTCB325 on 3.5-cm modified NGM agar plates (0.35% peptone) and (0.35 % TSA) respectively, which were cultured at 37°C overnight to have a partial lawn. The modified NGM plates were left to cool to room temperature for about 1 hour, and twenty young adult animals grown on *E. coli* OP50 were transferred to the center of each bacterial lawn after it. The number of animals on the bacterial lawns was counted at 12 and 24 hours after exposure.

Avoidance assays at 8% oxygen

Avoidance assays as described above were carried out in a hypoxia chamber. Briefly, after young gravid adult hermaphroditic animals were transferred to the avoidance plates, the plates were placed in the hypoxia chamber and the lids of the plates were removed. The chamber was purged with 8% oxygen (balanced with nitrogen) for 5 min at a flow rate of 25 L/min. The chamber was then sealed, and assays were carried out. Control plates were incubated at ambient oxygen.

Pharyngeal pumping rate assay

WT and *npr-15(tm12539)* animals were synchronized by placing 20 gravid adult worms on NGM plates seeded with *E. coli* OP50 and allowing them to lay eggs for 60 min at 20°C. The gravid adult worms were then removed, and the eggs were allowed to hatch and grow at 20°C until they reached the young adult stage. The synchronized worms were transferred to NGM plates fully seeded with P. aeruginosa for 24 hours at 25°C. Worms were observed under the microscope with a focus on the pharynx. The number of contractions of the pharyngeal bulb was counted over 60 s. Counting was conducted in triplicate and averaged to obtain pumping rates.

Defecation rate assay

WT and *npr-15(tm12539)* animals were synchronized by placing 20 gravid adult worms on NGM plates seeded with *E. coli* OP50 and allowing them to lay eggs for 60 min at 20°C. The gravid adult worms were then removed, and the eggs were allowed to hatch and grow at 20°C until they reached the young adult stage. The synchronized worms were transferred to NGM plates fully seeded with *P. aeruginosa* for 24 hours at 25°C. Worms were observed under a microscope at room temperature. For each worm, an average of 10 intervals between two defecation cycles was measured. The defecation cycle was identified as a peristaltic contraction beginning at the posterior body of the animal and propagating to the anterior part of the animal followed by feces expulsion.

Brood Size Assay

The brood size assay was done following the earlier described methods (70, 71). Ten L4 animals from egg-synchronized populations were transferred to individual NGM plates (seeded with *E. coli* OP50) (described above) and incubated at 20°C. The animals were transferred to fresh plates every 24 hours. The progenies were counted and removed every day.

C. elegans Longevity Assays

Longevity assays were performed on NGM plates containing live, UV-killed *E. coli* strains HT115 or OP50 as described earlier (20, 36, 66, 72, 73). Animals were scored as alive, dead, or gone each day. Animals that failed to display touch-provoked or pharyngeal movement were scored as dead. Experimental groups contained 60 to 100 animals and the experiments were performed in triplicate. The assays were performed at 20°C.

Intestinal Bacterial Loads Visualization and Quantification

Intestinal bacterial loads were visualized and quantified as described earlier (20, 72). Briefly, *P. aeruginosa*-GFP lawns were prepared as described above. The plates were cooled to ambient temperature for at least an hour before seeding with young gravid adult hermaphroditic animals and the setup was placed at 25°C for 24 hours. The animals were transferred from *P. aeruginosa*-GFP plates to the center of fresh *E. coli* plates for 10 min to eliminate *P. aeruginosa*-GFP on their body. The step was repeated two times more to further eliminate external *P. aeruginosa*-GFP left from earlier steps. Subsequently, ten animals were collected and used for fluorescence imaging to visualize the bacterial load while another ten were transferred into 100 µL of PBS plus 0.01% Triton X-100 and ground. Serial dilutions of the lysates (10^1-10^{10}) were seeded onto LB plates containing 50 µg/mL of kanamycin to select for *P. aeruginosa*-GFP cells and grown overnight at 37 °C. Single colonies were counted the next day and represented as the number of bacterial cells or CFU per animal.

Fluorescence Imaging

Fluorescence imaging were carried out as described previously (71). Briefly, animals were anesthetized using an M9 salt solution containing 50 mM sodium azide and mounted onto 2% agar pads. The animals were then visualized for bacterial load using a Leica M165 FC fluorescence stereomicroscope. The diameter of the intestinal lumen was measured using Fiji-ImageJ software. At least 10 animals were used for each condition.

RNA Sequencing and Bioinformatic Analyses

Approximately 40 gravid WT and *npr-15(tm12539)* animals were placed for 3 hours on 10-cm NGM plates (seeded with *E. coli* OP50) (described above) to have a synchronized population, which developed and grew to L4 larval stage at 20°C. Animals were washed off the plates with M9 and frozen in QIAzol by ethanol/dry ice and stored at –80 prior to RNA extraction. Total RNA was extracted using the RNeasy Plus Universal Kit (Qiagen, Netherlands). Residual genomic DNA was

removed using TURBO DNase (Life Technologies, Carlsbad, CA). A total of 6 µg of total RNA was reverse-transcribed with random primers using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The library construction and RNA sequencing in Illumina NovaSeq 6000 platform was done following the method described by (74) and (75) pair-end reads of 150 bp were obtained for subsequent data analysis. The RNA sequence data were analyzed using a workflow constructed for Galaxy (*https://usegalaxy.org*) as described (76) and was validated using Lasergene DNA star software. The RNA reads were aligned to the *C. elegans* genome (WS271) using the aligner STAR. Counts were normalized for sequencing depth and RNA composition across all samples. Differential gene expression analysis was then performed on normalized samples. Genes exhibiting at least two-fold change were considered differentially expressed. The differentially expressed genes were subjected SimpleMine tools from wormbase (*https://www.wormbase.org/tools/mine/simplemine.cgi*) to generate information such as wormBase ID and gene name, which are employed for further analyses. Gene ontology analysis was performed using the WormBase IDs in DAVID Bioinformatics Database (*https://david.ncifcrf.gov*) (77) and validated using a *C. elegans* data enrichment analysis tool (*https://wormbase.org/tools/enrichment/tea/tea.cgi*). Immune and age determination pathways were obtained using the Worm Exp version 1 (*http://wormexp.zoologie.uni-kiel.de/wormexp/*) (39) using the transcription factor target category. The Venn diagrams were obtained using the web tool InteractiVenn (*http://www.interactivenn.net*) (78) and bioinformatics and evolutionary genomics tool (*http://bioinformatics.psb.ugent.be/webtools/Venn/*). While neuron wiring was done using the database of synaptic connectivity of *C. elegans* for computation (79) *http://ims.dse.ibaraki.ac.jp/ccep-tool/*.

RNA Isolation and Quantitative Reverse Transcription-PCR (qRT-PCR)

Animals were synchronized and total RNA extraction was done following the protocol described above. qRT-PCR was conducted using the Applied Biosystems One-Step Real-time PCR protocol using SYBR Green fluorescence (Applied Biosystems) on an Applied Biosystems 7900HT real-time PCR machine in 96-well-plate format. Twenty-five microliter reactions were analyzed as outlined by the manufacturer (Applied Biosystems). The relative fold changes of the transcripts were calculated using the comparative $CT(2^{-\Delta\Delta CT})$ method and normalized to pan-actin (*act-1, –3, –4*). The cycle thresholds of the amplification were determined using StepOnePlus™ Real-Time PCR System Software v2.3 (Applied Biosystems). All samples were run in triplicate. The primer sequences were available upon request and presented in Table S6.

Generation of transgenic *C. elegans*

To generate *npr-15* rescue animals, the DNA *npr*-15 alongside its promoter was amplified from the genomic DNA of Bristol N2 *C. elegans* adult worms. Plasmid pPD95_77_*Pnpr-15_npr-15* was constructed by linearization of plasmid pPD95_77 using HindIII and SalI restriction digestion enzymes. The amplified *Pnpr-15::npr-15* DNA was cloned behind its native promoter in the plasmid pPD95_77, between HindIII and SalI sites. For the neuronal rescue strain, the plasmid pPD95_77_*Prab-3_npr-15* was constructed by cloning amplified *npr-15* DNA into BamHI/KpnI digested pPD95_77_*Prab-3* under the promoter of *rab-3*. The constructs were purified and sequenced. Young adult hermaphrodite *npr-15(tm12539)* and WT *C. elegans* were transformed by microinjection of plasmids into the gonad as described (80, 81). Briefly, a mixture containing pPD95_77_*Pnpr-15_npr-15* (40 ng/µl) and *Pmyo-3*::mCherry (5 ng/µl) that drives the expression of mCherry to the muscle as a transformation marker were injected into the animals. For the neuronal rescue, a mixture containing pPD95_77_*Prab-3_npr-15* plasmids (40 ng/µl) and *Pmyo-3*::mCherry (5 ng/µl) that drives the expression of mCherry to the pharynx as a transformation marker were injected into the animals. To rescue *npr-15* in ASJ neurons, *Ptrx-1* promoter and *npr-15* were amplified from gDNA. The *npr-15* fragment was cloned downstream of the *Ptrx-1* by in-fusion PCR to obtain *Ptrx-1::npr-15*, which was cloned into pPD95.77 between the PstI and SmaI sites to generate npr-*15(tm12539);Ptrx-1::npr-15*. The ASJ neuron– specific rescue strain

15(tm12539);Ptrx-1::npr-15 was generated by injecting plasmid pPD95_77_ *Ptrx-1::npr-15* (25 ng/µl) with *Pmyo-2*::mCherry (10 ng/µl) as a co-injection marker into the *npr-15(tm12539)* and WT which were crossed to *npr-15(tm12539)* animals. Primers used in the generation of transgenic animals are shown in Table S6.

Quantification and Statistical Analysis

Statistical analysis was performed with Prism 8 version 8.1.2 (GraphPad). All error bars represent the standard deviation (SD). The two-sample t test was used when needed, and the data were judged to be statistically significant when $p < 0.05$. In the figures, asterisks (*) denote statistical significance as follows: ns, not significant, *, $p < 0.05$, **, $p < 0.001$, ***, $p < 0.0001$, as compared with the appropriate controls. The Kaplan-Meier method was used to calculate the survival fractions, and statistical significance between survival curves was determined using the log-rank test. All experiments were performed at least three times.

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Author contributions

B.O and A.A. conceived and designed the experiments. B.O. and A.F.B. performed the experiments. B.O. and A.A. analyzed the data and wrote the paper.

Declaration of interests

The authors declare no competing interests.

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Reviewer #1 (Public Review):

Summary:

Otarigho et al. presented a solid study revealing that in C. elegans, the neuropeptide Y receptor GPCR/NPR-15 mediates both molecular and behavioral immune responses to pathogen attack. Previously, three npr genes were found to be involved in worm defense. In this study, the authors screened mutants in the remaining npr genes against P. aeruginosa-mediated killing and found that npr-15 loss-of-function improved worm survival. npr-15 mutants also exhibited enhanced resistance to other pathogenic bacteria but displayed significantly reduced avoidance to S. aureus, independent of aerotaxis, pathogen intake and defecation. The enhanced resistance in npr-15 mutant worms was attributed to upregulation of immune and neuropeptide genes, many of which were controlled by the transcription factors ELT-2 and HLH-30. The authors found that NPR-15 regulates avoidance behavior via the TRPM gene, GON-2, which has a known role in modulating avoidance behavior through the intestine. The authors further showed that both NPR-15-dependent immune and behavioral responses to pathogen attack were mediated by the NPR-15-expressing neurons

ASJ. Overall, the authors discovered that the NPR-15/ASJ neural circuit may regulate distinct defense mechanisms against pathogens under different circumstances. This study provides novel and useful information to researchers in the fields of neuroimmunology and C. elegans research.

Strengths:

1. This study uncovered specific molecules and neuronal cells that regulate both molecular immune defense and behavior defense against pathogen attack and indicate that the same neural circuit may regulate distinct defense mechanisms under different circumstances. This discovery is significant because it not only reveals regulatory mechanisms of different defense strategies but also suggests how C. elegans utilize its limited neural resources to accomplish complex regulatory tasks.

2. Most conclusions in this study are supported by solid evidence, which are often derived from multiple approaches and/or experiments. Multiple pathogenic bacteria were tested to examine the effect of NPR-15 loss-of-function on immunity; the impacts of pharyngeal pumping and defecation on bacterial accumulation were ruled out when evaluating defense; RNA-seq and qPCR were used to measure gene expression; gene inactivation was done in multiple strains to assess gene function.

3. Gene differential expression, gene ontology and pathway analyses were performed to demonstrate that NPR-15 controls immunity through regulating immune pathways.

4. Elegant approaches were employed to examine avoidance behavior (partial lawn, full lawn, and lawn occupancy) and the involvement of neurons in regulating immunity and avoidance (the use of a diverse array of mutant strains).

5. Statistical analyses were appropriate and adequate.

Weaknesses:

1. The authors identified NPR-15 and ASJ neurons that are involved in both molecular and behavioral responses to pathogen attack. This finding, by itself, is significant. However, how the NPR-15/ASJ circuit regulates the interplay between the two defense strategies was not explored. Therefore, emphasizing the interplay in the title and the abstract is misleading.

2. Although the discovery of a single GPCR regulating both immunity and avoidance behavior is significant and novel, NPR-15 is not the first GPCR identified with these functions. Previously, the same lab reported that the GPCR OCTR-1 also regulates immunity and avoidance behavior through ASH and ASI neurons respectively (PMID: 29117551). This point was not mentioned in the current manuscript.

3. The authors discovered that NPR-15 regulates avoidance behavior via the TRPM gene, GON-2. Only two factors (GON-2 and GTL-2) were examined in this study, and GON-2 happens to function through the intestine. It is possible that NPR-15 may broadly regulate multiple effectors in multiple tissues. Confining the regulation to the amphid sensory neuron-intestinal axis, as stated in the title and elsewhere in the manuscript, is not accurate.

4. The C. elegans nervous system is simple, and hermaphrodites only have 302 neurons. Individual neurons possessing multiple regulatory functions is expected. Whether this is conserved in mammals and other vertebrates is unknown, because in higher animals, neurons and neuronal circuits could be more specialized.

5. A key question, that is, why would NPR-15 suppress immunity (which is bad for defense) but enhance avoidance behavior (which is good for defense), is not addressed or explained. This could be due to temporal regulation, for example, upon pathogen exposure, NPR-15

could regulate behavior to avoid the pathogen, but after infection, NPR-15 could suppress excessive immune responses or quench the responses for the resolution of infection.

6. The discussion appears timid in scope and contains some repetitive statements. Point 5 can be addressed in the Discussion.

Overall, the authors presented an impactful study that identified specific molecules and neuronal cells that regulate both molecular and behavioral immune responses to pathogen attack. Most conclusions are supported by solid evidence. However, some statements are overreaching, for example, regulation of the interplay between molecular and behavioral immune responses was emphasized but not explored. Nonetheless, this study reported a significant and novel discovery and has laid a foundation for investigating such an interplay in the future.

Reviewer #2 (Public Review):

Summary:

The authors are studying the behavioral response to pathogen exposure. They and others have previously described the role that the G-protein coupled receptors in the nervous system plays in detecting pathogens, and initiating behavioral patterns (e.g. avoidance/learned avoidance) that minimize contact. The authors study this problem in C. elegans, which is amenable to genetic and cellular manipulations and allow the authors to define cellular and signaling mechanisms. This paper extends the original idea to now implicate signaling and transcriptional pathways within a particular neuron (ASJ) and the gut in mediating avoidance behaviour.

Strengths:

The work is rigorous and elegant and the data are convincing. The authors make superb use of mutant strains in C. elegans, as well tissue specific gene inactivation and expression and genetic methods of cell ablation. to demonstrate how a gene, NPR15 controls behavioral changes in pathogen infection. The results suggest that ASJ neurons and the gut mediate such effects. I expect the paper will constitute an important contribution to our understanding of how the nervous system coordinates immune and behavioral responses to infection.

Fig. 1/S1. Authors selected a mutant for further study, npr-15, which showed resistance to various pathogens, and less colonization. Data are convincing. Data also suggest that in response to S. aureus, where wt animals exhibit avoidance behavior measured as numbers of animals that move off a focal spot of bugs, the npr-15 mutants do not. The effect was abrogated when a full lawn was used, at least for S. aureus, where there was no place to run. The conclusion is that the NPR-15 mediates behavioral changes resulting in pathogen avoidance.

Comments: There is some variance in lawn occupancy of wt strains between the different trials in WT animals (e.g. in Fig. 1: 25 for wt vs 60% for npr mutant; S1c 5% for wt and 60% for npr mutant). Does this reflect rates of migration or re-occupancy in WT? Does pathogen avoidance persist and/or the rate of avoidance differ in npr mutant worms, and if animals were exposed then re-exposed, could the authors to determine whether a learned avoidance was similarly affected by this mutation by assessing rate changes?

Fig. 2/S2. NPR inhibits expression of immune and aversion pathway genes (ELT-2, HLH-30, PMK-1, and DAF-2/DAF-16). No concerns.

Comment: Is there any difference in gene expression of animals that have migrated off the lawn to those remaining on the lawn (e.g. in partial lawn expts?)

Fig. 3/S3. Let-2RNAi or hlh-30 RNAi abrogates immunity in both WT and npr mutants. Similar effects with mutants. pmk and daf-16 inactivation were without effect.

Comment. No concerns but the P values in the legends are a pain to read. Why not put them in figures as in the above figures.

Fig. 4. Using neuronal and gut specific RNAi, the authors implicate the ASJ neurons in NPR-15 effects (ie in WT animals npr15 RNAi resulted in a pathogen resistance phenotype similar to that of the mutant animals. Specific expression of NPR-15 in the enhanced survival of the npr-15 mutants, an effect rescued by neuronal expression of NPR-15. Using strains lacking particular neurons, they found that strains lacking ASJ- strains phenocopies the npr mutant. Finally, sealing things nicely, they rescued NPR-15 in the mutant on an ASJ-specific Ptrx promoter.

Fig. 5. explores the dependence of pathogen avoidance on ASJ neurons and gut effects. Fig 5 shows that mutation of NPR in ASJ neuron alone phenocopies pathogen avoidance of the global npr mutant, indicating NPR expression in this and only this neurons is required. Fig. 5 also demonstrates that the loss of the ion channel GON-2 phenocopies the npr-15 mutant.

Comments: The authors suggest that the ASJ/NPR15 effect to limit avoidance acts via inhibition of GON-2 in the intestine. The observation that GON-2 inhibition effects on pathogen avoidance occur independently of neurons could suggest that it is a redundant way of accomplishing the same thing, which then makes one ask what the connection exists between the neuron and the gut. The effect of ASJ via NPR on pathogen avoidance is not neuropeptide dependent, which they show. So how does the neuronal-gut communication works. Specific Transmitters... perhaps. Since ASJ neurons control entry into dauer, perhaps isn't surprising that DAF-16 showed up as an NPR-15. induced factor (and dauer worms are resistant to a lot of stressors); that said dauer hormones might be involved as well. Is there any evidence that DAF-16 down-regulates GON-2 expression (see Murphy, Kenyon et al. 2005), and along these lines would GON-2 RNAi work in a DAF-16 mutant? I think addressing these issues are in my view the subject of future studies.

Weaknesses: The paper is solid and elegantly defines the genetic basis of behavioral avoidance via neurons and gut. The neuronal gut connection is shown, but how they are connected remains unsolved. I wouldn't suggest this is a weakness as much as an invitation for future work.