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HOST RESPONSE AND INFLAMMATION

Deficiency of Innate Immunity against *Pseudomonas aeruginosa* Enhances Behavioral Avoidance via the HECW-1/NPR-1 Module in *Caenorhabditis elegans*

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ABSTRACT To antagonize infection of pathogenic bacteria in soil and confer increased survival, *Caenorhabditis elegans* employs innate immunity and behavioral avoidance synchronously as the two main defensive strategies. Although both biological processes and their individual signaling pathways have been partially elucidated, knowledge of their interrelationship remains limited. The current study reveals that deficiency of innate immunity triggered by mutation of the classic immune gene *pmk-1* promotes avoidance behavior in *C. elegans* and vice versa. Restoration of *pmk-1* expression using the tissue-specific promoters suggested that the functional loss of both intestinal and neuronal *pmk-1* is necessary for the enhanced avoidance. Additionally, PMK-1 colocalized with the E3 ubiquitin ligase HECW-1 in OLL neurons and regulated the expressional level of the latter, which consequently affected the production of NPR-1, a G-protein-coupled receptor (GPCR) homologous to the mammalian neuropeptide Y receptor, in RMG neurons in a non-cell-autonomous manner. Collectively, our study illustrates that once the innate immunity is impaired when *C. elegans* antagonizes bacterial infection, the other defensive strategy of behavioral avoidance can be enhanced accordingly via the HECW-1/NPR-1 module, suggesting that GPCRs in neural circuits may receive the inputs from the immune system and integrate those two systems for better adapting to the real-time status.

KEYWORDS innate immunity, avoidance behavior, pathogenic bacterium, *P. aeruginosa* PA14, *C. elegans*, PMK-1, HECW-1/NPR-1

In nature, animals generally encounter ever-changing stressful environments that may pose serious risks for their survival. Accordingly, they have developed a series of independent and/or redundant strategies to jointly counteract the crisis. Although each of the biological processes may be intensively investigated, a subtle regulation of the different processes or the interrelationship among them remains mostly unclear. For example, *Caenorhabditis elegans* has developed two types of stressful responses, such as programmed cell death to remove the damaged cells and animal-wide resistance to thrive in the harmful stimuli. It was found that if the apoptosis machinery was blocked, a more global, animal-wide stress resistance would happen via an unexpected regulatory mechanism (1). Additionally, a kind of cross talk between the nervous system and immune response has been described ranging from lower animals to mammals. In mammals, acute psychological stress damages the immune functions of erythrocytes and T-lymphocyte subsets significantly, but it increases blood granulocytes and monocytes (2, 3). The molecular evidence illustrated that the neural input from stress could change the mRNA levels of 23 genes that function in immune response (4). Meanwhile, the central and autonomous

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nervous systems control the function of immune organs or cells. An experiment using the mouse model showed that the mucosal neurons could express neuromedin U (NMU), a regulator of type 2 innate immunity that activated the NMUR1-dependent production of innate inflammatory and the tissue repair cytokines in group 2 innate lymphoid cells (5, 6). Within a glial-group 3 innate lymphoid cell (ILC3)-epithelial cell unit in the intestinal mucosa, the glial cells sensed microenvironmental cues and secreted neurotrophic factors, which controlled the production of ILC3-derived innate interleukin-22 (IL-22) (7). Similarly, the pathogen-associated molecular patterns or the cytokines from immune cells also execute their effects on the nervous system, mainly via receptors, prostaglandins, the second messenger of nitric oxide, and so on (8). Despite much attention to interrelationships among the different biological processes being attracted, the structural and functional complexities in mammals, as well as their complex communication mechanisms, make research in this area challenging (9).

C. elegans, one of the most widely distributed multicellular animals, feeds on bacteria. However, bacterial infection is a common stress for the animal due to variety of pathogens in the ecological environment. Therefore, two major defense strategies (i.e., innate immunity and avoidance behavior) are used to confer increased survival upon bacterial infection (10, 11). Immune response eliminates the microbes that invade animal's body by expressing a set of immunity-related genes, which mainly depends on three classical signaling pathways: i.e., the p38 mitogen-activated protein kinase (p38 MAPK), transforming growth factor β (TGF-β)-like, and insulin receptor-like pathways (12–14). The other defense strategy is avoidance to keep the animals away from predators or tainted food, and this behavior is generally regulated by the specific sensory neurons, interneurons, or neuropeptides. Among them, AWB neurons have been described to be involved in escaping from serrawettin W2 produced by *Serratia marcescens* Db10 (12). Additionally, the sensory neuron ASJ, along with the interneurons RIM and RIC, is responsible for the avoidance of pyochelin and phenazine-1-carboxamide, two metabolites secreted by *Pseudomonas aeruginosa* PA14 (12, 15).

Recently, an increasing body of experimental evidence has demonstrated that both innate immunity and avoidance behavior in *C. elegans* are under the control of neural signaling. Either neuropeptide INS-7 or dopamine affects the immune responses via the insulin or p38 MAPK pathway (16, 17). Meanwhile, several G-protein-coupled receptors (GPCRs) (e.g., NPR-1 and OCTR-1) expressed in the specific neurons modulate sensitivity of the neurons or the unfolded protein response of non-nervous cells to inhibit immunity (18, 19). Under some other situations, innate immunity and avoidance behavior even share the same molecular signaling pathways. It was suggested that activation of the p38 signaling pathway in epidermis or the intestinal system triggers the immunity response against *P. aeruginosa* PA14, while TIR-1, NSY-1, and SEK-1 (components of the p38 pathway) in the sensory neuronal system were required for serotonin synthesis and learning-associated avoidance (20). Similarly, the insulin pathway DAF-2/DAF-16 showed a dual function in immunity and avoidance against *Bacillus thuringiensis* (21).

Since few previous reports have suggested a link between innate immunity and behavioral avoidance, we performed the present study dealing with the interrelationship between them. Our data revealed that a deficiency of innate immunity caused by *pmk-1*(*km25*) mutation could stimulate avoidance behavior in *C. elegans*. Furthermore, enhanced avoidance depended on the mutations of both intestinal and neuronal *pmk-1*. Particularly, functional loss of PMK-1 localized in the neurons (e.g., OLL) increased expression of the neuropeptide receptor NPR-1 via suppressing HECW-1, which consequently contributed to the behavioral enhancement when innate immunity was impaired by *pmk-1* mutation.

RESULTS

A deficiency of innate immunity caused by *pmk-1* mutation enhances behavioral avoidance. Although the TIR-1/NSY-1/SEK-1 pathway in the nervous system has been shown to be involved in learning-associated avoidance via modulating the tryptophan

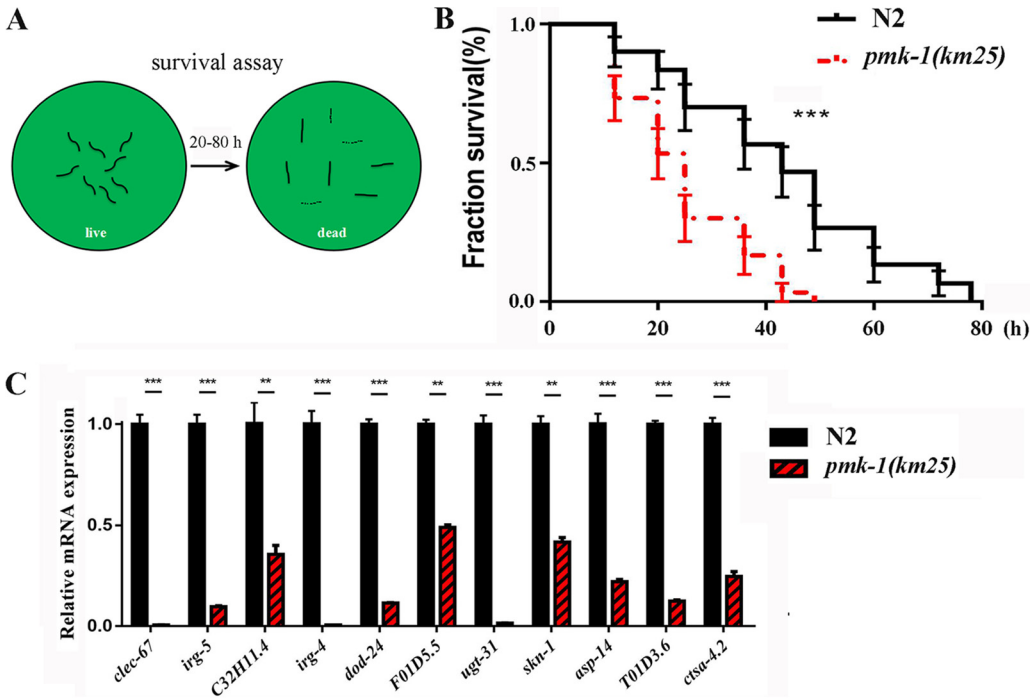

FIG 1 Mutation of *pmk-1* impaired innate immunity of *C. elegans* against the pathogenic bacterium *P. aeruginosa* PA14. (A) Graphic representation of the survival analysis for *C. elegans* infected by *P. aeruginosa* PA14. (B) Survival curves of the wild-type N2 worm and the *pmk-1*(*km25*) mutant in the survival assay. Error bars are presented as SE. (C) qPCR analysis of the innate immunity-related genes targeted by *pmk-1* when either N2 or *pmk-1*(*km25*) worms were infected with *P. aeruginosa* PA14 within 8 h. The results are presented as the mean ± SD from at least three independent experiments, and the internal reference gene was *act-1*. The statistical analysis of survival curves was performed by the log rank (Mantel-Cox) test, and the relative mRNA levels were analyzed by the Student's *t* test. Significance compared with wild-type N2 worms: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. ns, not significant ($P \geq 0.05$).

hydroxylase 1 (TPH-1), a rate-limiting enzyme in serotonin synthesis, another key component of the p38 signaling pathway, PMK-1, failed to have the same function in *C. elegans* (20). Instead, *pmk-1* is regarded as one of the most important genes in innate immunity against *P. aeruginosa* PA14 (22). Thus, the susceptibility to *P. aeruginosa* PA14 was first determined in the *pmk-1*(*km25*) mutant by assaying survival rates on the full bacterial lawn (Fig. 1A). Our experimental data showed that mortality was significantly higher in the *pmk-1*(*km25*) mutant than in the wild-type N2 worms at each time point (Fig. 1B). At the molecular level, mRNAs of the innate immunity-related genes, which had been reported as the targets of PMK-1 (23, 24), were all decreased after 8 h of exposure to *P. aeruginosa* PA14 (Fig. 1C), implying the innate immunity of *pmk-1* mutation was indeed impaired.

Next, the changes of avoidance behavior were observed when the innate immune response was decreased by *pmk-1* mutation. The avoidance assay was conducted on nematode growth medium (NGM) plates with the small lawn of *P. aeruginosa* PA14 (upper panel in Fig. 2A). It was found that, after exposure to *P. aeruginosa* PA14, the *pmk-1*(*km25*) mutant worms escaped faster from the bacterial lawn than the wild-type N2 worms (lower panel in Fig. 2A). The quantification of our observations also supported this conclusion because the *pmk-1*(*km25*) mutant had a higher avoidance index than N2 during 24 h (Fig. 2B). The increased avoidance behavior of the *pmk-1*(*km25*) mutant was likely not due to differences in locomotor activity or sensitivity to odors because the *pmk-1*(*km25*) worms had turning rates (25) (Fig. 2C) and chemotaxis indices (26, 27) for isoamyl alcohol and benzaldehdye similar to those of N2 worms (Fig. 2D). To further confirm the interrelationship between innate immunity and behavioral avoidance, *Chondrus crispus* water extract (CCWE) and kappa-carrageenan (K-CGN), which have previously been reported to enhance innate immunity of *C. elegans* by activating the *pmk-1*, *daf-16/daf-2*, and *skn-1* pathways (28), were added to the NGM plates, and the avoidance assay

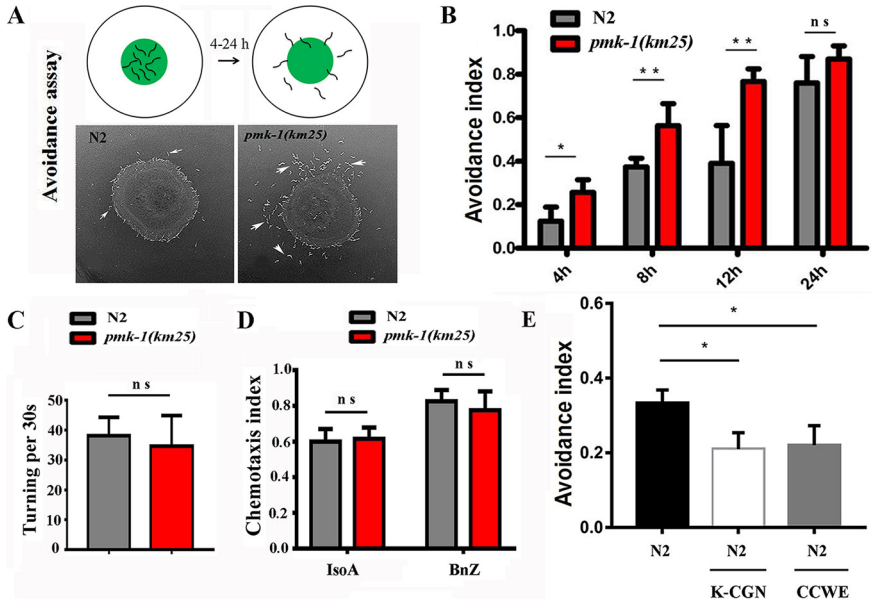

FIG 2 Deficiency of innate immunity by *pmk-1* mutation causes an enhanced avoidance behavior from *P. aeruginosa* PA14. (A) Graphic representation of the avoidance assay and the phenotypic observation to the wild-type N2 and *pmk-1*(*km25*) mutant worms that escaped from *P. aeruginosa* PA14. White arrows indicate the worms out of the bacterial lawn. (B) Quantitative analysis of the avoidance index of N2 and *pmk-1*(*km25*) worms at different time points. (C) Turning frequency/30 s of N2 and *pmk-1*(*km25*) worms. At least 10 worms were scored in each independent experiment. (D) Chemotaxis index of isoamyl alcohol and benzaldehyde. The chemotaxis index was calculated as (no. of animals at attractant − no. of animals at counterattractant)/total no. of animals. (E) Avoidance index of N2 worms at 8 h on the blank NGM, NGM plus K-CGN, or NGM plus CCWE. K-CGN and CCWE are two agonists of innate immunity in *C. elegans*. Results are presented as the mean ± SD from at least three independent experiments. The avoidance index was statistically analyzed using two-way analysis of variance (ANOVA) with repeated measures in panel B and one-way ANOVA in panels C to E. Significance compared with wild-type N2 worms: *, $P < 0.05$; **, $P < 0.01$. ns, not significant ($P \geq 0.05$).

was performed again. In contrast to the results from the *pmk-1*(*km25*) mutant with an elevated avoidance behavior, the worms treated with either K-CGN or CCWE had lower avoidance indices compared to N2 worms without any treatment (Fig. 2E). To identify other essential factors that affect the interaction between innate immunity and avoidance, N2 and *pmk-1*(*km25*) worms were exposed to a series of different bacterial lawns, such as the food bacterium *Escherichia coli* OP50, *E. coli* OP50 supplemented with 9% NaCl, the wild-type *P. aeruginosa* PA14 strain, inedible *P. aeruginosa* PA14 (10 mg/μl aztreonam-treated bacteria) (29), and dead *P. aeruginosa* PA14 (killed by being heated at 100°C for 15 min). Consistent with the results described above, when exposed to the wild-type *P. aeruginosa* strain PA14, which could infect the host worms and effectively induce innate immunity, a stronger avoidance could be observed in the *pmk-1*(*km25*) mutant than in N2. However, the nonpathogenic *E. coli* OP50 strain, the inedible *P. aeruginosa* PA14, the dead *P. aeruginosa* PA14 cells, and hypertonic stress (9% NaCl) showed little effect on the avoidance of *pmk-1*(*km25*) worms (Fig. 3A to C). Additionally, the enhanced avoidance was not limited to the pathogenic *P. aeruginosa* PA14, because a similar avoidance effect was also observed when the *pmk-1*(*km25*) mutant encountered *Staphylococcus aureus* (ATCC 25923) (Fig. 3A and B).

Collectively, our data demonstrated that a deficiency of innate immunity caused by *pmk-1* mutation could enhance behavioral avoidance, implying an interrelationship between those two biological processes.

Enhancement of avoidance is dependent on intestinal and neuronal *pmk-1* mutations. Since intestinal PMK-1 has been described to contribute more to innate immunity against *P. aeruginosa* PA14, we fused the promoter of *pmk-1* in-frame with mCherry to determine its transcriptional expression in worms. Red fluorescence was observed in both intestine and head neurons (Fig. 4A), which was consistent with a previous report (30). However, the

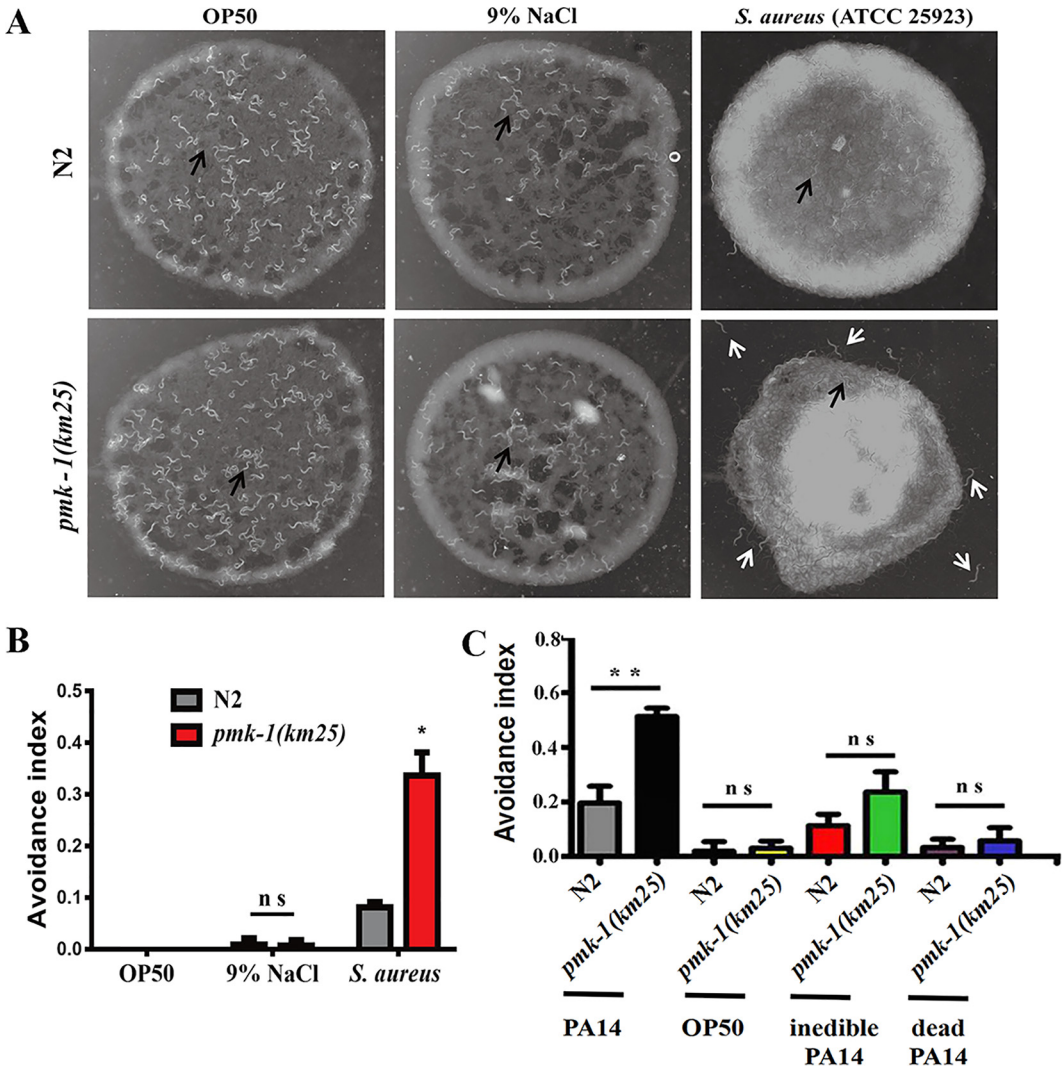

FIG 3 Analysis of the potential factors that affect enhancement of avoidance caused by *pmk-1* mutation. (A) Phenotypic observation of N2 and *pmk-1*(*km25*) worms to determine if the worms escape from the nonpathogenic *E. coli* OP50 strain, a general stress of hypertonicity, and another pathogenic bacterium, *S. aureus* ATCC 25923. The young adult worms of N2 and the *pmk-1*(*km25*) mutant fed on the NGM plates with *E. coli* OP50, *E. coli* OP50 supplemented with 9% NaCl, and *S. aureus* ATCC 25923, respectively. Black arrows indicate the worms on the bacterial lawn; white arrows indicate the worms off the lawn. (B) Quantitative analysis to the avoidance index of N2 and the *pmk-1*(*km25*) mutant under the conditions described above. (C) Avoidance index of N2 and *pmk-1*(*km25*) mutant worms fed with nonpathogenic *E. coli* OP50 or virulent, inedible, or dead *P. aeruginosa* PA14 at 8 h. Results are presented as the mean ± SD from at least three independent experiments. The avoidance index was statistically analyzed using Student's *t* test. Significance compared with wild-type N2 worms: *, $P < 0.05$; **, $P < 0.01$. ns, not significant ($P \geq 0.05$).

expression in intestine seemed much stronger than that in neurons. Therefore, we further explored the functional localization responsible for the interaction between immunity and avoidance by the tissue-specific rescue of PMK-1. Our experimental data show that overexpression of *pmk-1* in the wild-type N2 strongly suppressed avoidance behavior (Fig. 4B). However, the supplementary expression of *pmk-1* driven by its own promoter, which could drive the intestinal and neural expression synchronously, led to relatively normal behavior and almost rescued the phenotype of *pmk-1*(*km25*). The other two promoters of *vha-6* and *unc-119* were then used to drive *pmk-1* expression in intestines or neurons specifically (20, 31). Neither of *Pvha-6*::*pmk-1*; *pmk-1*(*km25*) and *Punc119*::*pmk-1*; *pmk-1*(*km25*) transgenic lines exhibited an avoidance index as high as the *pmk-1*(*km25*) line (Fig. 4B), suggesting that suppression of both intestinal and neuronal *pmk-1* expression should be necessary for enhancement of avoidance behavior triggered by the impaired immunity.

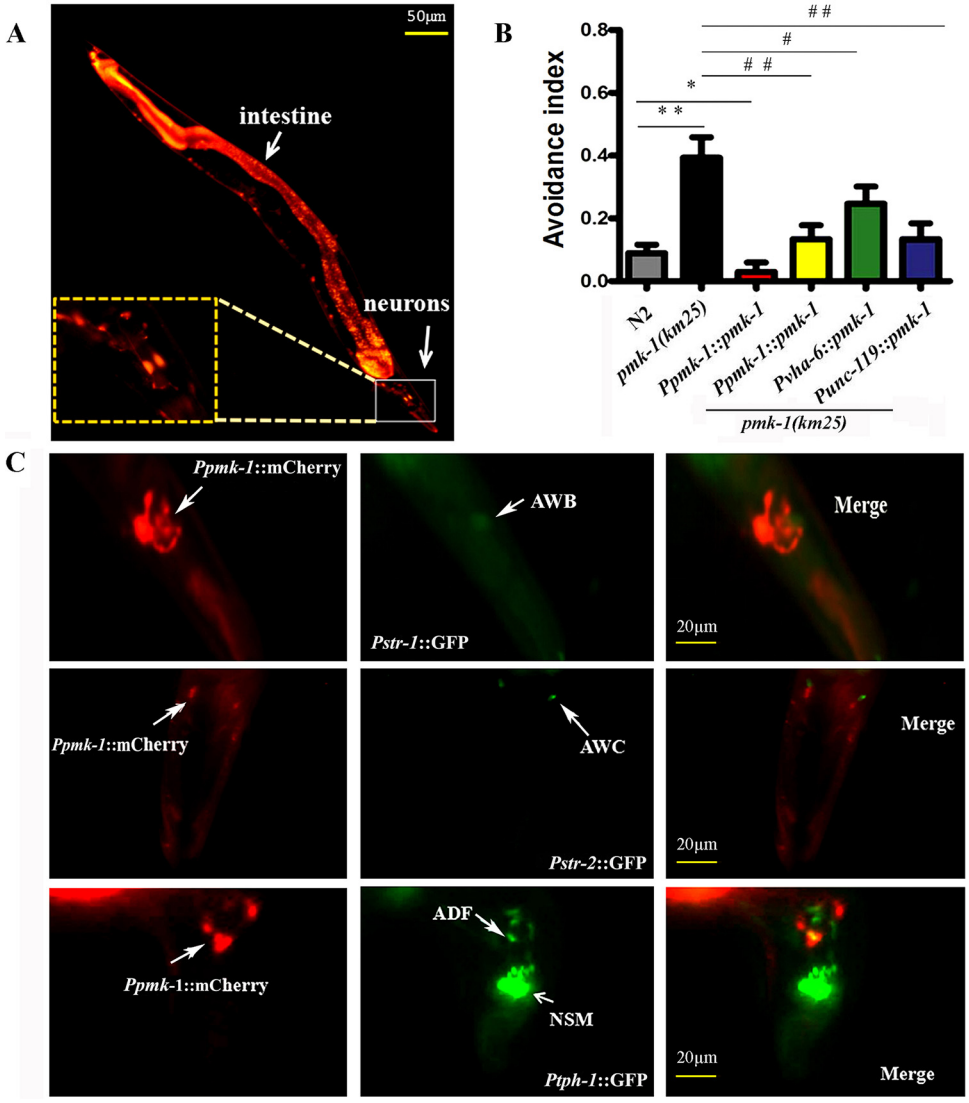

FIG 4 The *pmk-1* mutation in both intestine and neurons is required for enhancement of avoidance behavior. (A) Red fluorescence from *Ppmk-1*::mCherry was observed in both intestine and neurons. (B) Avoidance assay after the overexpression of *pmk-1* in N2 and the complementary expression of *pmk-1* driven by tissue-specific promoters (*pmk-1*, *vha-6*, or *unc-119*) in the *pmk-1*(*km25*) mutant worms. (C) Determination of the neuron types where *pmk-1* was localized. Results are presented as the mean ± SD from at least three independent experiments. The avoidance index was statistically analyzed using one-way ANOVA. Significance compared with wild-type N2 worms: *, $P < 0.05$; **, $P < 0.01$. Significance compared with the other specific control *pmk-1*(*km25*) worms: #, $P < 0.05$; ##, $P < 0.01$.

To further analyze the neuronal localization of *pmk-1*, we compared the expression of PMK-1 with the known chemosensory neurons that were necessary for the learning-associated avoidance, including AWB, AWC, and ADF (25). AWB and AWC were obviously not colocalized with the red fluorescence from *Ppmk-1*::mCherry (Fig. 4C). One ADF neuron of a pair neurons partly colocalized with *Ppmk-1*::mCherry (Fig. 4C). However, considering the symmetry of two neurons in *C. elegans*, it seemed a bit indistinct to conclude *pmk-1* functioned in ADF neurons.

HECW-1 in OLL neurons colocalized with PMK-1 and regulated by PMK-1. In addition to the chemosensory neurons AWB, AWC, and ADF, the OLL neurons have also been reported to play an important role in avoidance behavior against *P. aeruginosa* PA14 via the conserved HECT domain-containing E3 ubiquitin ligase HECW-1 (32). Interestingly, when we expressed *Phecw-1*::GFP and *Ppmk-1*::mCherry within N2 worms synchronously, it was found that both of them indeed colocalized well in OLL neurons (Fig. 5A to D).

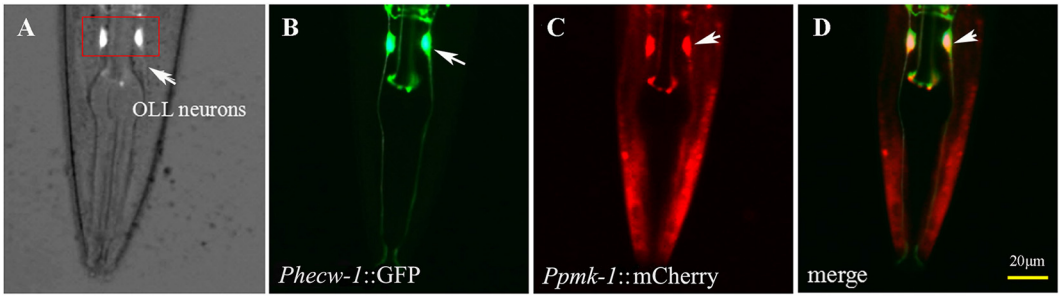

FIG 5 *pmk-1* colocalizes with *hecw-1* in OLL neurons. (A to D) *Phecw-1*::GFP and *Ppmk-1*::mCherry colocalize in OLL neurons.

Next, we investigated whether the expression of HECW-1 in OLL neurons could be affected upon *pmk-1* mutation by comparing the fluorescent changes of *Phecw-1*::GFP between N2 and the *pmk-1*(*km25*) mutant. As expected, the fluorescence of the *pmk-1* (*km25*) mutant was weaker in OLL neurons than that of N2 worms (Fig. 6A), which was validated by our quantitative analysis (Fig. 6B). The quantitative PCR (qPCR) data showed that the mRNA levels of *hecw-1* were significantly lower in the *pmk-1*(*km25*) mutant too (Fig. 6C), suggesting *pmk-1* positively regulated *hecw-1* in OLL neurons. To explore the genetic interaction of *pmk-1* and *hecw-1* in the same biological process, the *pmk-1* (*km25*); *hecw-1* (*ok1347*) double mutant was generated, and the avoidance behaviors of N2, *pmk-1*(*km25*), *hecw-1*(*ok1347*), and *pmk-1*(*km25*); *hecw-1*(*ok1347*) worms were then assayed. The *hecw-1* (*ok1347*) mutant, when exposed to *P. aeruginosa* PA14, escaped faster from the bacterial lawn than N2, similarly to the *pmk-1*(*km25*) mutant. Furthermore, the *pmk-1*(*km25*); *hecw-1* (*ok1347*) double mutant showed a similar phenotype to *pmk-1*(*km25*) and *hecw-1*(*ok1347*) (Fig. 6D), suggesting that *hecw-1* should act downstream of *pmk-1*.

Involvement of the HECW-1/NPR-1 module in the enhanced avoidance triggered by *pmk-1* mutation. During the regulation of lawn avoidance, HECW-1 negatively controls the neuropeptide receptor NPR-1, which integrates the signals from various sensory neurons and in turn modulates the chemosensory responses (32, 33). Since our data demonstrated that HECW-1 functions downstream of PMK-1, we next examined whether the HECW-1/NPR-1 module is involved in the impaired immunity and enhanced avoidance induced by *pmk-1* mutation. In the avoidance behavior assay of N2, the *pmk-1* and *npr-1* single mutants, and the *pmk-1*; *npr-1* double mutant, it was found that *npr-1*(*ad609*) and *npr-1*(*ur89*) worms had lower avoidance indices than N2, as previously reported (32) (Fig. 7A). However, when *npr-1* was knocked out in the *pmk-1* mutation background, the enhanced avoidance of *pmk-1*(*km25*) was diminished (Fig. 7A), suggesting a genetic interaction between *pmk-1* and *npr-1* and suggesting the latter might function downstream of *pmk-1* in the signaling pathway. Our qPCR result supported this hypothesis, because the *pmk-1*(*km25*) mutant had significantly higher level of *npr-1* mRNA than the wild-type N2 worms (Fig. 7B).

In order to identify the neurons where *npr-1* played its role, we rescued the expression of *npr-1* using cDNA behind the different tissue-specific promoters in the background of *pmk-1*(*km25*); *npr-1*(*ad609*). Compared to the lower avoidance index in the *pmk-1*(*km25*); *npr-1*(*ad609*) mutant, the complementary expression of *npr-1* driven by its own promoter, in several neurons, including RMG, URX, AQR, and AVA (34, 35), reversed the enhanced avoidance phenotype of *pmk-1*(*km25*). A similar phenotype was observed under the control of *flp-21* promoter, which drove gene expression in neurons such as RMG, URX, ASJ, ASK, FLP, and URA. On the contrary, no effects on avoidance were detected when using the *gcy-32* or *flp-8* promoter, which activated gene transcription in URX, AQR, and AVA neurons (Fig. 7C). Together, these data indicate that NPR-1 in RMG neurons contributed to the enhanced avoidance induced by a deficiency of innate immunity caused by *pmk-1* mutation. However, the *pmk-1* mutant with expression in RMG neurons rescued using the promoter of *flp-21* still retained a similar avoidance index to the *pmk-1*(*km25*) mutant (Fig. 7D).

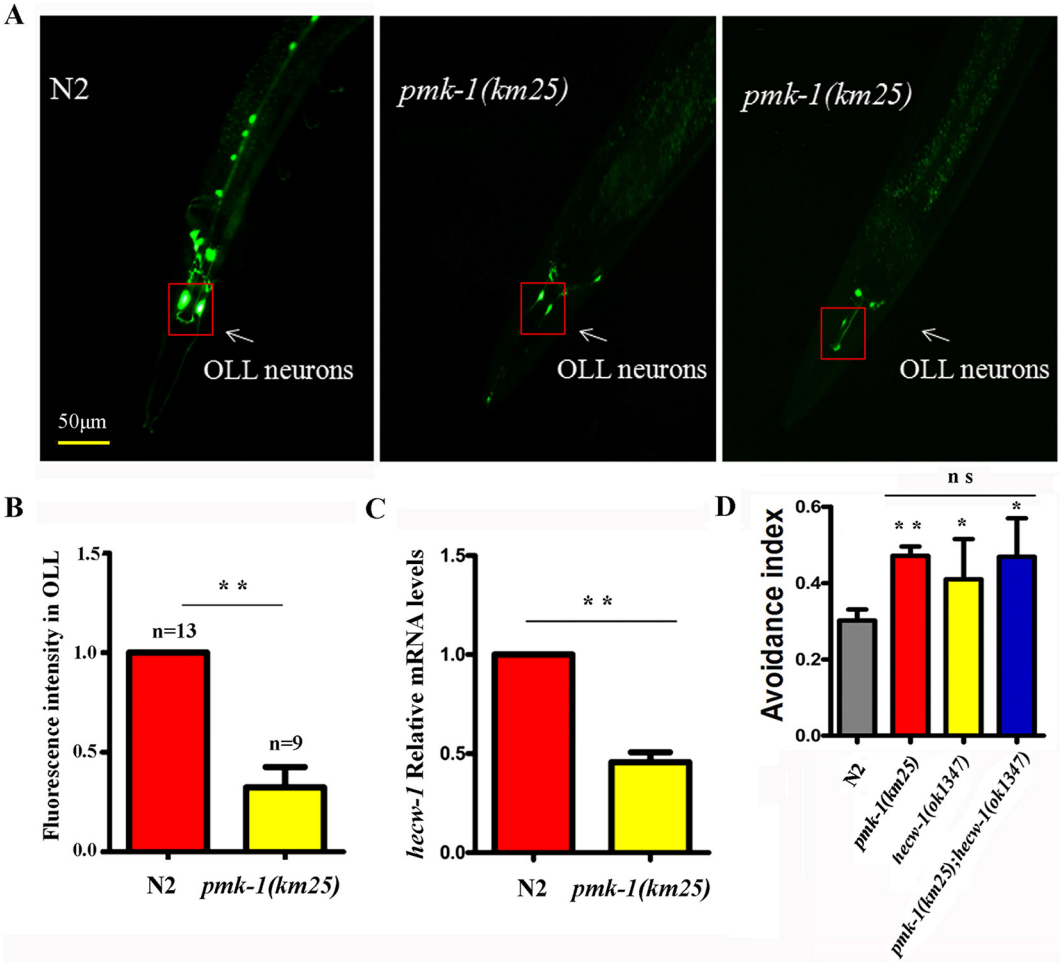

FIG 6 *hecw-1* functions downstream of *pmk-1* to regulate the enhanced avoidance in *pmk-1*(*km25*) mutant worms. (A) The wild-type N2 worms showed a much stronger GFP signal in OLL neurons than *pmk-1*(*km25*) mutant worms. (B) Quantitative analysis of the fluorescence intensity of *Phecw-1*::GFP in OLL neurons of N2 and *pmk-1*(*km25*) worms. (C) The mRNA levels of *hecw-1* in N2 and *pmk-1*(*km25*) worms. (D) Avoidance index of N2, *pmk-1*(*km25*), *hecw-1*(*ok1347*), and *pmk-1*(*km25*); *hecw-1*(*ok1347*) worms that were infected by *P. aeruginosa* PA14 after 8 h. Results are presented as the mean ± SD from at least three independent experiments. The statistical differences were analyzed using Student's *t* test (B and C) and one-way ANOVA (D). Significance compared with wild-type N2 worms: *, $P < 0.05$; **, $P < 0.01$. ns, not significant ($P \geq 0.05$).

DISCUSSION

Bacteria are not only the food source for worms, but they are also a great threat challenging the survival of *C. elegans*. It has been shown that a few bacterial pathogens emit several attractive molecules for olfactory trapping of their host (26, 36), but more commonly, a behavioral response (e.g., avoidance) helps the worms escape the pathogenic bacteria and decreases the risk of infection. NPR-1, a neuropeptide receptor, has been found necessary to induce the pathogenic avoidance independent of associated learning. The gene *npr-1* encodes a GPCR homologous to the mammalian neuropeptide Y receptor, and the polymorphisms of NPR-1 cause the different food-related behaviors, such as aggregation, aerotaxis, and locomotion (37–40). Thus, the mutant CB4856 (Hawaiian strain), which exhibited decreased NPR-1 activity, was found to have a slower speed of lawn leaving and therefore suffered a higher death rate, indicating involvement of this neuropeptide receptor in accelerating pathogenic avoidance (33). However, another interesting phenomenon in our current work has been revealed—that the threat of immune deficiency activates the neuropeptide receptor NPR-1 to strengthen the machinery of avoidance.

In *C. elegans*, the NSY-1/SEK-1/PMK-1 MAPK pathway, which is orthologous to the mammalian ASK1/MKK3-p38 MAPK pathway, participates in both innate immunity and

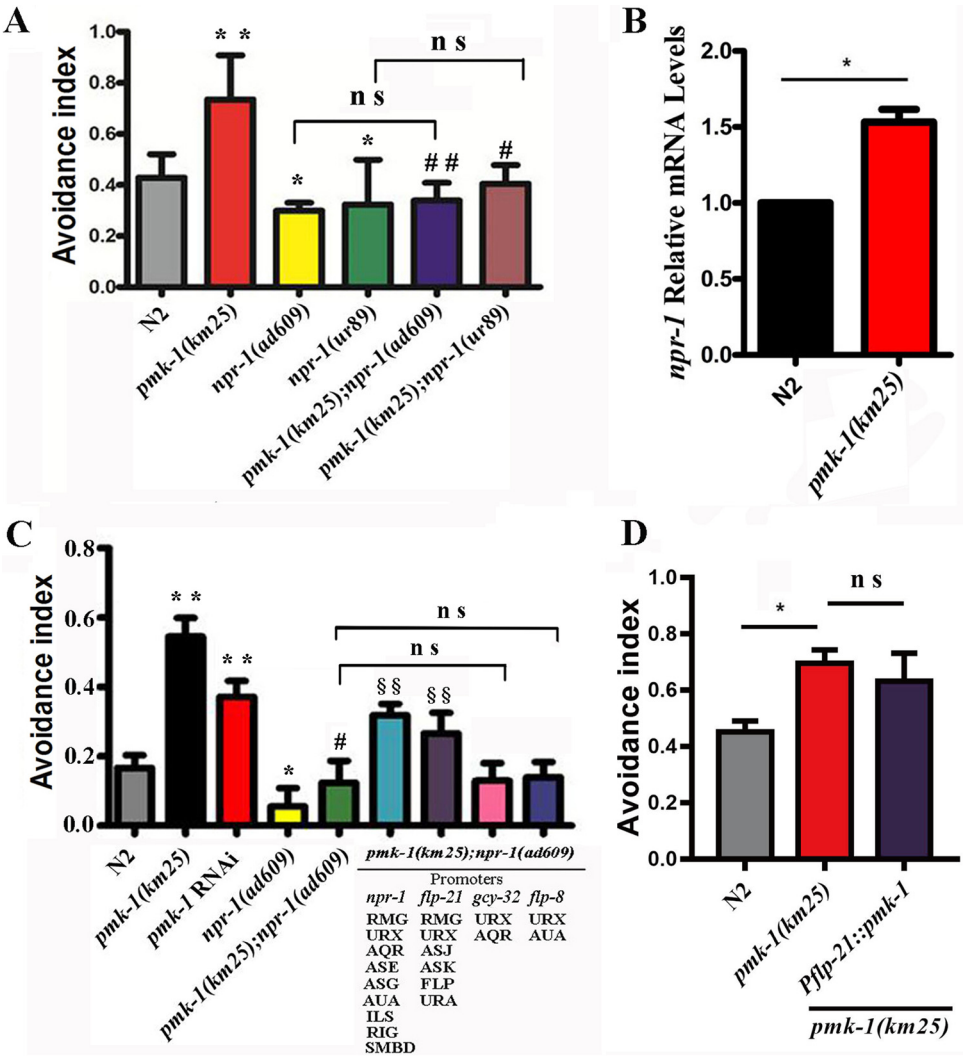

FIG 7 Involvement of the HECW-1/NPR-1 module in the enhanced avoidance caused by *pmk-1* mutation. (A) The mutation of *npr-1* in the *pmk-1*(*km25*) background could diminish the enhanced avoidance behavior of *pmk-1*(*km25*) worms. (B) Increase in *npr-1* mRNA levels in *pmk-1*(*km25*) worms. (C) Complementary expression of wild-type *npr-1* cDNA in the *pmk-1*(*km25*); *npr-1*(*ad609*) double mutant driven by the tissue-specific promoters (*npr-1*, *gcy-32*, *flp-8*, or *flp-21*). (D) Avoidance index of N2 and the *pmk-1*(*km25*) and *Pflp-21*::*pmk-1*; *pmk-1*(*km25*) mutants. Results are presented as the mean ± SD from at least three independent experiments. Statistical differences were analyzed using Student's *t* test (B) and one-way ANOVA (A, C, and D). Significance compared with wild-type N2 worms: *, $P < 0.05$; **, $P < 0.01$. Significance compared with the control *pmk-1* (*km25*) worms: #, $P < 0.05$; ##, $P < 0.01$. Significance compared with the control *pmk-1*(*km25*); *npr-1*(*ad609*) worms: §§, $P < 0.01$. ns, not significant ($P \geq 0.05$).

aversive behavior (24, 41, 42), and exerts its functions in a tissue-specific manner. Activation of the p38 MAPK pathway in intestine and epidermis is responsible for the innate immune response antagonizing *P. aeruginosa* PA14 (22). While the TIR-1/NSY-1/SEK-1 pathway in the sensory neuronal system has been proven to control serotonin synthesis via modulating the expression of TPH-1 and hence shows an effect on the learning-associated avoidance (20), PMK-2, instead of PMK-1, functions downstream of TIR-1/NSY-1/SEK-1 in behavioral avoidance. Since PMK-1 failed to play the same role in regulating avoidance, it prompted us to achieve the immunodeficiency status in *C. elegans* after *pmk-1* mutation and to observe the potential interrelationship between innate immunity and avoidance behavior. Our current data support that promotion of behavioral avoidance can be triggered by the impaired immunity in the *pmk-1*(*km25*) mutant. Instead, stimulation of innate immunity with K-CGN or CCWE treatment suppresses avoidance behavior, probably due to energy saving. Furthermore, *pmk-1* in OLL neurons

regulates the expression of *hecw-1* and *npr-1*. In fact, the role of NPR-1 in the interactions of innate immunity and avoidance is consistent with the previous report that *npr-1* in sensory neurons negatively controls the immune response of nonneuronal tissues (19). However, this avoidance assay in our study likely underestimates the increased avoidance of the *pmk-1* mutant, especially when the immune impairment of the *pmk-1*(*km25*) mutant deteriorated further and finally slowed movement due to toxicity as the infection period extended.

Though loss of *pmk-1* or other key immune genes seldom occurs under natural conditions, the immune system can be compromised by a variety of factors, such as malnutrition, congenital disorders and other diseases, or even immunosuppressive agents. In the case of immune deficiency, the real-time status of the immune system may be perceived via the GPCRs in neural circuits based on our results, and in turn complementary strategies, like enhancement of avoidance behavior in *C. elegans*, are developed to counter the pathogenic threats. Thus, such a subtle regulation will undoubtedly confer better survival in the context of immunodeficiency, making evolutionary sense.

In summary, our available data provide experimental evidence for a potential interrelationship between innate immunity and behavioral avoidance in *C. elegans* and further suggest that the HECW-1/NPR-1 module contributes to avoidance enhancement when innate immunity is impaired by mutation of *pmk-1*.

MATERIALS AND METHODS

Strains of *C. elegans*. The *C. elegans* strains were generally grown and maintained as described previously (43). In brief, they were cultivated at 20°C on NGM seeded with *E. coli* OP50. Before each assay, the worms were synchronized using a hypochlorite treatment and then grown on the well-nourished NGM at 20°C until the young adult stage. After being washed three times with M9 buffer, those young adult worms were collected and used for the next assays.

The following strains were obtained from the Caenorhabditis Genetics Center (CGC): the wild-type Bristol strain N2 and the *pmk-1*(*km25*), *npr-1*(*ad609*), *npr-1*(*ur89*), *hecw-1*(*ok1347*), *str-1p*::GFP(*kyIs104*), [*str-2*::GFP + *lin-15*(+)](*kyIs140*), and [*tph-1*::GFP + *rol-6*(*su1006*)](*mgIs42*) mutant strains. Additionally, the CX9592 *npr-1* (*ad609*) X, *kyEx2061* [*npr-1*::*npr-1 SL2* GFP, *ofm-1*::DsRed], CX9395 *npr-1*(*ad609*) X, *kyEx1965* [*gcy-32*::*npr-1 SL2* GFP, *ofm-1*::DsRed], CX9633 *npr-1*(*ad609*) X, *kyEx2096* [*flp-8*::*npr-1 SL2* GFP, *ofm-1*::DsRed], CX9396 *npr-1*(*ad609*) X, and *kyEx1966* [*flp-21*::*npr-1 SL2* GFP, *ofm-1*::DsRed] strains were the kind gifts from Miriam B. Goodman.

Avoidance assay. Before the avoidance assay, the small lawn of *P. aeruginosa* PA14 was prepared by incubating this bacterium on NGM plates at 37°C for 24 h and at 25°C for 12 h. Then, 40 to 60 worms were added to the center of bacterial lawn. Within 24 h, the worms were scored as on or off the lawn. The avoidance index was calculated by dividing the number of worms off the lawn by the total numbers of worms.

Survival analysis. The survival assay was performed on the full lawn of *P. aeruginosa* PA14 supplemented with 50 μg/ml 5-fluorodeoxyuridine (FUdR) to avoid egg hatching. About 100 young adult animals were added to each plate and were infected by this pathogenic bacterium within 80 h. The worms were gently touched with a platinum wire to score them as live or dead under the microscope every 20 h. The survival rates were calculated as live animals/total animals.

Quantitative real-time PCR. After the worm samples were washed with M9 buffer several times to remove bacteria, the total RNA was isolated by TRIzol reagent (Tiangen Co., Tianjin, China). Synthesis of cDNA was performed using the random primers with the PrimeScript RT reagent kit (TaKaRa, Dalian, China). The SYBR green JumpStart *Taq* Ready Mix for qPCR kit (Sigma-Aldrich Co., USA) was employed for qPCR analysis following the manufacturer's instructions. The housekeeping genes *act-1* and *csq-1* (44) were used as the internal controls. The PCR amplification protocol consisted of 40 cycles of 94°C for 10 s, 60°C for 10 s, and 72°C for 10 s. PCRs were performed on an ABI PRISM 7000 real-time PCR device (Applied Biosystems, Foster City, CA). Fold change was calculated using the threshold cycle ($2^{-\Delta\Delta CT}$) method (45). The real-time PCR experiments were repeated three times for each reaction using independent RNA samples.

Fluorescence localization of neurons. To construct the recombinant vector *Ppmk-1*::mCherry, the *pmk-1* promoter was amplified using the genomic DNA as the template and cloned into the vector pCFJ104 with In-Fusion HD cloning kits (TaKaRa) replacing the original promoter of *myo-3*. The other transgenic lines, *kyIs104* [*str-1p*::GFP], *kyIs140* [*str-2*::GFP + *lin-15*(+)], and *mgIs42* [*tph-1*::GFP + *rol-6* (*su1006*)], were purchased from CGC for colocalization analysis with AWB, AWC, and ADF neurons, respectively. *Ppmk-1*::mCherry was then microinjected into the three transgenic lines described above. To colocalize *pmk-1* with *hecw-1* in OLL neurons, the promoter of *hecw-1* was also cloned into the vector pPD95_75 to construct *Phecw-1*::GFP by a similar method. The constructed *Phecw-1*::GFP was then mixed with *Ppmk-1*::mCherry and comicroinjected into the gonads of the wild-type N2 worms. The F2 progeny with both GFP and mCherry were selected and observed using the fluorescence microscope Nikon Eclipse Ni (Tokyo, Japan). The software ImageJ was used to quantify the mean fluorescence intensity.

Complementary tissue-specific expression. For complementary expression, the cDNA sequence of *pmk-1* was amplified from the cDNA library of N2. This target sequence was then fused to the promoter of *pmk-1* that had been obtained via the same method described above. Tissue-specific expression of *pmk-1* was achieved using the promoters of *vha-6* and *unc-119*, which drove *pmk-1* expression in the intestine and neurons, respectively (20, 31).

Statistical analysis. Each experiment was performed with at least three independent replicates. All of the data are expressed as the mean ± standard deviation (SD) or the mean ± standard error (SE) in survival curves. The statistical comparisons were generally performed using GraphPad Prism 7 (GraphPad, La Jolla, CA); the specific methods are described in the corresponding figure legends.

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The authors declare they have no conflicts of interest.

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