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Neural inhibition of dopaminergic signaling enhances immunity in a cell non-autonomous manner

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SUMMARY

The innate immune system is the frontline of host defense against microbial infections, but its rapid and uncontrolled activation elicits microbicidal mechanisms that have deleterious effects [1, 2]. Increasing evidence indicates that the metazoan nervous system, which responds to stimuli originating from both the internal and the external environment, functions as a modulatory apparatus that controls not only microbial killing pathways but also cellular homeostatic mechanisms [3–5]. Here, we report that dopamine signaling controls innate immune responses through a D1-like dopamine receptor, DOP-4, in *Caenorhabditis elegans*. Chlorpromazine inhibition of DOP-4 in the nervous system activates a microbicidal PMK-1/p38 mitogen-activated protein kinase signaling pathway that enhances host resistance against bacterial infections. The immune inhibitory function of dopamine originates in CEP neurons and requires active DOP-4 in downstream ASG neurons. Our findings indicate that dopamine signaling from the nervous system controls immunity in a cell non-autonomous manner and identifies the dopaminergic system as a potential therapeutic target for not only infectious diseases but also a range of conditions that arise as a consequence of malfunctioning immune responses.

RESULTS AND DISCUSSION

The ability of an organism to survive microbial infections requires tightly controlled activation of immune responses. Excessive inflammation can lead to conditions such as Crohn's disease, rheumatoid arthritis, Alzheimer's disease, and many other diseases that involve aberrant inflammation. Indeed, to survive an infection and return to homeostasis, animals appear to rely on reflex circuits that prevent inflammation when triggered by bacterial products or inflammatory cues [6, 7]. This rapid and precise communication between the nervous and immune systems serves as an ancestral mechanism to control immune homeostasis in metazoans.

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AUTHOR CONTRIBUTIONS

X.C. and A.A. conceived and designed experiments. X.C. performed experiments. X.C. and A.A. analyzed the data and wrote the paper.

Increasing evidence highlights the importance of the vagus nerve circuit in the mediation of anti-inflammatory effects [8]. Recent studies have demonstrated that electroacupuncture requires intact sciatic and vagus nerves to inhibit inflammation by promoting the release of dopamine in the adrenal medulla [9, 10]. Dopamine is a well-characterized catecholamine neurotransmitter that is involved in diverse functions, including movement, reward, pain perception, and drug addiction [11–13]. It has also been shown to target a number of dopamine receptors in a variety of immune cells [14, 15]. However, the precise dopaminergic neural circuits potentially involved in the control of immunity remain unknown due in part to the complexity of the nervous and immune systems of most model organisms. To dissect dopaminergic neural circuits capable of regulating immunity, we took advantage of the simple nervous system of the nematode *Caenorhabditis elegans*, which comprises only 302 neurons.

Recent findings indicate that several dopamine antagonists can activate a conserved p38/PMK-1 MAPK immune pathway in *C. elegans* [16]. As an initial step to determine whether drugs that target the dopaminergic nervous system may control immunity at the cell non-autonomous level, we treated wild-type animals with the dopamine antagonist chlorpromazine during exposure to the human opportunistic pathogen *Pseudomonas aeruginosa*. As shown in Figure 1A, animals treated with chlorpromazine exhibited enhanced resistance to *P. aeruginosa*-mediated killing. In contrast, wild-type animals showed a small but significantly enhanced susceptibility to the pathogen when treated with dopamine (Figure 1B). We also studied whether the enhanced resistance to *P. aeruginosa*-mediated killing induced by treatment with chlorpromazine correlated with higher levels of active PMK-1. Western blot analysis indicated that animals treated with chlorpromazine had higher levels of active PMK-1 (Figure 1C). Furthermore, two genes that are markers of the PMK-1 pathway, *F35E12.5* and *lys-2*, were up-regulated in response to the chlorpromazine treatment (Figure 1D), suggesting that chemical inhibition of dopamine signaling protects *C. elegans* against pathogenic bacteria by inducing the expression of immune-related genes.

Dopamine released from pre-synaptic vesicles must engage receptors expressed in downstream neurons to perform its physiological functions *in vivo*. To identify the dopamine receptor/s that may be involved in the control of immune responses, we examined the susceptibility to *P. aeruginosa* of four strains of *C. elegans* lacking dopamine receptors. We found that only *dop-4(tm1392)* animals, which carry a 1086-bp deletion in the *dop-4* gene, showed enhanced resistance to *P. aeruginosa* infection compared with wild-type animals (Figure 2A and Figure S1). We also found that *dop-4(tm1392)* animals do not show a significant lifespan extension on heat-killed bacteria compared to wild-type animals (Figure 2B), indicating that *dop-4* may modulate immunity without affecting longevity. To rule out the possibility of an allelic effect caused by the *dop-4(tm1392)* mutation, we examined the susceptibility to *P. aeruginosa*-mediated killing of *dop-4(ok1321*) animals, which harbor a 1766-bp deletion in the *dop-4* locus (Figure S1). Both *dop-4(tm1392*) and *dop-4(ok1321*) animals exhibited similar resistance to killing by *P. aeruginosa* (Figure 2C), suggesting that *dop-4* may be involved in dopamine-mediated immune regulation. Consistent with this idea, *dop-4* was required for the enhanced susceptibility to *P. aeruginosa*-mediated killing induced by dopamine treatment (Figure 2D). In addition, the enhanced resistance to *P. aeruginosa*-mediated killing of *dop-4(tm1392*) animals did not differ from that of *dop-4(tm1392*)

animals treated with chlorpromazine (Figure 2E). In contrast, treatment with chlorpromazine enhanced resistance to *P. aeruginosa* infection in animals carrying mutations in other dopamine receptors (Figure S2), suggesting that chlorpromazine enhances immunity primarily by inhibiting the D1-like dopamine receptor DOP-4.

Because avoidance to *P. aeruginosa* is part of the *C. elegans* defense response against this pathogen [17, 18], we performed a so-called "full-lawn" survival assay that uses plates that are completely covered in bacteria, a condition that eliminates pathogen avoidance. We found that *dop-4(tm1392*) animals exhibited enhanced resistance to *P. aeruginosa* infection compared with wild-type animals on full-lawn plates (Figure 2F). In addition, we monitored the lawn occupancy of wild-type and *dop-4(tm1392*) animals on partial-lawn plates, which are normally used for survival assays. The nematode distribution on the pathogen lawn did not differ significantly between wild-type and *dop-4(tm1392*) animals (Figure 2G), indicating that the enhanced resistance of *dop-4(tm1392*) animals to *P. aeruginosa* infection was not due to enhanced pathogen avoidance. We further questioned whether the enhanced resistance to *P. aeruginosa* infection correlated with reduced bacterial accumulation in the intestine. To address this question, *dop-4(tm1392*) animals were infected with *P. aeruginosa* labelled with GFP. The *dop-4(tm1392*) animals showed a significant reduction of fluorescence intensity within the intestine (Figure 2H). In addition, there were fewer bacterial cells in the intestine of the *dop-4* mutant compared with wild-type animals (Figure 2I). To study whether a reduced feeding behavior in *dop-4(tm1392)* animals may account for the reduced bacterial burden observed, we studied the pumping rate of the animals and the accumulation of fluorescent beads in the intestine. We observed that over 30 seconds or 2 minutes the pumping rate of *dop-4(tm1392)* is not lower than that of wild type animals (Figure S2E and S2F). In addition, the accumulation of fluorescent beads in the gut of *dop-4(tm1392)* and wild-type animals was comparable, while the accumulation of fluorescent beads in the feeding mutant *eat-2(ad465)* was lower (Figure S2G). These results indicate that *dop-4(tm1392)* animals do not exhibit any feeding deficiency and that the dose of infecting bacteria they received is comparable to that of wild type animals. Thus, we reasoned that the *dop-4* mutation leads to the activation of microbial killing mechanisms that result in reduced bacterial burden and enhanced resistance to infection.

Based on these results, we hypothesized that DOP-4 acts as a negative regulator of immunity, at least in part by inhibiting the p38/PMK-1 MAPK pathway. Consistent with this idea, we observed that *dop-4(tm1392)* animals had higher levels of active PMK-1 compared with wild-type animals (Figure 3A). Inhibition of the *pmk-1* gene by RNAi also completely suppressed the enhanced resistance to *P. aeruginosa* of *dop-4(tm1392)* animals (Figure 3B). Furthermore, PMK-1-dependent genes were up-regulated in *dop-4(tm1392)* animals relative to wild-type animals in the absence or presence of an infection (Figure 3C and Figure S3A). The levels of *pmk-1* in *dop-4(tm1392)* and wild-type animals were comparable (Figure S3B), indicating that DOP-4 controls PMK-1 activation rather than expression. To study the *in vivo* role of DOP-4 in the regulation of PMK-1, we used a transgenic transcriptional reporter strain of PMK-1 activity that expresses GFP under the control of the promoter of *F35E12.5* [19]. We observed higher levels of GFP in *dop-4(tm1392);*, P*F35E12.5::gfp* than in P*F35E12.5::gfp* animals (Figure 3D). We also found that animals lacking *dop-4* exhibited higher levels of GFP than control animals when infected with *P. aeruginosa* (Figure S3C),

indicating that DOP-4 functions as a negative regulator of immunity by preventing the expression of PMK-1-dependent genes in the intestine. It has been shown that the *C. elegans* transcription factor SKN-1 is activated by PMK-1 in response to stressful conditions [20]. SKN-1 is orthologous to mammalian Nrf transcription factors and functions to activate the oxidative stress response during pathogen infection [21]. Consistent with the notion that SKN-1 functions downstream of PMK-1, we found that RNAi of *skn-1* also abolished the enhanced resistance of *dop-4* animals (Figure 3E). In addition, genes controlled by SKN-1 were up-regulated in *dop-4(tm1392)* animals (Figure 3F).

It has been reported that *dop-4* is expressed in a series of neurons, including ASG, AVL, and pharyngeal neurons, as well as in intestinal cells [22]. To identify the tissue in which DOP-4 functions to regulate immune responses, we employed nematode strains that were engineered to promote RNAi only in neural or intestinal cells. We observed that inhibition of *dop-4* in the nervous system but not in the intestine increased the resistance of the nematodes to *P. aeruginosa* infection (Figure 4A and Figure S4A), indicating that DOP-4 functions in the nervous system to control immunity. As a first step to identify the specific dopaminergic neurons responsible for the observed immune regulation, we performed targeted killing of CEP neurons. We focused on these cells because they are the only dopaminergic neurons in *C. elegans* hermaphrodites that are exposed to the pseudocoelomic body fluid, from where they can directly communicate through neuroendocrine signals with tissues involved in defense responses. The killing of CEP neurons was achieved by expressing the two subunits of the *C. elegans* caspase CED-3 (p15 and p17) under the promoters of *dat-1* and *ace-1*, respectively. Because the expression patterns of these two genes overlap solely in the four CEP neurons, only in these neurons could the two subunits of CED-3 form a functional module to induce programmed cell death. The ablation was conducted using strain BY250, in which all dopaminergic neurons are labeled with GFP. We observed that only CEP neurons, but not other dopaminergic neurons, were ablated, confirming the efficacy of this cell-specific killing approach (Figure 4B). We found that CEP(-) animals showed enhanced resistance to *P. aeruginosa*-mediated killing compared with control animals (Figure 4C). In addition, the lawn occupancy of wild-type and CEP(-) animals was comparable at different time points (Figure S4B), which indicated that the pathogen resistance of CEP(-) animals is not due to enhanced pathogen avoidance. These data suggest that CEP neurons participate in immune modulation and that dopamine secreted from CEP neurons can induce downstream immune inhibition. Therefore, we explored whether treatment with exogenous dopamine could suppress the enhanced resistance to *P. aeruginosa* infection of CEP(-) animals. Indeed, CEP(-) animals treated with dopamine exhibited susceptibility to *P. aeruginosa*-mediated killing similar to that of wild-type animals (Figure 4D). No difference in the lawn occupancy of animals treated with chlorpromazine or dopamine was observed, indicating that the drugs affect immunity without altering pathogen avoidance (Figure S4C). We also observed that the ablation of CEP neurons could not further increase the resistance to *P. aeruginosa* of *dop-4(tm1392)* animals (Figure 4E). The lack of synergism between the *dop-4* mutation and CEP ablation suggests that dopamine released from CEP neurons may function through the dopamine receptor DOP-4 to inhibit downstream immune pathways. In support of this idea, we found that PMK-1-dependent genes were up-regulated in CEP(-) animals (Figure 4F). Therefore, we predicted that

expression of *dop-4* in neurons downstream CEP would suppress the enhanced resistance to *P. aeruginosa* of *dop-4(tm1392)* animals. Because ASG neurons are the only *dop-4*-expressing cells reported to have direct synaptic connectivity with CEP neurons, we tested whether *dop-4* is required in these cells. We found that single-neuron rescue of *dop-4* in ASG neurons could at least partially suppress the enhanced resistance to *P. aeruginosa*-mediated killing *of dop-4* animals (Figure 4G).

In the present study, we have shown that dopamine signaling negatively regulates the innate immune response through a D1-like dopamine receptor, DOP-4, by down-regulating the p38/PMK-1 MAPK pathway. Furthermore, a putative neural circuit involving dopaminergic neurons CEP and DOP-4-expressing neurons ASG has been shown to control the immune response against pathogen infections (Figure 4H). The ablation of CEP neurons conferred a similar enhanced resistance to infection, as observed in the *dop-4* mutant animals. In addition, exogenous dopamine suppressed the enhanced resistance to pathogen infection caused by CEP ablation. The identification of this neural circuit and the demonstration that chemical inhibition of dopamine signaling in the nervous system can control immune pathways at the cell non-autonomous level provides proof of concept for the use of neural interventions to control infections and conditions that involve aberrant immune functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Inhibition of dopamine signaling protects against bacterial infections
- Chlorpromazine enhances immunity by inhibiting a D1-like dopamine receptor
- Dopamine signaling regulates p38 MAP kinase activity
- Dopaminergic neurons control immunity in the C. elegans intestine

In Brief

Cao and Aballay show that chemical inhibition of the neurotransmitter dopamine in the nervous system enhances a signaling pathway that functions in the intestine to fight bacterial infections. The identification of this neural circuit provides proof of concept for the use of neural interventions to control infections and the immune system.

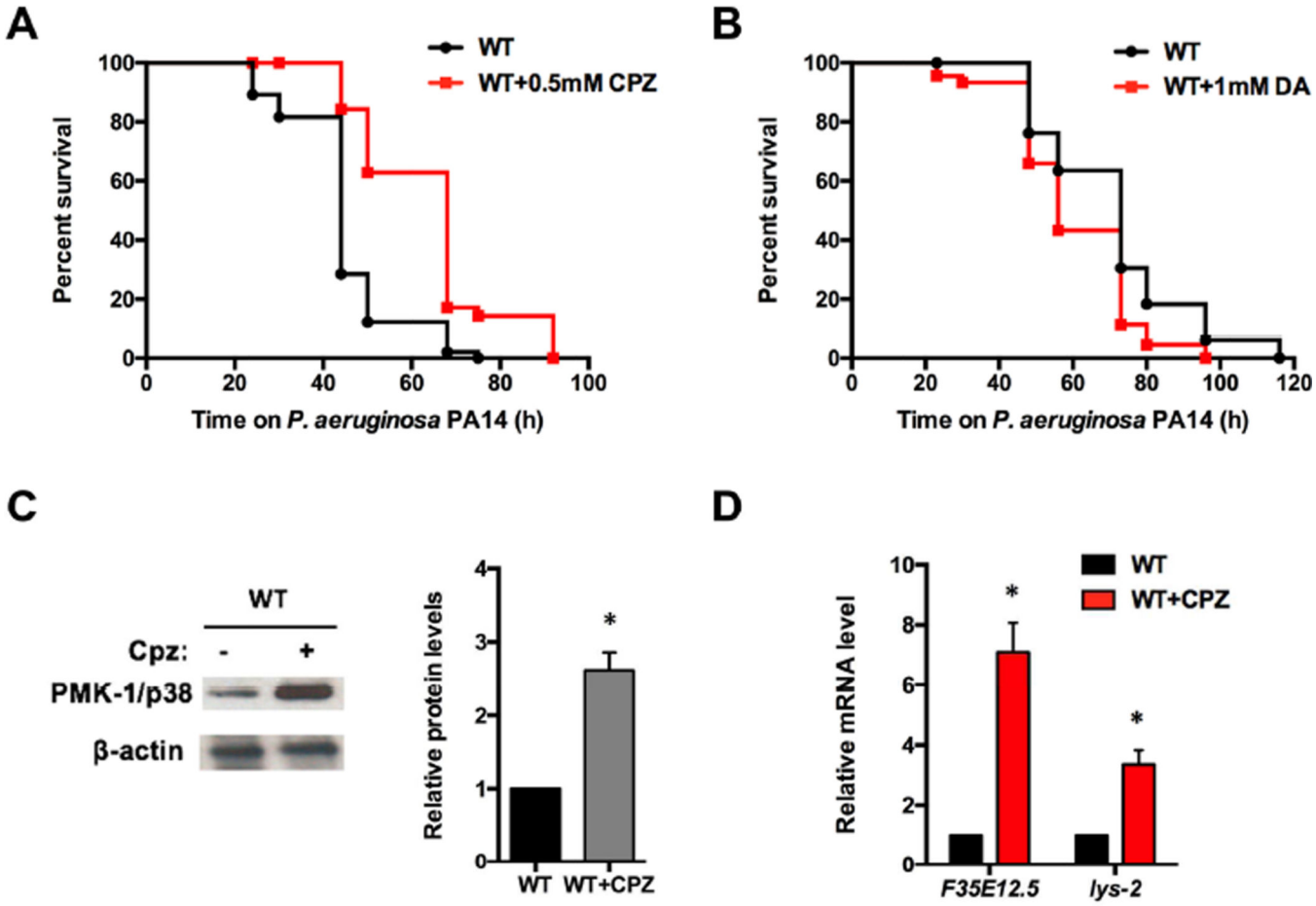

Figure 1. Chemical Targeting of Dopamine Signaling Affects Immune Responses

(A) Wild-type animals were exposed to *P. aeruginosa* PA14 in the presence or absence of the dopamine antagonist chlorpromazine (CPZ) and scored for survival. WT vs. WT+CPZ: $p < 0.001$. (B) Wild-type animals were exposed to *P. aeruginosa* PA14 in the presence or absence of dopamine (DA) and scored for survival. WT vs. WT+DA: $p < 0.01$. (C) Western blot analysis of active PMK-1/p38 levels in wild-type animals treated with or without chlorpromazine. Image quantification was performed using the software program ImageJ. (D) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of *F35E12.5* and *lys-2* gene expression in wild-type animals treated with chlorpromazine compared with control animals. All bars represent means ± SEM. *, $p < 0.05$.

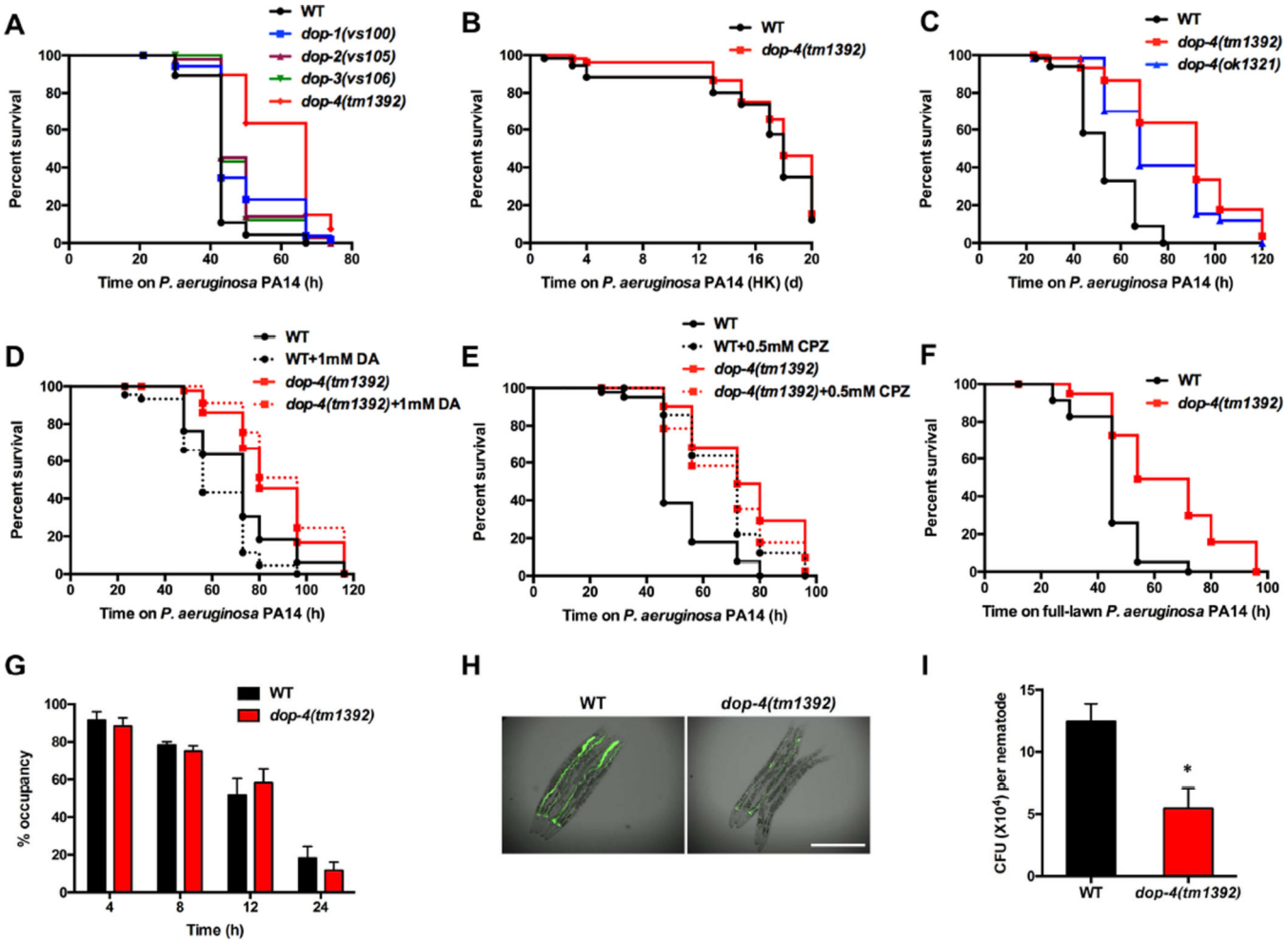

Figure 2. The D1-like Dopamine Receptor DOP-4 Controls Immunity against *P. aeruginosa* Infection

(A) Wild-type animals and dopamine receptor mutants were exposed to *P. aeruginosa* PA14 and scored for survival. WT vs. *dop-4(tm1392)*: $p < 0.0001$. (B) Wild-type and *dop-4(tm1392)* animals were exposed to heat-killed *P. aeruginosa* PA14 and scored for survival. WT vs. *dop-4(tm1392)*: $p > 0.1$. (C) Wild-type, *dop-4(tm1392)*, and *dop-4(ok1321)* animals were exposed to *P. aeruginosa* PA14 and scored for survival. WT vs. *dop-4(tm1392)*: $p < 0.001$; WT vs. *dop-4(ok1321)*: $p < 0.001$. (D) Wild-type and *dop-4(tm1392)* animals were exposed to *P. aeruginosa* PA14 in the presence or absence of dopamine and scored for survival. WT vs. WT+DA: $p < 0.01$; *dop-4(tm1392)* vs. *dop-4(tm1392)*+DA: $p > 0.1$. (E) Wild-type and *dop-4(tm1392)* animals were exposed to *P. aeruginosa* PA14 in the presence or absence of chlorpromazine and scored for survival. WT vs. WT+CPZ: $p < 0.001$; *dop-4(tm1392)* vs. *dop-4(tm1392)*+CPZ: $p > 0.1$. (F) Wild-type and *dop-4(tm1392)* animals were exposed to a full lawn of *P. aeruginosa* PA14 and scored for survival. WT vs. *dop-4(tm1392)*: $p < 0.001$. (G) Wild-type and *dop-4(tm1392)* animals were cultured on partial pathogen lawns, and the percentage of animals on the pathogen lawn was calculated for each time point. (H) Wild-type and *dop-4(tm1392)* animals were exposed to *P. aeruginosa* PA14-GFP for 30 hours and visualized using a Leica M165 FC

fluorescence stereomicroscope. Scale bar = 0.5 mm. (I) Wild-type and *dop-4(tm1392)* animals were exposed to *P. aeruginosa* PA14-GFP for 30 hours, and the colony forming units (CFU) were counted. Ten animals were used for each condition. All bars represent means ± SEM; *, $p < 0.05$. See also Figure S1 for allele information and Figure S2 for additional chlorpromazine treatments and feeding behaviors.

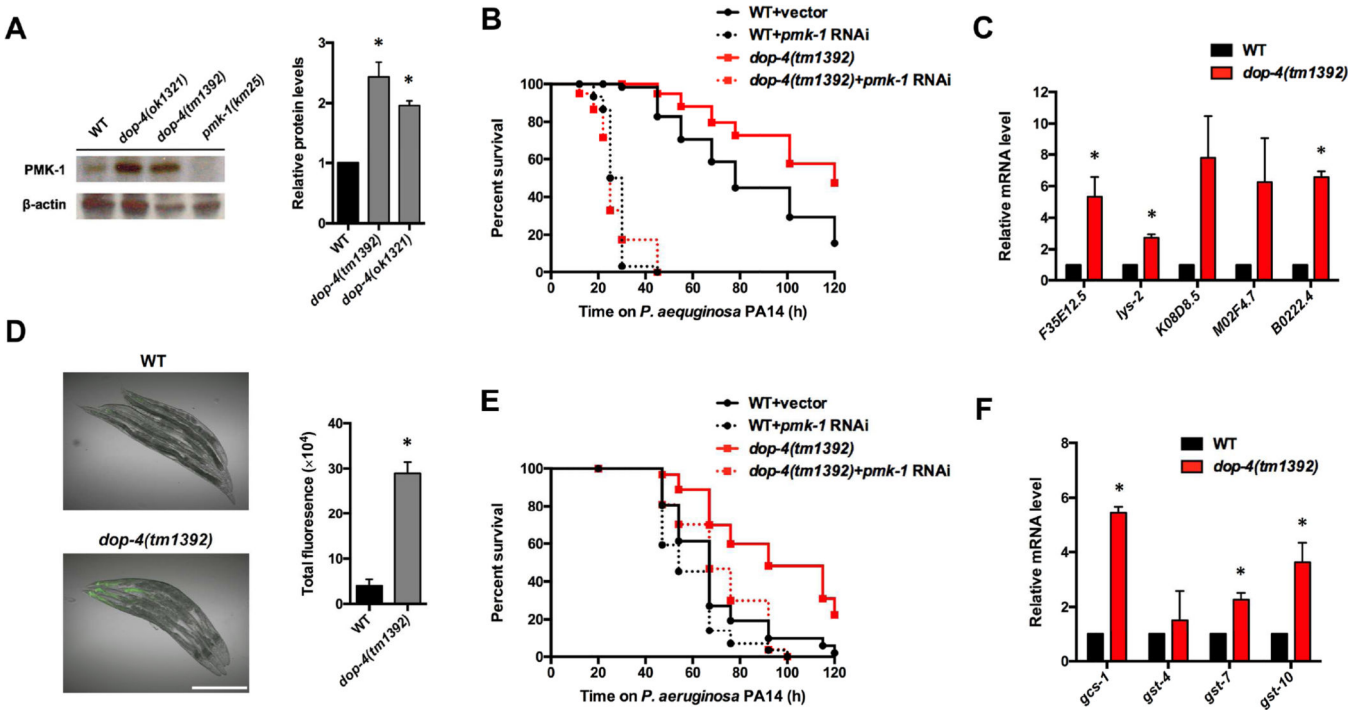

Figure 3. A conserved p38/PMK-1 MAP kinase pathway is required for the enhanced resistance to *P. aeruginosa* infection of *dop-4* animals

(A) Western blot analysis of active p38 levels of wild-type, *dop-4(ok1321)*, and *dop-4(tm1392)* animals. Image quantification was performed using the software program ImageJ. (B) Wild-type and *dop-4(tm1392)* animals fed with *E. coli* strain HT115 carrying a vector control or expressing double-stranded RNA (dsRNA) targeting *pmk-1* were exposed to *P. aeruginosa* PA14 and scored for survival. WT+*pmk-1* RNAi vs. *dop-4(tm1392)*+*pmk-1* RNAi: $p > 0.1$. (C) Quantitative RT-PCR analysis of *pmk-1*–regulated genes in *dop-4(tm1392)* compared with wild-type animals fed on *E. coli* OP50. (D) Fluorescence image of wild-type and *dop-4(tm1392)* animals expressing *F35E12.5*::*gfp*. Young adult animals were cultured on *E. coli* OP50 and visualized using a Leica M165 FC fluorescence stereomicroscope. Scale bar = 0.5 mm. Fluorescence was quantified using software ImageJ. (E) Wild-type and *dop-4(tm1392)* animals fed with *E. coli* strain HT115 carrying a control vector or expressing dsRNA targeting *skn-1* were exposed to *P. aeruginosa* PA14 and scored for survival. WT+*skn-1* RNAi vs. *dop-4(tm1392)*+*skn-1* RNAi: $p > 0.1$. (F) Quantitative RT-PCR analysis of *skn-1*–regulated genes in *dop-4(tm1392)* compared with wild-type animals fed on *E. coli* OP50. All bars represent means ± SEM; *, $p < 0.05$. See also Figure S3 for expression level of *pmk-1*–regulated genes upon *P. aeruginosa* infection.

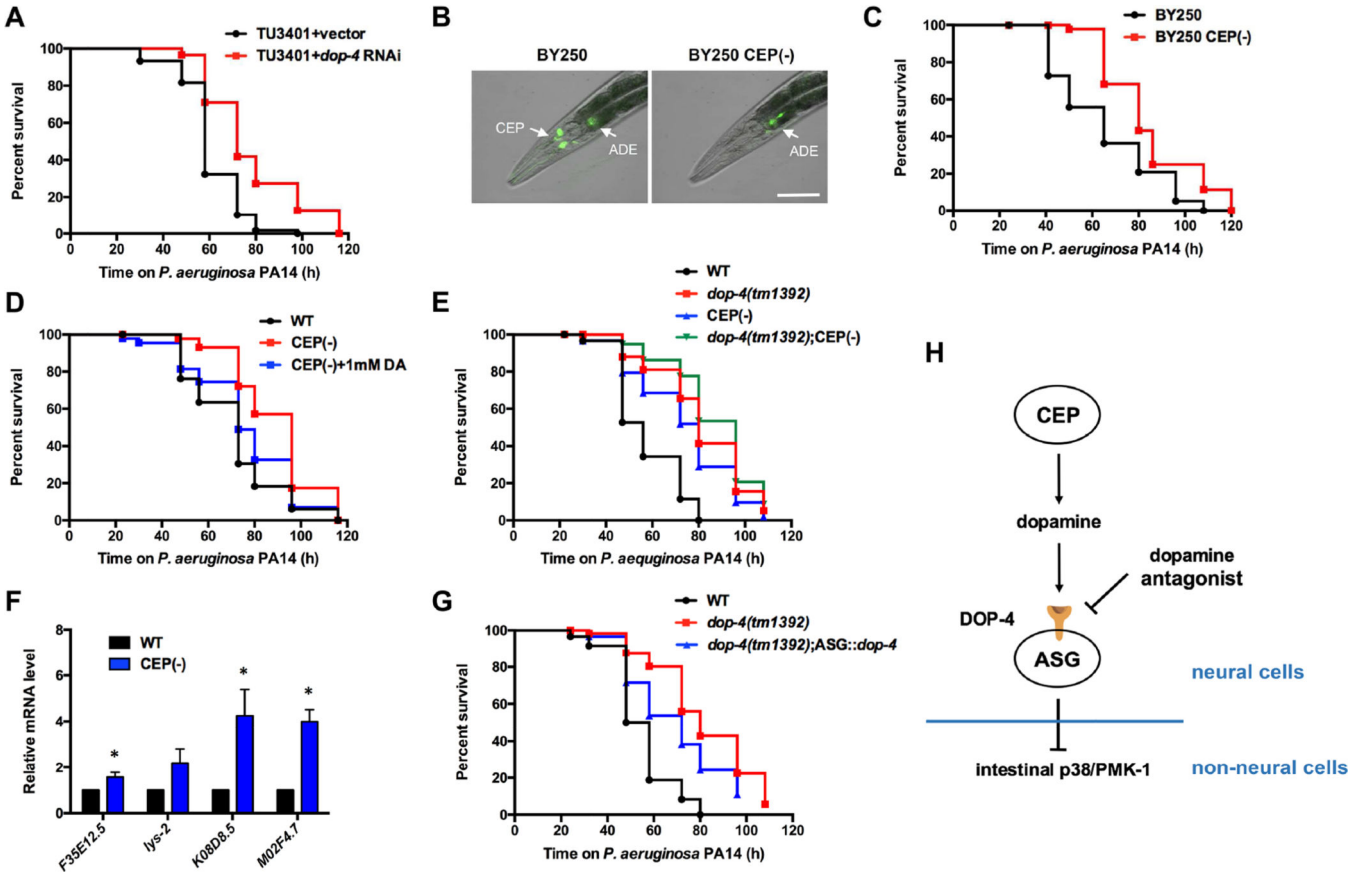

Figure 4. Dopaminergic CEP neurons control immunity through DOP-4 expressed in ASG neurons

(A) The neural-specific RNAi strain TU3401 fed with vector control or *dop-4* RNAi bacteria was exposed to *P. aeruginosa* PA14 and scored for survival. TU3401+vector vs. TU3401+*dop-4* RNAi: $p < 0.01$. (B) Fluorescence image showing the targeted ablation of CEP neurons. Scale bar = 20 μm. (C) BY250 and BY250 CEP(-) animals were exposed to *P. aeruginosa* PA14 and scored for survival. BY250 vs. BY250 CEP(-): $p < 0.01$. (D) Wild-type, CEP(-) and CEP(-) animals treated with dopamine were exposed to *P. aeruginosa* PA14 and scored for survival. WT vs. CEP(-): $p < 0.01$; WT vs. CEP(-)+DA: $p > 0.1$. (E) Wild-type, *dop-4(tm1392)*, CEP(-), and *dop-4(tm1392)*;CEP(-) animals were exposed to *P. aeruginosa* PA14 and scored for survival. WT vs. *dop-4(tm1392)*;CEP(-): $p < 0.001$; *dop-4(tm1392)* vs. *dop-4(tm1392)*;CEP(-): $p > 0.1$. (F) Quantitative RT-PCR analysis of *pmk-1*–regulated genes in CEP(-) compared with wild-type animals. Bars represent means ± SEM. *, $p < 0.05$. (G) Wild-type, *dop-4(tm1392)*, and *dop-4(tm1392)*;P*gcy-15*::*dop-4(tm1392)* animals were exposed to *P. aeruginosa* PA14 and scored for survival. *dop-4(tm1392)* vs. *dop-4(tm1392)*;P*gcy-15*::*dop-4(tm1392)*: $p < 0.05$. (H) Proposed model of dopamine regulation of innate immunity. See also Figure S4.