

Extra View

Neural regulation of immunity

Role of NPR-1 in pathogen avoidance and regulation of innate immunity

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Key words: neurons, innate immunity, infection, inflammation, G-protein coupled receptors, GPCR, npr-1

The nervous and immune systems consist of complex networks that have been known to be closely interrelated. However, given the complexity of the nervous and immune systems of mammals, including humans, the precise mechanisms by which the two systems influence each other remain understudied. To cut through this complexity, we used the nematode *Caenorhabditis elegans* as a simple system to study the relationship between the immune and nervous systems using sophisticated genetic manipulations. We found that *C. elegans* mutants in G-protein coupled receptors (GPCRs) expressed in the nervous system exhibit aberrant responses to pathogen infection. The use of different pathogens, different modes of infection and genome-wide microarrays highlighted the importance of the GPCR NPR-1 in avoidance to certain pathogens and in the regulation of innate immunity. The regulation of innate immunity was found to take place at least in part through a mitogen-activated protein kinase signaling pathway similar to the mammalian p38 MAPK pathway. Here, the results that support the different roles of the NPR-1 neural circuit in the regulation of *C. elegans* responses to pathogen infection are discussed.

Introduction

A wealth of data indicates that the nervous system receives inputs from infected local sites and integrates them to coordinate appropriate immune responses.¹⁻³ It has also been postulated that the immune system may function as a "sixth sense", recognizing microorganisms and microbial toxins that cannot be seen, heard, tasted, touched or smelled.² However, given the complexity of the nervous and immune systems of mammals, including humans, the precise mechanisms by which the two systems influence each other remain understudied. While the mammalian nervous system contains hundreds of billions of neurons, the simple nervous system of *C. elegans* only contains 302 neurons and 56 glial cells,

which represent 37% of all somatic cells in an adult hermaphrodite. The simplicity of the *C. elegans* nervous system, together with the great deal of information about the morphology and synaptic connectivity of every neuron in the adult organism, provide an excellent system to study neural circuits that regulate behaviors and different organismal processes, including innate immunity.

C. elegans is a 1 millimeter long nematode that in the laboratory is cultivated on agar plates containing *Escherichia coli*. In nature, *C. elegans* lives in soils and composts rich in microorganisms, and while it lacks adaptive immunity, it has evolved mechanisms to respond to different microorganisms in different ways. For example, *C. elegans* is capable of sensing some bacterial compounds and avoid certain pathogens.^{4,5} The nematode can also respond to pathogen exposure with an inducible innate immune system that comprises conserved effectors such as antimicrobial peptides, lectins, lysozymes.⁶⁻¹⁰ A recent study indicates that the *C. elegans* nervous system may not only regulate avoidance to certain pathogens but also innate immune responses that are independent of the behavioral avoidance to pathogens.¹¹

Mutants in GPCR-Encoding Genes Exhibit Aberrant Responses to Pathogen Infection

GPCRs constitute the largest family of transmembrane signaling proteins that are present in the cell surface of all multicellular organisms where they regulate host physiological processes including homeostasis, metabolism, neurotransmission, cardiovascular function and immune function. Thus, GPCRs represent the most widely targeted pharmacological protein class, accounting for the targets of about one-third of all approved drugs.^{12,13} To study the role of GPCRs in the regulation of innate immune response, the susceptibility of forty *C. elegans* strains carrying mutations in GPCRs to the human opportunistic pathogen *Pseudomonas aeruginosa* strain PA14 was studied. Depending on the statistical analysis used, three or five mutants exhibited enhanced resistance to *P. aeruginosa* while only one or two mutants exhibited enhanced susceptibility to *P. aeruginosa*.¹¹

The strain that consistently exhibited enhanced susceptibility to *P. aeruginosa*-mediated killing was *npr-1(ad609)*, which carries a loss-of-function allele of a gene that encodes NPR-1, a G protein-coupled receptor related to mammalian neuropeptide

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Submitted: 01/30/09; Accepted: 02/04/09

Previously published online as a *Cell Cycle* E-publication:
<http://www.landesbioscience.com/journals/cc/article/8074>

Y receptors.¹⁴ In nature, NPR-1 is found in two allelic forms that differ in a single amino acid at position 215, NPR-1(215V) and NPR-1(215F).¹⁴ The NPR1(215V) allele, which is found in the standard laboratory strain N2, has high activity whereas the NPR-1(215F) allele has low activity.^{15,16} The lifespan of *npr-1(ad609)* animals grown on agar plates covered with killed *E. coli* or *P. aeruginosa* was not different than that of N2 animals,¹¹ indicating that *npr-1(ad609)* animals are deficient in host-defense responses to live *P. aeruginosa*.

Hyperoxia Avoidance Overrides Avoidance to *P. aeruginosa* in Animals with Low NPR-1 Activity

Genetic studies indicate that *npr-1* is part of a neural circuit that integrates behavioral responses to environmental oxygen, bacteria and other animals.^{15,17-21} While N2 animals avoid oxygen levels above 10% when *E. coli* is absent and fail to avoid high oxygen in the presence of *E. coli*, *npr-1(ad609)* animals have strong hyperoxia avoidance in the absence or presence of *E. coli*.¹⁸ As a result, *npr-1(ad609)* animals show a preference for regions in the bacterial lawn rich in metabolically active bacteria that lower oxygen levels¹⁵ (Fig. 1). When *npr-1(ad609)* animals are grown at high densities, they also tend to aggregate into feeding groups to decrease local oxygen concentrations.¹⁸

Since *C. elegans* naturally exhibits an avoidance behavior towards *P. aeruginosa*,^{5,22,23} it is possible that the reduced lifespan of *npr-1(ad609)* animals infected by *P. aeruginosa* is due to a higher exposure to bacteria. As illustrated in Figure 1, while N2 animals can freely enter and exit the *P. aeruginosa* lawn, *npr-1(ad609)* animals spend most of the time on the border of the lawn and in full contact with bacteria.¹¹ *C. elegans* spends less time on the *P. aeruginosa* lawn than on the *E. coli* lawn. To allow *npr-1(ad609)* animals to move more freely on the plates, the infections were performed at 8% oxygen, a favorable oxygen environment that suppresses most behavioral phenotypes of *npr-1* mutants. Under 8% oxygen, *npr-1(ad609)* animals do not exhibit a preference for regions in the agar plates with lower oxygen levels such as the bacterial border, and are capable of leaving the *P. aeruginosa* lawn. At this low oxygen level, *npr-1(ad609)* animals are more resistant to *P. aeruginosa*-mediated killing than at 21% oxygen, indicating that the preference for low oxygen has a deleterious effect on *C. elegans* survival. Additional experiments using agar plates that were either partially covered in *P. aeruginosa* (Fig. 1, partial lawn) or completely covered in *P. aeruginosa* (Fig. 1, full lawn) highlighted the importance of avoidance to *P. aeruginosa*.¹¹ While N2 animals grown on full lawns of *P. aeruginosa* died at a higher rate than N2 animals grown on partial lawns of *P. aeruginosa*, *npr-1(ad609)* animals were equally susceptible to *P. aeruginosa* when grown on full lawns or partial lawns.¹¹ Since *C. elegans* does not avoid *P. aeruginosa* strains PAK1 or PA01,²³ it would be interesting to study the susceptibility of *npr-1(ad609)* animals to those strains.

Avoidance to *P. aeruginosa* is Not the only Determinant of Pathogen Susceptibility in *npr-1* Animals

Even under 8% oxygen or on full lawns of *P. aeruginosa*, two conditions that control for pathogen avoidance, *npr-1(ad609)*

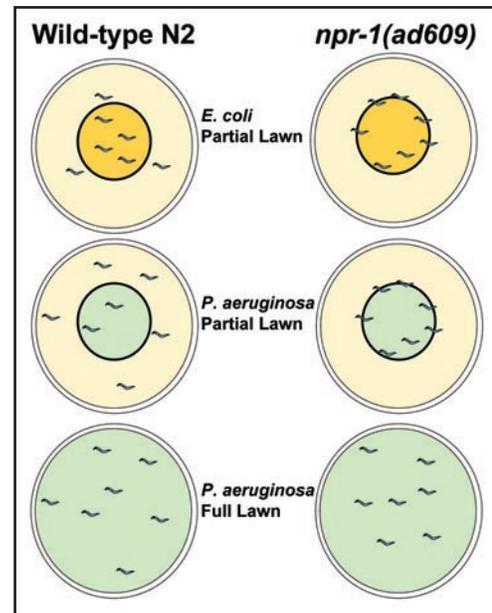


Figure 1. Hyperoxia avoidance overrides avoidance to *P. aeruginosa*. *P. aeruginosa* strain PA14 is known to negatively affect the attraction response of *C. elegans* to *E. coli* OP50, which is the food source of nematodes in the laboratory. As a consequence, animals grown on agar plates containing lawns of *P. aeruginosa* PA14 spend more time outside the bacterial lawn than animals grown on plates containing lawns of *E. coli*. Moreover, *npr-1(ad609)* animals have a strong preference for low oxygen concentrations such as those that exist at the edge of the bacterial lawn, and spend more time on the bacteria lawn than wild-type N2 animals. To expose *npr-1(ad609)* and N2 animals to comparable amounts of *P. aeruginosa*, full lawns of *P. aeruginosa* are used.

animals were found to be significantly more susceptible to *P. aeruginosa*-mediated killing than N2 animals.¹¹ After our study was published, another group of investigators published data that confirm the importance of avoidance to *P. aeruginosa* and show small residual differences between N2 and *npr-1(ad609)* animals under conditions that control for avoidance to *P. aeruginosa*.²⁴ A caveat of these experiments is the use of the drug 5-fluorodeoxyuridine (FUdR) in the assays. FUdR is an inhibitor of DNA synthesis that is used to prevent progeny formation to avoid the transfer of the nematodes to fresh plates every day.²⁵ FUdR could be affecting the virulence of *P. aeruginosa* as well as different aspects of *C. elegans* physiology that may contribute to susceptibility to *P. aeruginosa*. For example, *P. aeruginosa*-infected animals become immobile and die, and in a high number of cases, animals become laden with eggs and embryos hatched internally.²⁶ Since FUdR prevents progeny formation, and embryo hatching from gravid adults increases their susceptibility to *P. aeruginosa*,²⁶ the use of the drug eliminates a component of *P. aeruginosa*-mediated killing.

The enhanced susceptibility to *P. aeruginosa* of *npr-1(ad609)* animals compared to that of N2 animals, when infections were performed at 8% oxygen or on full lawns of *P. aeruginosa*,¹¹ indicate that pathogen avoidance cannot account for all of the difference between the two *C. elegans* strains. To provide further evidence that *npr-1(ad609)* animals succumb to pathogen infection faster than N2 animals by mechanisms that are independent

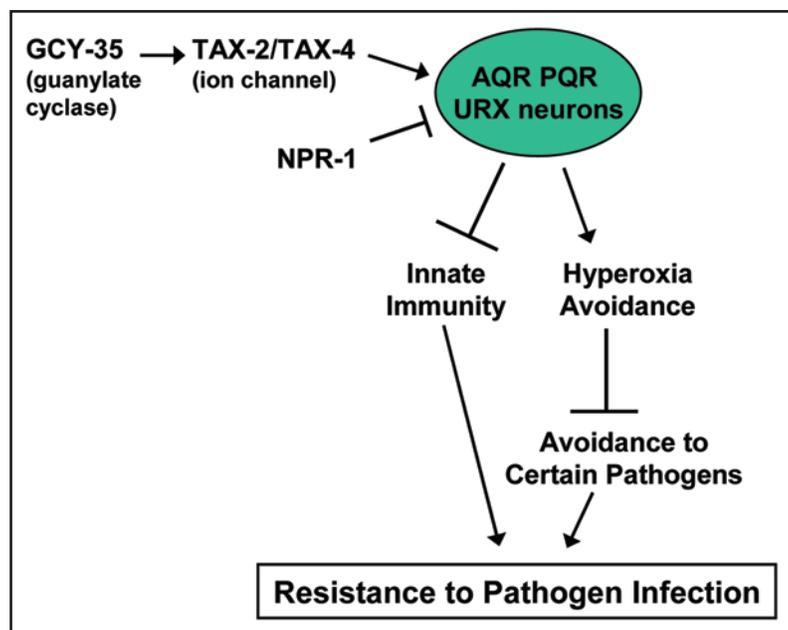


Figure 2. Model of the role of the NPR-1 neural circuit in resistance to pathogen infection in *C. elegans*. NPR-1 inhibits the activity of at least AQR, PQR, URX neurons that suppress pathogen resistance by at least two mechanisms. While GCY-35, TAX-2 and TAX-4 are required for the activation of AQR, PQR and URX neurons, NPR-1 inhibits their activity. When the inhibitory function of NPR-1 is affected, AQR, PQR and URX neurons promote hyperoxia avoidance which suppresses pathogen avoidance and results in a reduced resistance to infection. Additionally, when the NPR-1-mediated inhibition of AQR, PQR and URX neurons is affected, innate immunity is suppressed.

of pathogen avoidance, we used the pathogen *Salmonella enterica*. As in mammalian hosts, a small inoculum of *S. enterica* is capable of establishing a persistent infection in *C. elegans*.²⁷ In contrast to *P. aeruginosa*-mediated killing, *S. enterica*-mediated killing does not require constant exposure to bacteria and cannot be prevented by transferring the infected animals to plates containing *E. coli*. Indeed, it is known that 5 hours of exposure to *S. enterica* is sufficient to cause a similar rate of killing to the one obtained when *C. elegans* is in contact with *S. enterica* during the entire assay.²⁷ In addition, it has been demonstrated that *S. enterica* does not elicit an avoidance behavior.²⁸ Thus, the observed increased susceptibility of *npr-1(ad609)* to *S. enterica*,¹¹ is consistent with a role of NPR-1 in the regulation of immune responses that are independent of pathogen avoidance.

The NPR-1 Neural Circuit Regulates Innate Immunity

Several transcriptional profiling analyses have demonstrated that *C. elegans* responds to pathogen attack by differentially regulating the expression of conserved immune effectors including antimicrobial peptides, lysozymes and lectins. Some of the genes that are markers of innate immunity are under the regulation of the TGF β -related gene *dbl-1*,^{6,29} the FOXO transcription factor DAF-16,³⁰ the intestinal GATA transcription factor ELT-2,^{7,8} and MAPK pathways.^{9,31} To identify possible genes and pathways involved in innate immunity that may be regulated by NPR-1, we performed a full-genome microarray analysis.¹¹ The data show that

most of the genes that are misregulated in *npr-1(ad609)* animals correspond to markers of innate immune responses that are regulated by DBL-1, DAF-16, and the conserved PMK-1/P38 MAPK signaling pathway.¹¹ A significant enrichment in intestinally-expressed genes was also found.¹¹ Differential expression of genes that are markers of innate immunity may be a consequence of different exposure to pathogens. However, *npr-1(ad609)* and N2 animals were infected on plates completely covered by *P. aeruginosa*, ruling out this possibility. Another independent comparison of gene expression between N2 animals and animals carrying a polymorphism in *npr-1* support the idea that NPR-1 regulates the expression of immune genes in a manner that is independent of pathogen avoidance.³²

The difference in pathogen susceptibility between the commonly used N2 strain and the wild isolate CB4856 has been attributed to a polymorphism in *npr-1*.²⁴ A genome-wide microarray study involving CB4856 supports the idea that NPR-1 regulates the expression of immune genes in a manner that is independent of pathogen avoidance.³² The microarray analysis shows that the pattern of expression of immune genes over time differs between N2 and CB4856. In addition, some of the immune genes are solely expressed in either N2 or in CB4856.³² Since N2 only avoids oxygen levels above 10% when *E. coli* is absent and fails to avoid high oxygen in the presence of *E. coli*,¹⁸ it is unlikely that a different exposure to *E. coli* could account for differences in the expression of innate immunity genes observed between N2 and CB4856. In addition, since genes that are markers of *C. elegans* innate immunity were identified essentially by comparing the expression profile of animals infected with pathogens to that of animals exposed to *E. coli*, different exposure to *E. coli* should not result in misregulation of immune genes.

Genetic studies have shown that the NPR-1 inhibition of neural activation requires the soluble guanylyl cyclase GCY-35 and TAX-2 and TAX-4, which are two subunits of a cGMP-gated-ion-channel.^{14,19,33} Consistent with the idea that NPR-1 regulates innate immunity, it was found that a *gcy-35* mutation in *npr-1(ad609)* animals rescues the altered expression of 10 genes that are markers of *C. elegans* immune response. In addition, the observed low levels of active PMK-1 in *npr-1(ad609)* animals compared to N2 animals grown on *E. coli* further indicate that NPR-1 regulates innate immune responses that are independent of pathogen avoidance (Fig. 2).

Conclusions

Several lines of evidence indicate that animals deficient in the activity of NPR-1 are more susceptible to the human pathogen *P. aeruginosa* strain PA14 due to two factors: decreased pathogen avoidance and decreased innate immune responses. The use of different oxygen concentrations, different bacteria and different modes of infection provide additional evidence that the NPR-1 neural circuit regulates, at least in part, innate immune responses in *C. elegans*. *C. elegans* neurons are known to express numerous

secreted peptides of the TGFbeta family, the insulin family and neuropeptide families.³⁴⁻³⁹ This myriad of secreted factors has the potential to act at a distance to modulate various physiological processes by regulating the function of neural and non-neural cells throughout the animal. Taken together, the studies discussed here show that specific genes and neurons in the nervous system of *C. elegans* control immune responses, indicating that cell non-autonomous signals from different neurons may act on non-neural tissues to regulate innate immunity. It is plausible that the role of NPR-1 in innate immunity, together with the postulated trade-off between dispersal and competitive ability,⁴⁰ conspire to maintain *npr-1* polymorphisms.

Acknowledgements

A.A. is funded by The Whitehead Scholars Program and NIH grant GM070977.

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