Neuropeptide Receptors NPR-1 and NPR-2 Regulate *Caenorhabditis elegans* Avoidance Response to the Plant Stress Hormone Methyl Salicylate

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ABSTRACT Methyl salicylate (MeSa) is a stress hormone released by plants under attack by pathogens or herbivores . MeSa has been shown to attract predatory insects of herbivores and repel pests. The molecules and neurons underlying animal response to MeSa are not known. Here we found that the nematode *Caenorhabditis elegans* exhibits a strong avoidance response to MeSa, which requires the activities of two closely related neuropeptide receptors NPR-1 and NPR-2. Molecular analyses suggest that NPR-1 expressed in the RMG inter/motor neurons is required for MeSa avoidance. An NPR-1 ligand FLP-18 is also required. Using a rescuing *npr-2* promoter to drive a *GFP* transgene, we identified that NPR-2 is expressed in multiple sensory and interneurons. Genetic rescue experiments suggest that NPR-2 expressed in the AIZ interneurons is required for MeSa avoidance. We also provide evidence that the AWB sensory neurons might act upstream of RMGs and AIZs to detect MeSa. Our results suggest that NPR-2 has an important role in regulating animal behavior and that NPR-1 and NPR-2 act on distinct interneurons to affect *C. elegans* avoidance response to MeSa.

KEYWORDS *C. elegans*; methyl salicylate; *npr-1*; *npr-2*; interneuron

PLANTS emit odorants that can affect animal behaviors. The identification of the molecules and neurons regulating these behaviors remains a central task for understanding an animal's nervous system.

Methyl salicylate (MeSa) is a volatile stress hormone released by plants when infected by pathogens (Park *et al.* 2007) or attacked by herbivores (van den Boom *et al.* 2004). Besides enhancing the systemic acquired resistance of the affected plants, MeSa could be sensed by adjacent plants as a warning signal for the infection (Park *et al.* 2007).

Ecological experiments also uncovered interesting effects of MeSa on animal behaviors. For example, MeSa is released by some plants as a pest repellent (Hardie *et al.* 1994; Jayasekara *et al.* 2005) and as an attractant for beneficial insects as well (James 2003; De Boer and Dicke 2004; James and Price 2004; Zhu and Park 2005). It is not clear what molecules and neural mechanisms determine whether an animal is attracted to or repelled by MeSa.

Caenorhabditis elegans has been an efficient model for studying the molecular and neural mechanisms underlying odorant-elicited behaviors (Bargmann 2006, 2012). Specifically, the detailed description of the neural connections by reconstructing serial-section electron microscopic pictures of the animals (White *et al.* 1986) provides a unique map for dissecting the neural correlates of each individual behavior. A combination of the neural connection diagram with molecular analyses will likely eventually lead to a systematic understanding of the neural regulation of a behavior, from key regulatory molecules to signaling integration in neural circuits. Several examples of such efforts include the dissection of a hub-and-spoke circuit that controls *C. elegans* social behavior (Macosko *et al.* 2009), the thermotaxis circuit (Kimata *et al.* 2012), the circuit that generates long-lasting roaming and dwelling states (Flavell *et al.* 2013), the mechanosensation circuit (Chalfie *et al.*

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1985), and the behavioral quiescence circuit (Choi *et al.* 2013).

We found that *C. elegans* strongly avoids MeSa, suggesting that this animal can be used for studying the molecular and neuronal mechanisms underlying the behavioral effects of MeSa. In this study we identified multiple genes important for the behavior and analyzed in detail how neuropeptide receptors NPR-1 and NPR-2 and neuropeptide FLP-18 act in different neurons to affect the avoidance behavior.

Materials and Methods

Strains are listed in Supporting Information, File S1.

MeSa avoidance assay

C. elegans avoidance to MeSA was performed using a previously described odortaxis assay (Bargmann *et al.* 1993) with modifications. Synchronized young adults were washed with M9 twice and H_2O once and placed on the midline of a 9-cm NGM plate without food. On the assay plate, 2 μl ethanol and 2 μl MeSa (Sigma, cat. no. M2047-100ML), respectively, were spotted in a small area (<3 mm in diameter and 0.5 cm from the periphery) at opposite ends. One microliter of NaN_3 (1.0 M) was spotted at these sites to paralyze animals that locomoted nearby. The plate was loosely sealed with paraffin membrane and kept in a 20° incubator for 4 hr (the standard exposure time in our study). The number of animals on the MeSa side (A) and the ethanol side (B) was scored under a dissecting microscope. The MeSa avoidance index is calculated as the ratio of (B minus A) divided by (B plus A). Animals climbing up the side of the plate were excluded from the analysis. A positive avoidance index indicates that the animals avoid MeSa while a negative index indicates that the animals are attracted to MeSa. A total of 30–200 animals were tested in each assay and the experiments were repeated at least three times for each strain. We found that 2 μl ethanol alone had no obvious effects on the behaviors of wild-type, *npr-1*, and *npr-2* animals (J. Luo and L. Ma, unpublished observations).

Molecular biology

To construct the *npr-1p*::*npr-1*::*GFP* transgene, a PCR-amplified full-length *npr-1* genomic DNA (gDNA) together with an *npr-1* promoter (2 kb upstream of the *npr-1* start codon) was subcloned to the pPD95.79 vector in-frame with *GFP* using *Xma*I/*Age*I restriction sites.

To construct the *flp-18p*::*flp-18* transgene, a PCR-amplified full-length *flp-18* gDNA with a *flp-18* promoter (1.7 kb upstream of the *flp-18* start codon) and a 3′ UTR fragment (0.8 kb downstream of the *flp-18* stop codon) was subcloned to the pMD18-T vector (Sino Biological).

To construct the *npr-2p*::*npr-2*::*GFP* transgene, a PCR-amplified full-length *npr-2* gDNA was subcloned to the pPD95.79 vector in-frame with *GFP* using *Bam*HI/*Age*I restriction sites. An *npr-2* promoter (2 kb upstream of the start codon of *npr-2*) was subcloned to the pPD95.79::*npr-2* backbone using *Pst*I/*Bam*HI restriction sites.

We inserted a PCR-amplified full-length *npr-1* gDNA fragment to pPD95.79 in-frame with *GFP* using *Xma*I/*Age*I restriction sites. The resulting pPD95.79::*npr-1* and pPD95.79::*npr-2* (see above) were used as backbones for constructing transgenes for neuron-specific rescue experiments.

For control transgenes, we tested *myo-3p*::*GFP* alone and *myo-3p*::*GFP* with *npr-1p*::*GFP*, *flp-18p*::*GFP* or *npr-2p*::*GFP*. We found no obvious effects of these transgenes on the MeSa avoidance responses in wild-type, *flp-18(gk3063)*, *npr-1(ky13)*, and *npr-2(ok419)* animals.

Neuron-specific promoters are listed in Table S1 (for *npr-1* transgenes), Table S2 (for *flp-18* transgenes), and Table S3 (for *npr-2* transgenes). Promoters were amplified from wild-type animals using primers listed in Table S4.

Transgene experiments

Germline transgene experiments were performed as described (Mello *et al.* 1991). Transgene mixtures contain 5–10 ng/μl transgene and 20 ng/μl pPD95.86::*GFP* (*myo-3p*::*GFP*) plasmid (which expresses GFP in body-wall muscles) as co-injection marker.

Identification of npr-2-expressing neurons

Two transgenic lines expressing GFP under control of the 2 kb rescuing *npr-2* promoter were generated. GFP-positive neurons were identified using a ×100 DIC/fluorescent objective of a Leica 5000B inverted microscope (Figure S2A) or a Leica TCS SP5 II laser confocal microscope (see Figure 4C) and compared to anatomical and morphological characteristics described in WormAtlas (www.wormatlas.org). We used DiI-stained sensory neurons (Tong and Burglin 2010) as landmarks to facilitate the verification of the identities of *npr-2*-expressing neurons (Figure S2A).

Statistical analysis

A two-tailed unpaired Student's *t*-test was used for single comparison. The Bonferroni correction after one-way ANOVA was used for multiple comparisons. *fat-4(wa14)*, *odr-4(n2144)*, *egl-4(n478)*, *odr-3(n2150)*, and *che-1(e1034)* mutants were found to be significantly different from wild-type animals in the MeSa avoidance response by Student *t*-test at the error rate of 1% (J. Luo and L. Ma, unpublished observations). In addition, *fat-4(wa14)*, *odr-4(n2144)*, *egl-4(n478)*, and *che-1(e1034)* were found to be significantly different from wild type based on the Benjamini–Hochberg procedure (also called false discovery rate method) (reviewed by Fay and Gerow 2013) at the false discovery rate of 1%.

Results

C. elegans exhibits a strong avoidance response to MeSa

To understand the molecular mechanism underlying the biological effect of MeSa on animal behaviors, we examined how *C. elegans* responds to different doses of MeSa using a previously described odortaxis assay (Bargmann *et al.*

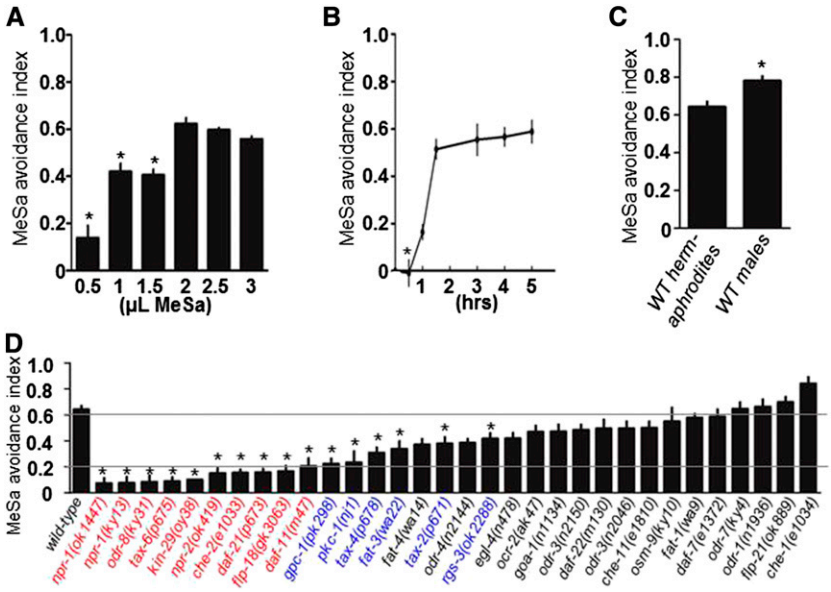

Figure 1 *C. elegans* exhibits a strong avoidance response to MeSa that requires activities of multiple genes. (A) Wild-type animals exhibited dose-dependent avoidance responses after 8-hr exposure to MeSa. Statistics: different from the index of 2 μl MeSa. Error bars: standard errors. $*P < 0.01$ (Bonferroni correction after one-way ANOVA). (B) Wild-type animals exhibited a maximal avoidance response after 1.5- to 5-hr exposure to 2 μl MeSa. Statistics: different from the index at 4 hr. Error bars: standard errors. $*P < 0.01$ (Bonferroni correction after one-way ANOVA). (C) MeSa avoidance response of wild-type male animals. Statistics: different from hermaphrodites. Error bars: standard errors. $*P < 0.05$ (Student's *t*-test). (D) MeSa avoidance responses of *C. elegans* behavioral mutants. Three groups of mutants were identified in the screen: mutants with strong (index ≤ 0.2, red), moderate (0.2 < index < 0.6, blue) and no apparent defects (black). Statistics: different from wild type. Error bars: standard errors. $*P < 0.01$ (Bonferroni correction after one-way ANOVA).

1993). After an 8-hr exposure to MeSa, wild-type animals exhibit a dose-dependent avoidance response, reaching a maximal response at 2 μl MeSa or higher doses (Figure 1A). Using 2 μl MeSa as the standard dose, we found that wild-type animals exhibit a gradually increased avoidance as the MeSa exposure time increases, reaching a maximal response between 1.5 and 5 hr (Figure 1B). Animals sense MeSa as a volatile because MeSa spotted on the inside of the Petri dish lid that did not contact the agar medium also caused a strong avoidance response (Figure S1A). We found that wild-type males have a slightly stronger MeSa avoidance response compared to hermaphrodites (Figure 1C), implying that sex might affect an animal's response to MeSa. In plants MeSa is converted to the biologically active defense hormone salicylic acid by the SA-binding protein 2 (SABP2, a MeSa esterase) to induce systemic acquired resistance (Kumar and Klessig 2008). We failed to identify a homolog of SABP2 in the *C. elegans* genome (BLAST, www.wormbase.org), suggesting that MeSa hydrolysis might not be required for the avoidance response.

A screen for genes required for the MeSa avoidance behavior

To identify genes required for MeSa avoidance, we screened 32 *C. elegans* mutants with previously described or putative behavioral defects, in which 30 distinct genes were affected (Figure 1D). We identified mutants with strong (index ≤ 0.2, red), moderate (0.2 < index < 0.6, blue), or no apparent (index > 0.6, black) defects (Figure 1D). Nine genes with strong effects on MeSa avoidance include the neuropeptide receptor genes *npr-1* (de Bono and Bargmann 1998) and *npr-2* (de Bono and Bargmann 1998), the ER-associated Ufm1-specific protease 2 gene *odr-8* (Dwyer *et al.* 1998; Chen *et al.* 2014), the calcineurin A subunit gene *tax-6* (Kuhara *et al.* 2002), the serine/threonine kinase gene *kin-29* (Lanjuin and Sengupta 2002), the WD40 domain protein-encoding gene *che-2* (Lewis and Hodgkin 1977; Fujiwara *et al.* 1999), the HSP90 chaperone gene *daf-21* (Vowels and Thomas 1994; Birnby *et al.* 2000), the neuropeptide gene *flp-18* (Rogers *et al.* 2003), and the receptor guanylyl cyclase gene *daf-11* (Vowels and Thomas 1994; Birnby *et al.* 2000).

We also identified six genes with moderate effects on the MeSa avoidance response (Figure 1D, blue). These genes encode a G protein gamma-subunit (*gpc-1*) (Jansen *et al.* 2002), a protein kinase C (*pkc-1*) (Okochi *et al.* 2005; Sieburth *et al.* 2007), two subunits of a cGMP-gated channel (*tax-4* and *tax-2*) (Coburn and Bargmann 1996; Komatsu *et al.* 1996), a delta-6 fatty acid desaturase (*fat-3*) (Watts and Browse 2002), and a regulator of G protein signaling (*rgs-3*) (Ferkey *et al.* 2007).

The neuropeptide receptor gene npr-1 is expressed in the RMG neurons to regulate the MeSa avoidance behavior

npr-1 acts in the RMG inter/motor neurons at the center of a hub-and-spoke neural circuit (the RMG circuit hereafter) to regulate *C. elegans* social feeding behavior (de Bono and Bargmann 1998; Macosko *et al.* 2009) and is a key regulatory gene for ethanol adaptation (Davies *et al.* 2004), pathogen susceptibility (Styer *et al.* 2008; Reddy *et al.* 2009), and behavioral quiescence (Choi *et al.* 2013). *npr-2* is the closest paralog of *npr-1* (de Bono and Bargmann 1998) with previously unknown functions in animal behaviors. We postulate that a detailed analysis of *npr-1* and *npr-2* in regulating the MeSa avoidance behavior might provide novel insights into functions of neuropeptide receptors.

To verify the role of *npr-1* in the MeSa avoidance response, we tested the Hawaiian *C. elegans* strain CB4856 that carries a hypomorphic allele of *npr-1* (de Bono and Bargmann 1998) and three *npr-1* loss-of-function mutants, *npr-1(ky13)*, *npr-1(ad609)* (de Bono and Bargmann 1998),

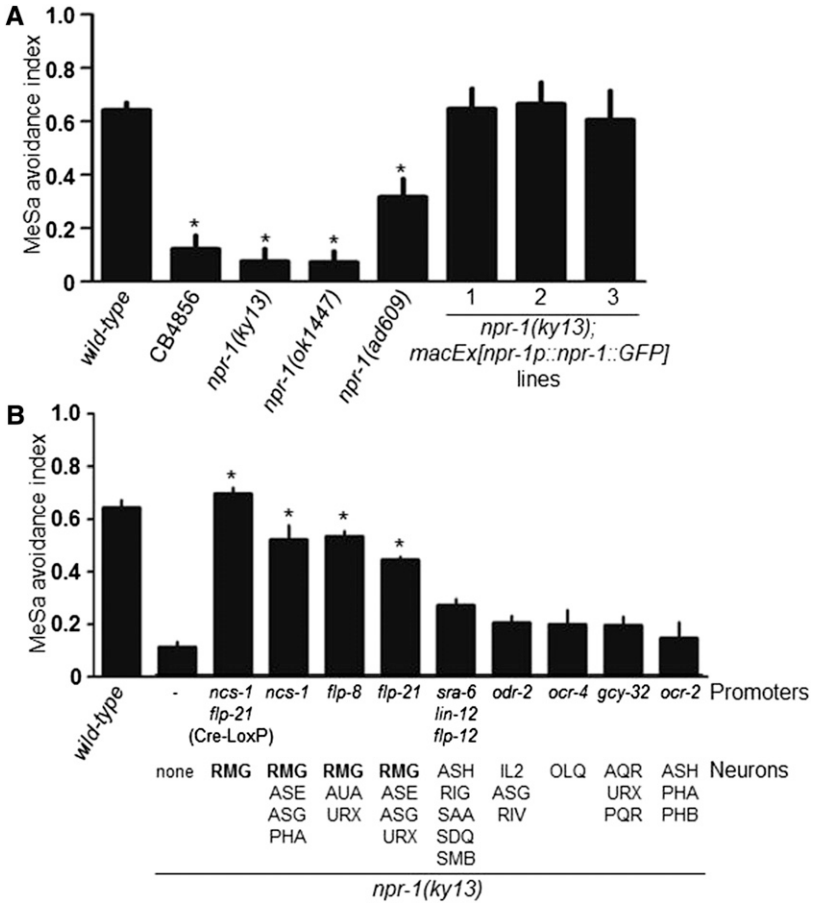

Figure 2 *npr-1* expressed in the RMG inter/motor neurons is required for *C. elegans* avoidance response to MeSa. (A) MeSa avoidance responses of different *npr-1* mutants and *npr-1(ky13)* mutants expressing an *npr-1*::*GFP* transgene under control of an endogenous *npr-1* promoter. Statistics: different from wild type. Error bars: standard errors. $*P < 0.01$ (Bonferroni correction after one-way ANOVA). (B) MeSa avoidance responses of *npr-1(ky13)* mutants expressing an *npr-1*::*GFP* transgene under control of neuron-specific promoters. Target neurons are listed. Three lines were assayed for each transgene. Statistics: different from *npr-1(ky13)*. Error bars: standard errors. $*P < 0.01$ (Bonferroni correction after one-way ANOVA).

and *npr-1(ok1447)* (Stawicki *et al.* 2013). We found that all strains exhibited apparent MeSa avoidance defects (Figure 2A), in which CB4856, *npr-1(ky13)*, and *npr-1(ok1447)* animals had similarly strong defects while *npr-1(ad609)* animals had a moderate defect. An *npr-1*::*GFP* transgene under control of a 2-kb *npr-1* endogenous promoter completely rescued the defective MeSa avoidance of *npr-1(ky13)* mutants (Figure 2A), demonstrating that *npr-1* is essential for the behavior. The moderate effect of *npr-1(ad609)* on the MeSa avoidance response implies that NPR-1(ad609) might retain a residual activity for mediating this behavior. Alternatively an unknown mutation in the *npr-1(ad609)* strain might have modified the MeSa avoidance behavior.

To identify *npr-1*-expressing neurons involved in the behavior, we performed transgene rescue experiments using previously described neuron-specific promoters (Table S1). *npr-1* transgene expression in the RMG neurons could significantly rescue the defective MeSa avoidance of *npr-1(ky13)* mutants (Figure 2B), while transgene expression in *npr-1*-expressing neurons other than RMGs failed to rescue (Figure 2B). Using a Cre-LoxP transgene combination (Macosko *et al.* 2009) to express an *npr-1* transgene specifically in RMGs, we found that the defective MeSa avoidance of *npr-1(ky13)* mutants was also completely rescued (Figure 2B). Therefore, *npr-1* likely acts in RMGs to regulate the MeSa avoidance.

The neuropeptide FLP-18 is required for the MeSa avoidance behavior

flp-18 and *flp-21* were previously shown to encode FMRFamide-related ligands for NPR-1 (Kubiak *et al.* 2003; Rogers *et al.* 2003). We found that *gk3063* (Figure 3A), a deletion mutation of *flp-18* caused a strong MeSa avoidance defect (Figure 1D and Figure 3B). This defect could be rescued by a *flp-18* transgene (Figure 3B). To test whether FLP-18 expressed by different neurons (Rogers *et al.* 2003) might differentially affect the MeSa avoidance, we expressed a *flp-18* transgene under control of various neuron-specific promoters (Table S2). We found that each transgene rescued the defective MeSa avoidance of *flp-18(gk3063)* mutants to a level similar to that of wild type (Figure 3C), suggesting indistinguishable roles for FLP-18-expressing neurons in mediating the MeSa avoidance. Different from *flp-18*, a *flp-21(ok889)* deletion mutation did not obviously affect the MeSa avoidance (Figure 1D). Therefore FLP-18 activity might be specifically required for the behavior.

The neuropeptide receptor gene npr-2 acts in the AIZ interneurons to regulate the MeSa avoidance behavior

Among mutants exhibiting strong MeSa avoidance defects (Figure 1D) is a deletion mutant (*ok419*) (Figure 4A) of the neuropeptide receptor gene *npr-2* (de Bono and Bargmann 1998). *npr-2(ok419)* mutants were previously found to have

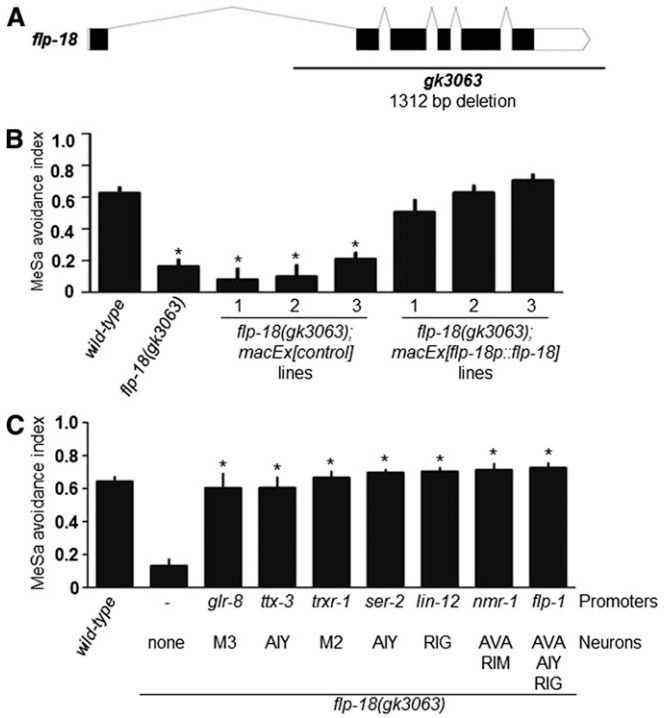

Figure 3 *flp-18* is required for *C. elegans* avoidance response to MeSa. (A) *flp-18* gene structure (designed using the Exon-Intron Graphic Maker software at www.wormweb.org based on gene sequence information at www.wormbase.org) and the *gk3063* deletion mutation. (B) MeSa avoidance responses of *flp-18(gk3063)* mutants expressing a *myo-3p*::*GFP* control transgene or a *flp-18p*::*flp-18* transgene. Three lines were assayed for each transgene. Statistics: different from wild type. Error bars: standard errors. *$P < 0.01$ (Bonferroni correction after one-way ANOVA). (C) MeSa avoidance response of *flp-18(gk3063)* mutants expressing a *flp-18* transgene in different *flp-18*-expressing neurons. Three lines were assayed for each transgene. Statistics: different from *flp-18(gk3063)*. Error bars: standard errors. *$P < 0.01$ (Bonferroni correction after one-way ANOVA).

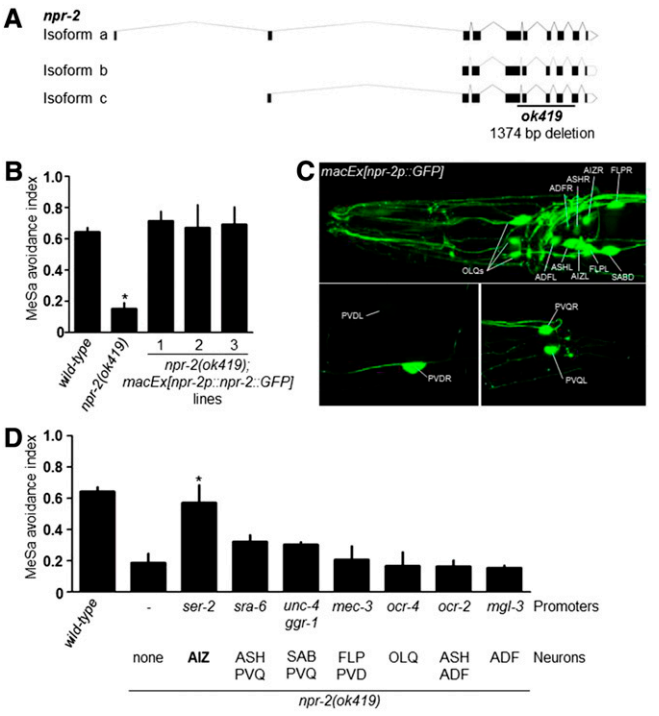

Figure 4 *npr-2* is expressed in multiple neurons and functions in the AIZ interneurons to regulate the MeSa avoidance response. (A) *npr-2* gene structure (designed using the Exon-Intron Graphic Maker software at www.wormweb.org based on gene sequence information at www.wormbase.org). The *ok419* mutation deletes a region including exon 5 (partial), exons 6, 7, and 8, and exon 9 (partial) of *npr-2* isoform a. (B) MeSa avoidance response of *npr-2(ok419)* mutants expressing an *npr-2*::*GFP* transgene under control of an endogenous *npr-2* promoter. Statistics: different from wild type. Error bars: standard errors. *$P < 0.01$ (Bonferroni correction after one-way ANOVA). (C) A *GFP* transgene under control of the endogenous *npr-2* promoter is expressed in multiple sensory neurons and interneurons. (D) MeSa avoidance responses of *npr-2 (ok419)* mutants expressing an *npr-2*::*GFP* transgene under control of different neuron-specific promoters. Target neurons are indicated. Three lines were assayed for each transgene. Statistics: different from *npr-2 (ok419)*. Error bars: standard errors. *$P < 0.05$ (Bonferroni correction after one-way ANOVA).

increased intestinal fat storage (Cohen *et al.* 2009). However behavioral defects have not been described. A transgene expressing an NPR-2::GFP fusion protein under control of a 2-kb endogenous *npr-2* promoter rescued the defective MeSa avoidance in *npr-2(ok419)* mutants (Figure 4B), suggesting that *npr-2* is required for the behavior.

To identify *npr-2*-expressing cells, we generated transgenic animals expressing GFP (Figure 4C) under control of the rescuing *npr-2* promoter and identified a total of 15–17 GFP-positive neurons that likely express *npr-2* (Figure 4C and Figure S2A). These neurons include sensory neurons OLQs (2–4), ASHL/R, ADFL/R, FLPL/R, and PVDL/R and interneurons AIZL/R, SABD, and PVQL/R (Figure 4C, Figure S2A, and Figure S2B). The expressivity of the *npr-2p*::*GFP* transgene in each class of neurons ranges from 62.5% for the AIZ interneurons and 100% for the FLP sensory neurons (Figure S2B). The variable penetrance of GFP expression in these neurons might be caused by transgene mosaicism or variable GFP expression intensity.

In *C. elegans*, FLPs and PVDs are the sensory neurons that mediate harsh touch stimuli (Way and Chalfie 1989). *npr-2(ok419)* mutants exhibited a grossly normal response to harsh touch (J. Luo and L. Ma, unpublished observations), suggesting that *npr-2* might not be essential for harsh touch sensation.

To identify *npr-2*-expressing neurons involved in the MeSa avoidance, we performed neuron-specific transgene rescue experiments. We tested eight promoters (Table S3) that drive transgene expression in each or a combination of the *npr-2*-expressing neurons. Only a *ser-2* promoter that was previously shown to drive expression in the AIZ interneurons (Tsalik *et al.* 2003) but not in any other *npr-2*-expressing neurons could strongly rescue the defective MeSa avoidance (Figure 4D), while other promoters (Table S3) that do not drive transgene expression in AIZs had no

apparent rescuing effects (Figure 4D). Hence *npr-2* might act in the AIZ interneurons to regulate the MeSa avoidance.

npr-1 loss-of-function mutants exhibit social feeding (de Bono and Bargmann 1998), a behavior regulated by the RMG circuit (Macosko *et al.* 2009). We found that *npr-2(ok419)* mutants did not exhibit an obvious social feeding (Figure S1B).

The AWB sensory neurons might be required for detecting MeSa

In *C. elegans* the AWB sensory neurons primarily detect repulsive volatiles (Bargmann 2006), raising the question of whether AWBs are responsible for sensing MeSa. To test this, we examined the *lim-4(ky403)* mutants, in which the AWB neurons are transformed to the AWC cell fate (Sagasti *et al.* 1999), and other mutants with different sensory neuron fate transformations (Hobert 2010). The results showed that only the *lim-4(ky403)* mutants are strongly defective in the MeSa avoidance (Figure 5A), implying that AWBs might be the sensory neurons primarily responsible for MeSa detection. We examined the *C. elegans* wiring diagram (White *et al.* 1986) (www.wormweb.org) and found that AWBs synapse onto AIZs and connect with RMGs by a gap junction (Figure 5B). Such a wiring diagram implies a neural pathway that consists of AWBs, AIZs, and RMGs for mediating *C. elegans* avoidance response to MeSa.

Finally we tested six other neuropeptide receptors and found that none was required for the MeSa avoidance (Figure 5C). NPR-5 and NPR-4 (not available in this study) were previously found to be FLP-18 receptors as well (Cohen *et al.* 2009). That *npr-5* mutants were not apparently defective in the MeSa avoidance supports the notion the FLP-18 might act though NPR-1 or other receptors to regulate this behavior.

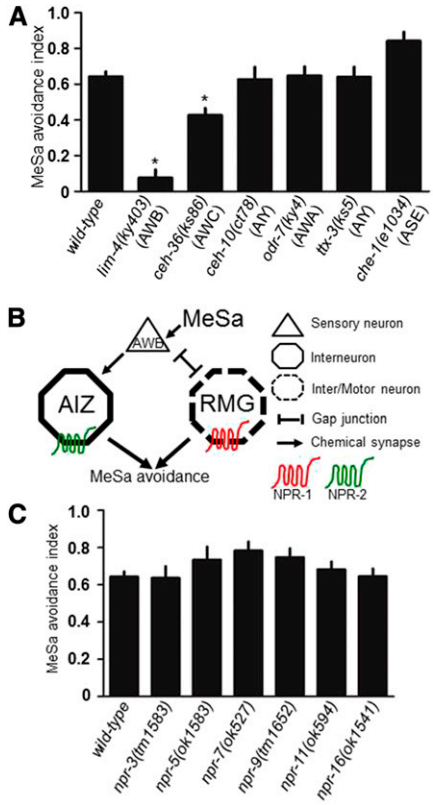

Figure 5 The AWB sensory neurons might be required for detecting MeSa. (A) MeSa avoidance responses of terminal selector gene mutants. Affected neurons are indicated. Statistics: different from wild type. Error bars: standard errors. $*P < 0.01$ (Bonferroni correction after one-way ANOVA). (B) A simplified wiring diagram of the neurons that likely mediate the MeSa avoidance response. Neuron classes, NPR-1 (red) and NPR-2 (green) are indicated. (C) MeSa avoidance responses of deletion mutants of six other GPCRs.

Discussion

In this study we provide evidence that *C. elegans* exhibits a strong avoidance response to the plant stress hormone methyl salicylate, which requires activities of two neuropeptide receptor genes *npr-1* and *npr-2*. We propose a model that the AWB sensory neurons act upstream to detect MeSa and transmit the odorant signals to NPR-1-expressing RMG inter/motor neurons and NPR-2-expressing AIZ interneurons. Our study suggests a novel function of NPR-2 in regulating *C. elegans* response to natural odorants.

MeSa has a myriad of effects on plants and animals. As a volatile, MeSa is released by plants to elicit systemic acquired resistance upon pathogen infection (Park *et al.* 2007), to attract predators of herbivores (James 2003; De Boer and Dicke 2004; James and Price 2004; Zhu and Park 2005) and to repel pests (Hardie *et al.* 1994; Jayasekara *et al.* 2005). MeSa is widely used as a refreshing odorant in food (Lewis 1989) and hygiene products (Lachenmeier *et al.* 2013). MeSa is also an ingredient in over-the-counter topical creams for the relief of musculoskeletal aches and pains (Chan 1996a,b; Higashi *et al.* 2010). Excess intake of MeSa could be life threatening probably due to severe, rapid-onset salicylate poisoning (Chan 1996a,b; Davis 2007). The broad effects of MeSa on animals warrant a detailed study of the underlying biological mechanisms.

How sensory neurons detect MeSa is unclear. MeSa was shown to have both stimulatory and inhibitory effects on human transient receptor potential V1 (TRPV1), in which the inhibitory effect of MeSa on capsaicin-induced TRPV1 activation was proposed to underlie the analgesic effects of MeSa (Ohta *et al.* 2009). Five genes (*osm-9*, *ocr-1*, *ocr-2*, *ocr-3*, and *ocr-4*) encode TRPV channels in the *C. elegans* genome (Tobin *et al.* 2002) and it appears that none is expressed in the AWB sensory neurons (Colbert *et al.* 1997; Tobin *et al.* 2002). In addition, *osm-9* and *ocr-2* mutants had grossly normal MeSa avoidance responses (Figure 1D). Therefore, TRPV channels might not be the MeSa receptors in AWBs. Recently EpOR1, a seven-transmembrane odorant receptor expressed in antennae sensory neurons of the tortricid moth *Epiphyas postvittana*, was shown to exhibit high sensitivity to MeSa when expressed in the insect sf9 cells (Jordan *et al.* 2009). We failed to identify

a *C. elegans* protein similar to EpOR1 (BLAST, www.wormbase.org). *C. elegans* has >1000 G protein-coupled receptors, among which >500 might function as chemosensory receptors (Bargmann 2006). A future survey of AWB-specific GPCRs that are required for the MeSa avoidance response might lead to the identification of a MeSa receptor in *C. elegans*.

NPR-1 regulates *C. elegans* social feeding behavior (de Bono and Bargmann 1998; Macosko *et al.* 2009), oxygen sensing (Cheung *et al.* 2005), acute response to ethanol (Davies *et al.* 2004), pathogen susceptibility (Styer *et al.* 2008; Reddy *et al.* 2009), and behavioral quiescence (Choi *et al.* 2013). We found that RMG-expressed NPR-1 is required for *C. elegans* avoidance to MeSa, verifying the key role of the RMG inter/motor neurons in NPR-1-regulated behaviors (Macosko *et al.* 2009; Choi *et al.* 2013). As a close paralog of NPR-1 (de Bono and Bargmann 1998), NPR-2 acts in the AIZ interneurons to regulate *C. elegans* avoidance to MeSa (Figure 4). AIZs also have a well-defined function in mediating cryophilic migration (Mori and Ohshima 1995; Kimata *et al.* 2012) and are involved in chemotaxis to NaCl (Iino and Yoshida 2009) and aversive olfactory learning (Ha *et al.* 2010). These findings suggest that AIZs might function like RMGs as an integrating site for various sensory stimuli. If so, NPR-2 might be a regulator of these behaviors.

Besides *npr-1*, *npr-2*, and *flp-18*, we identified 12 other genes required for the MeSa avoidance response (Figure 1D and Figure S1C). These genes encode a G protein gamma-subunit (*gpc-1*) (Jansen *et al.* 2002) and a G protein beta-subunit-like (*che-2*) (Fujiwara *et al.* 1999), an Ufm1 protease subunit required for GPCR maturation (*odr-8*) (Dwyer *et al.* 1998; Chen *et al.* 2014), an AMP/SNF kinase essential for GPCR expression (*kin-29*) (Lanjuin and Sengupta 2002), a protein kinase C involved in neuropeptide secretion (*pkc-1*) (Okochi *et al.* 2005; Sieburth *et al.* 2007), and five factors downstream of G protein signaling (*daf-11*, *tax-4*, *tax-2*, *fat-3*, and *rgs-3*) (Coburn and Bargmann 1996; Komatsu *et al.* 1996; Birnby *et al.* 2000; Watts and Browse 2002; Bargmann 2006; Ferkey *et al.* 2007). *tax-6* encodes a calcineurin A protein (Kuhara *et al.* 2002) that genetically interacts with G proteins (Lee *et al.* 2004), while *daf-21* encodes an HSP90 chaperone that functions similarly to *daf-11* in the dauer pathway (Vowels and Thomas 1994; Birnby *et al.* 2000). Therefore G protein signaling is likely essential for the MeSa avoidance response, consistent with our findings that NPR-1, NPR-2, and FLP-18 play critical roles in this behavior and implying that an unknown GPCR might be the chemosensory receptor for MeSa.

In short, we found that NPR-1 and NPR-2 regulate *C. elegans* avoidance response to MeSa. We identified a neuronal pathway from the AWB sensory neurons to the RMG inter/motor neurons and AIZ interneurons as a possible regulatory pathway for this behavior. Future identification of the MeSa receptor and dissection of the detailed neural circuit will provide novel insight into the effects of MeSa on animals.

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Literature Cited

Bargmann, C. I., 2006 Chemosensation in *C. elegans* (October 25, 2006), WormBook, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.123.1, http://www.wormbook.org.

Bargmann, C. I., 2012 Beyond the connectome: how neuromodulators shape neural circuits. BioEssays 34: 458–465.

Bargmann, C. I., E. Hartwieg, and H. R. Horvitz, 1993 Odorant-selective genes and neurons mediate olfaction in *C. elegans*. Cell 74: 515–527.

Birnby, D. A., E. M. Link, J. J. Vowels, H. Tian, P. L. Colacurcio *et al.*, 2000 A transmembrane guanylyl cyclase (DAF-11) and Hsp90 (DAF-21) regulate a common set of chemosensory behaviors in *Caenorhabditis elegans*. Genetics 155: 85–104.

Chalfie, M., J. E. Sulston, J. G. White, E. Southgate, J. N. Thomson *et al.*, 1985 The neural circuit for touch sensitivity in *Caenorhabditis elegans*. J. Neurosci. 5: 956–964.

Chan, T. Y., 1996a Medicated oils and severe salicylate poisoning: quantifying the risk based on methyl salicylate content and bottle size. Vet. Hum. Toxicol. 38: 133–134.

Chan, T. Y., 1996b Potential dangers from topical preparations containing methyl salicylate. Hum. Exp. Toxicol. 15: 747–750.

Chen, C., E. Itakura, K. P. Weber, R. S. Hegde, and M. de Bono, 2014 An ER complex of ODR-4 and ODR-8/Ufm1 specific protease 2 promotes GPCR maturation by a Ufm1-independent mechanism. PLoS Genet. 10: e1004082.

Cheung, B. H., M. Cohen, C. Rogers, O. Albayram, and M. de Bono, 2005 Experience-dependent modulation of *C. elegans* behavior by ambient oxygen. Curr. Biol. 15: 905–917.

Choi, S., M. Chatzigeorgiou, K. P. Taylor, W. R. Schafer, and J. M. Kaplan, 2013 Analysis of NPR-1 reveals a circuit mechanism for behavioral quiescence in *C. elegans*. Neuron 78: 869–880.

Coburn, C. M., and C. I. Bargmann, 1996 A putative cyclic nucleotide-gated channel is required for sensory development and function in *C. elegans*. Neuron 17: 695–706.

Cohen, M., V. Reale, B. Olofsson, A. Knights, P. Evans *et al.*, 2009 Coordinated regulation of foraging and metabolism in *C. elegans* by RFamide neuropeptide signaling. Cell Metab. 9: 375–385.

Colbert, H. A., T. L. Smith, and C. I. Bargmann, 1997 OSM-9, a novel protein with structural similarity to channels, is required for olfaction, mechanosensation, and olfactory adaptation in *Caenorhabditis elegans*. J. Neurosci. 17: 8259–8269.

Davies, A. G., J. C. Bettinger, T. R. Thiele, M. E. Judy, and S. L. McIntire, 2004 Natural variation in the *npr-1* gene modifies ethanol responses of wild strains of *C. elegans*. Neuron 42: 731–743.

Davis, J. E., 2007 Are one or two dangerous? Methyl salicylate exposure in toddlers. J. Emerg. Med. 32: 63–69.

De Boer, J. G., and M. Dicke, 2004 The role of methyl salicylate in prey searching behavior of the predatory mite *phytoseiulus persimilis*. J. Chem. Ecol. 30: 255–271.

de Bono, M., and C. I. Bargmann, 1998 Natural variation in a neuropeptide Y receptor homolog modifies social behavior and food response in *C. elegans*. Cell 94: 679–689.

Dwyer, N. D., E. R. Troemel, P. Sengupta, and C. I. Bargmann, 1998 Odorant receptor localization to olfactory cilia is mediated by ODR-4, a novel membrane-associated protein. Cell 93: 455–466.

Fay, D. S., and K. Gerow, 2013 A biologist's guide to statistical thinking and analysis (July 9, 2013), WormBook, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.159.1, http://www.wormbook.org.

Ferkey, D. M., R. Hyde, G. Haspel, H. M. Dionne, H. A. Hess *et al.*, 2007 *C. elegans* G protein regulator RGS-3 controls sensitivity to sensory stimuli. Neuron 53: 39–52.

Flavell, S. W., N. Pokala, E. Z. Macosko, D. R. Albrecht, J. Larsch *et al.*, 2013 Serotonin and the neuropeptide PDF initiate and extend opposing behavioral states in *C. elegans*. Cell 154: 1023–1035.

Fujiwara, M., T. Ishihara, and I. Katsura, 1999 A novel WD40 protein, CHE-2, acts cell-autonomously in the formation of *C. elegans* sensory cilia. Development 126: 4839–4848.

Ha, H. I., M. Hendricks, Y. Shen, C. V. Gabel, C. Fang-Yen *et al.*, 2010 Functional organization of a neural network for aversive olfactory learning in *Caenorhabditis elegans*. Neuron 68: 1173–1186.

Hardie, J., R. Isaacs, J. A. Pickett, L. J. Wadhams, and C. M. Woodcock, 1994 Methyl salicylate and (-)-(1R,5S)-myrtenal are plant-derived repellents for black bean aphid,*Aphis fabae Scop. (Homoptera: Aphididae)*. J. Chem. Ecol. 20: 2847–2855.

Higashi, Y., T. Kiuchi, and K. Furuta, 2010 Efficacy and safety profile of a topical methyl salicylate and menthol patch in adult patients with mild to moderate muscle strain: a randomized, double-blind, parallel-group, placebo-controlled, multicenter study. Clin. Ther. 32: 34–43.

Hobert, O., 2010 Neurogenesis in the nematode *Caenorhabditis elegans* (October 4, 2010), WormBook, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.12.2, http://www.wormbook.org.

Iino, Y., and K. Yoshida, 2009 Parallel use of two behavioral mechanisms for chemotaxis in *Caenorhabditis elegans*. J. Neurosci. 29: 5370–5380.

James, D. G., 2003 Field evaluation of herbivore-induced plant volatiles as attractants for beneficial insects: methyl salicylate and the green lacewing, *Chrysopa nigricornis*. J. Chem. Ecol. 29: 1601–1609.

James, D. G., and T. S. Price, 2004 Field-testing of methyl salicylate for recruitment and retention of beneficial insects in grapes and hops. J. Chem. Ecol. 30: 1613–1628.

Jansen, G., D. Weinkove, and R. H. Plasterk, 2002 The G-protein gamma subunit *gpc-1* of the nematode *C.elegans* is involved in taste adaptation. EMBO J. 21: 986–994.

Jayasekara, T. K., P. C. Stevenson, D. R. Hall, and S. R. Belmain, 2005 Effect of volatile constituents from *Securidaca longepedunculata* on insect pests of stored grain. J. Chem. Ecol. 31: 303–313.

Jordan, M. D., A. Anderson, D. Begum, C. Carraher, A. Authier *et al.*, 2009 Odorant receptors from the light brown apple moth (*Epiphyas postvittana*) recognize important volatile compounds produced by plants. Chem. Senses 34: 383–394.

Kimata, T., H. Sasakura, N. Ohnishi, N. Nishio, and I. Mori, 2012 Thermotaxis of *C. elegans* as a model for temperature perception, neural information processing and neural plasticity. Worm 1: 31–41.

Komatsu, H., I. Mori, J. S. Rhee, N. Akaike, and Y. Ohshima, 1996 Mutations in a cyclic nucleotide-gated channel lead to abnormal thermosensation and chemosensation in *C. elegans*. Neuron 17: 707–718.

Kubiak, T. M., M. J. Larsen, S. C. Nulf, M. R. Zantello, K. J. Burton *et al.*, 2003 Differential activation of "social" and "solitary" variants of the *Caenorhabditis elegans* G protein-coupled receptor NPR-1 by its cognate ligand AF9. J. Biol. Chem. 278: 33724–33729.

Kuhara, A., H. Inada, I. Katsura, and I. Mori, 2002 Negative regulation and gain control of sensory neurons by the *C. elegans* calcineurin TAX-6. Neuron 33: 751–763.

Kumar, D., and D. F. Klessig, 2008 The search for the salicylic acid receptor led to discovery of the SAR signal receptor. Plant Signal. Behav. 3: 691–692.

Lachenmeier, D. W., Y. B. Monakhova, M. Markova, T. Kuballa, and J. Rehm, 2013 What happens if people start drinking mouthwash as surrogate alcohol? A quantitative risk assessment. Food Chem. Toxicol. 51: 173–178.

Lanjuin, A., and P. Sengupta, 2002 Regulation of chemosensory receptor expression and sensory signaling by the KIN-29 Ser/Thr kinase. Neuron 33: 369–381.

Lee, J., C. Jee, H. O. Song, J. Bandyopadhyay, J. I. Lee *et al.*, 2004 Opposing functions of calcineurin and CaMKII regulate G-protein signaling in egg-laying behavior of *C.elegans*. J. Mol. Biol. 344: 585–595.

Lewis, J. A., and J. A. Hodgkin, 1977 Specific neuroanatomical changes in chemosensory mutants of the nematode *Caenorhabditis elegans*. J. Comp. Neurol. 172: 489–510.

Lewis, R. J., 1989 *Food Additives Handbook*, Van Nostrand Reinhold, New York.

Macosko, E. Z., N. Pokala, E. H. Feinberg, S. H. Chalasani, R. A. Butcher *et al.*, 2009 A hub-and-spoke circuit drives pheromone attraction and social behaviour in *C. elegans*. Nature 458: 1171–1175.

Mello, C. C., J. M. Kramer, D. Stinchcomb, and V. Ambros, 1991 Efficient gene transfer in *C.elegans*: extrachromosomal maintenance and integration of transforming sequences. EMBO J. 10: 3959–3970.

Mori, I., and Y. Ohshima, 1995 Neural regulation of thermotaxis in *Caenorhabditis elegans*. Nature 376: 344–348.

Ohta, T., T. Imagawa, and S. Ito, 2009 Involvement of transient receptor potential vanilloid subtype 1 in analgesic action of methylsalicylate. Mol. Pharmacol. 75: 307–317.

Okochi, Y., K. D. Kimura, A. Ohta, and I. Mori, 2005 Diverse regulation of sensory signaling by *C. elegans* nPKC-epsilon/eta TTX-4. EMBO J. 24: 2127–2137.

Park, S. W., E. Kaimoyo, D. Kumar, S. Mosher, and D. F. Klessig, 2007 Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. Science 318: 113–116.

Reddy, K. C., E. C. Andersen, L. Kruglyak, and D. H. Kim, 2009 A polymorphism in *npr-1* is a behavioral determinant of pathogen susceptibility in *C. elegans*. Science 323: 382–384.

Rogers, C., V. Reale, K. Kim, H. Chatwin, C. Li *et al.*, 2003 Inhibition of *Caenorhabditis elegans* social feeding by FMRFamide-related peptide activation of NPR-1. Nat. Neurosci. 6: 1178–1185.

Sagasti, A., O. Hobert, E. R. Troemel, G. Ruvkun, and C. I. Bargmann, 1999 Alternative olfactory neuron fates are specified by the LIM homeobox gene *lim-4*. Genes Dev. 13: 1794–1806.

Sieburth, D., J. M. Madison, and J. M. Kaplan, 2007 PKC-1 regulates secretion of neuropeptides. Nat. Neurosci. 10: 49–57.

Stawicki, T. M., S. Takayanagi-Kiya, K. Zhou, and Y. Jin, 2013 Neuropeptides function in a homeostatic manner to modulate excitation-inhibition imbalance in *C. elegans*. PLoS Genet. 9: e1003472.

Styer, K. L., V. Singh, E. Macosko, S. E. Steele, C. I. Bargmann *et al.*, 2008 Innate immunity in *Caenorhabditis elegans* is regulated by neurons expressing NPR-1/GPCR. Science 322: 460–464.

Tobin, D., D. Madsen, A. Kahn-Kirby, E. Peckol, G. Moulder *et al.*, 2002 Combinatorial expression of TRPV channel proteins de-

fines their sensory functions and subcellular localization in *C. elegans* neurons. Neuron 35: 307–318.
Tong, Y. G., and T. R. Burglin, 2010 Conditions for dye-filling of sensory neurons in *Caenorhabditis elegans*. J. Neurosci. Methods 188: 58–61.
Tsalik, E. L., T. Niacaris, A. S. Wenick, K. Pau, L. Avery *et al.*, 2003 LIM homeobox gene-dependent expression of biogenic amine receptors in restricted regions of the *C. elegans* nervous system. Dev. Biol. 263: 81–102.
van den Boom, C. E., T. A. van Beek, M. A. Posthumus, A. de Groot, and M. Dicke, 2004 Qualitative and quantitative variation among volatile profiles induced by *Tetranychus urticae* feeding on plants from various families. J. Chem. Ecol. 30: 69–89.
Vowels, J. J., and J. H. Thomas, 1994 Multiple chemosensory defects in *daf-11* and *daf-21* mutants of *Caenorhabditis elegans*. Genetics 138: 303–316.
Watts, J. L., and J. Browse, 2002 Genetic dissection of polyunsaturated fatty acid synthesis in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA 99: 5854–5859.
Way, J. C., and M. Chalfie, 1989 The *mec-3* gene of *Caenorhabditis elegans* requires its own product for maintained expression and is expressed in three neuronal cell types. Genes Dev. 3: 1823–1833.
White, J. G., E. Southgate, J. N. Thomson, and S. Brenner, 1986 The structure of the nervous system of the nematode *Caenorhabditis elegans*. Philos. Trans. R. Soc. Lond. B Biol. Sci. 314: 1–340.
Zhu, J., and K. C. Park, 2005 Methyl salicylate, a soybean aphid-induced plant volatile attractive to the predator *Coccinella septempunctata*. J. Chem. Ecol. 31: 1733–1746.

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GENETICS

Supporting Information
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Neuropeptide Receptors NPR-1 and NPR-2 Regulate *Caenorhabditis elegans* Avoidance Response to the Plant Stress Hormone Methyl Salicylate

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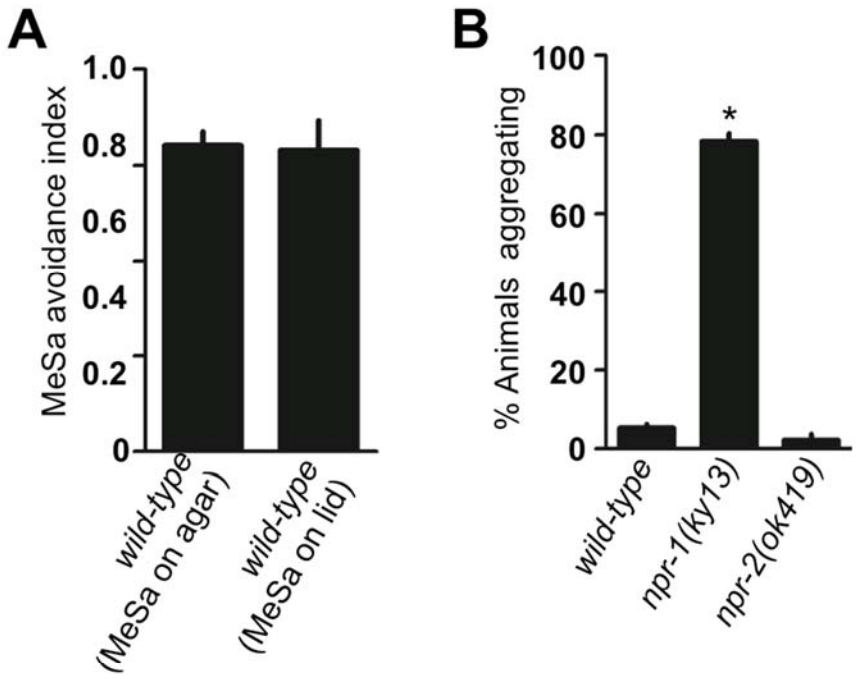
Figure S1



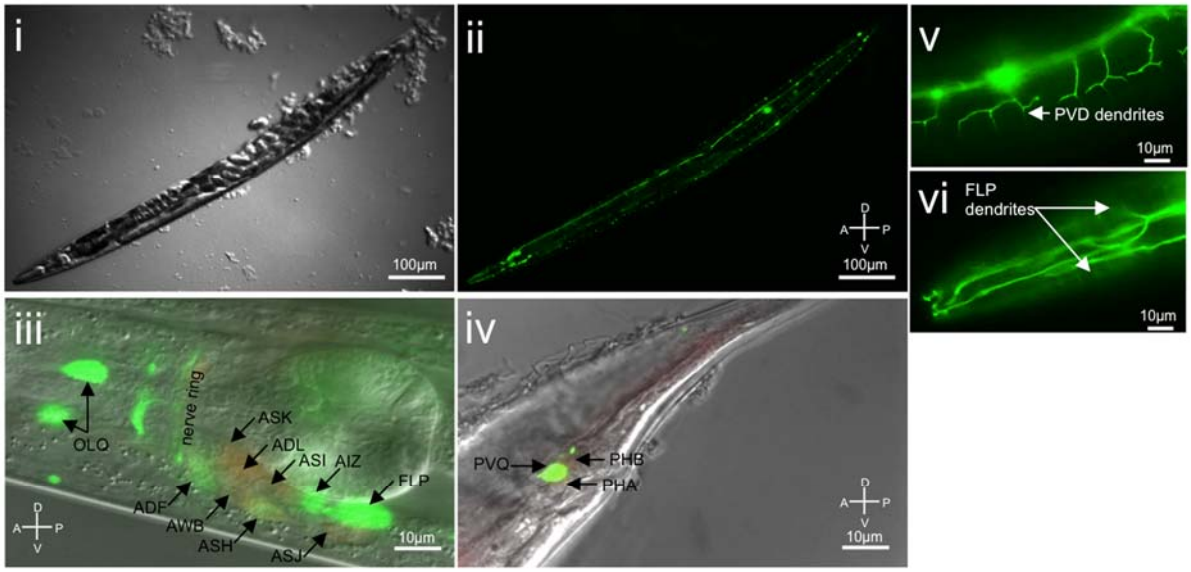
C

Gene	Protein identity/function
npr-1	Neuropeptide receptor
npr-2	Neuropeptide receptor
flp-18	Neuropeptide
odr-8	Ufm1 protease subunit for GPCR maturation
tax-6	Calcineurin A, G protein-interacting
kin-29	AMP/SNF kinase, essential for GPCR expression
che-2	G protein beta-like WD40 domain
daf-21	HSP90 chaperone, genetically similar to *daf-11*
daf-11	Receptor guanylate cyclase, downstream of GPCR
gpc-1	G proetin gamma-subunit
pkc-1	Protein kinase C required for neuropeptide secretion
tax-4	cGMP-gated channel subunit
tax-2	cGMP-gated channel subunit
fat-3	Lipid biosynthesis, downstream of GPCR
rgs-3	Regulator of G protein signaling

Figure S1 ***C. elegans*** **avoids MeSa as a volatile and** ***npr-2*** **is not required for social feeding.**
(A) *C. elegans* exhibits a similar avoidance response to MeSa spotted on either the agar or the petri dish lid. Error bars: standard errors.
(B) *npr-2(ok419)* mutants do not exhibit social feeding. The behavior was scored as described (DE BONO and BARGMANN 1998; MACOSKO *et al.* 2009). Statistics: different from wild type. Error bars: standard errors. *: p<0.01 (Student's t-test).
(C) List of genes important for the MeSa avoidance response. Genes with strong effects are in red. Genes with moderate effects are in blue.

Figure S2

A



B

	OLQs	ADFs	ASHs	AIZs	FLPs	SABD	PVDs	PVQs
worm01	O	O	O	X	O	O	O	O
worm02	X	O	X	O	O	X	O	O
worm03	O	O	O	O	O	X	O	O
worm04	X	O	O	O	O	O	O	O
worm05	O	O	O	O	O	O	O	O
worm06	O	O	O	X	O	O	O	O
worm07	O	O	O	X	O	O	O	O
worm08	O	O	O	O	O	X	O	O
worm09	X	O	O	X	O	O	O	O
worm10	X	X	X	X	O	O	O	O
worm11	O	O	X	O	O	O	O	O
worm12	O	O	X	O	O	X	O	O
worm13	O	X	O	O	O	O	X	X
worm14	O	O	O	X	O	O	O	O
worm15	O	O	O	O	O	O	O	O
worm16	O	O	O	O	O	O	O	O
Total	12	14	12	10	16	12	15	15
% GFP +	**75%**	**87.5%**	**75%**	**62.5%**	**100%**	**75%**	**93.75%**	**93.75%**

Figure S2 *npr-2* is expressed in sensory neurons and interneurons.
(A) An *npr-2p::GFP* transgenic animal in low (i, ii) and high magnifications (iii- vi) showing GFP-positive neurons (green) and DiI-labeled neurons (light red). ASH is labeled by GFP and DiI. The highly branched dendrites of PVD and FLP sensory neurons are shown in v and vi. A: anterior; P: posterior; D: dorsal; V: ventral.
(B) Penetrance of *npr-2p::GFP* transgene expression in neurons. 16 animals were scored. O: GFP-positive; X: GFP-negative.

Table S1 List of promoters for *npr-1* transgenes.

Promoters	***npr-1*-expressing neurons**
npr-1 (MACOSKO *et al.* 2009)	All
ncs-1 (MACOSKO *et al.* 2009)	ASE, ASG, RMG, PHA
flp-21 (MACOSKO *et al.* 2009)	ASE, ASH, RMG, URX
flp-8 (MACOSKO *et al.* 2009)	AUA, URX, RMG
gcy-32 (MACOSKO *et al.* 2009)	AQR, URX, PQR
ocr-2 (TOBIN *et al.* 2002)	ASH, PHA, PHB
ocr-4 (TOBIN *et al.* 2002)	OLQ
odr-2 (CHOU *et al.* 2001)	IL2, ASG, RIV
sra-6 (TROEMEL *et al.* 1995)	ASH
flp-12 (KIM and LI 2004)	SAA, SDQ, SMB
lin-12 (CHAO *et al.* 2005)	RIG

Table S2 List of promoters for *flp-18* transgenes.

Promoters	***flp-18*-expressing neurons**
flp-18 (ROGERS *et al.* 2003)	All
glr-8 (BROCKIE *et al.* 2001)	M3
ttx-3 (HOBERT *et al.* 1997)	AIY
trxr-1 (LI *et al.* 2012)	M2
ser-2 (TSALIK *et al.* 2003)	AIY
lin-12 (CHAO *et al.* 2005)	RIG
nmr-1 (BROCKIE *et al.* 2001)	AVA, RIM
flp-1 (KIM and LI 2004; NELSON *et al.* 1998)	AIY, AVA, RIG

Table S3 List of promoters for *npr-2* transgenes.

Promoters	***npr-2*-expressing neurons**
npr-2	All
ser-2 (TSALIK *et al.* 2003)	AIZ
sra-6 (TROEMEL *et al.* 1995)	ASH, PVQ
ocr-2 (TOBIN *et al.* 2002)	ASH, ADF
mgl-3 (GREER *et al.* 2008)	ADF
mec-3 (WAY and CHALFIE 1989)	FLP, PVD
ocr-4 (TOBIN *et al.* 2002)	OLQ
unc-4 (MILLER and NIEMEYER 1995)	SAB
ggr-1(I. Katsura, personal communication, 06/2014)	PVQ

Table S4 List of transgene promoters and PCR primers.

Promoters	Forward primer	Reverse primer
flp-1	acgcgtcgacgagatctgaaatgctttccctg	cgcggatcccttggtagagcagagtcatg
flp-8	ctgcagctatgtgtgttccgtcttaagac	cccgggtccggagagcattttctacttg
flp-12	aactgcagtatcaatgccgagagtgtgg	tccccccgggagggcgatgatgacttgaac
flp-21	ctgcaggacatttctgtctgaacgctg	cccgggcagccgcatcgtctgaaaatg
gcy-32	aactgcagaaatgtcgatcacaattattgtcggac acc	tccccccgggtctataatacaatcgtgatcttcgctt cgg
ggr-1	ctgcaggagagcttatatctctgttc	ggatcctcaataattaaagtatgcagttg
glr-8	acgcgtcgactgttccagttgatgacaaacc	cgcggatcccatttcggaaagcgaaaagtg
lin-12	aactgcagggacacagatgatgatgcac	tccccccgggaacaaatcgtagggatccgc
mec-3	ctatcaaaatgaggagcgatgtg	tcgcactgagaggctttgac
mgl-3	ctgcagatagtgaagactcccgtatcag	ggatccatccggacaagcgatgac
ncs-1	aactgcagttctgagccattagccactg	tccccccgggcccttgcccatttcttcgc
nmr-1	acgcgtcgaccaaacatctggaggatcattcatc	cgcggatccctgatattcggaacatatctgtaac
npr-2	aaaactgcagaagtgttcacctgtcagcgttcgag	cgggatccacgtggcactcataatgacggatg
ocr-2	aagcttgacaaatgaatgaagttacagagc	ggatccggaacccatcttaatgatgtgatg
ocr-4	aactgcagtgttccaacaaaacctgccg	cgggatcccagcactagatgcattaccc
odr-2	aactgcagctagtcagcatttcaccctg	tccccccgggggaaggtgcatttctgtctg
ser-2	aactgcagctggtaagttgaacatggagtc	cgggatcctacctgactaaagtcgtcgttc
sra-6	acgcgtcgaccggatgtcaggtgatgtttg	gcggatccaacatctggagcagcacaac
trxr-1	acgcgtcgacgtagcgatggaccaaaagcaac	cgcggatccggtgagagatttcatgccatc
ttx-3	cgggatccttttcagatagcagatgattcacaag	ggggtaccttgaaaagtaggaagcattgatatcc
unc-4	aactgcagaatggtggctgaagtggaaacc	cgggatccgcagtgcaccgatcattttcac

File S1

Supplementary Materials and Methods

Strains

C. elegans strains were grown at 20°C unless otherwise indicated. N2 (Bristol) was the reference wild type strain (BRENNER 1974). Strains used in this study include:

MT2426 *goa-1(n1134) I* (SEGALAT *et al.* 1995)

PR671 *tax-2(p671) I* (COBURN and BARGMANN 1996)

CB1034 *che-1(e1034) fer-1(hc1) I* (LEWIS and HODGKIN 1977; UCHIDA *et al.* 2003)

RB770 *npr-11(ok594) I* (CHALASANI *et al.* 2010; COHEN *et al.* 2009; STYER *et al.* 2008)

RB1780 *rgs-3(ok2288) II* (this study)

DR476 *daf-22(m130) II* (GOLDEN and RIDDLE 1985)

MT5300 *odr-4(n2144) III* (DWYER *et al.* 1998)

PR678 *tax-4(p678) III* (KOMATSU *et al.* 1996)

CB1372 *daf-7(e1372) III* (SCHACKWITZ *et al.* 1996)

BW506 *ceh-10(ct78) III* (MANSER and WOOD 1990)

BX24 *fat-1(wa9) IV* (WATTS and BROWSE 2002)

BX30 *fat-3(wa22) IV* (WATTS and BROWSE 2002)

BX17 *fat-4(wa14) IV* (WATTS and BROWSE 2002)

CX2386 *odr-8(ky31) IV* (DWYER *et al.* 1998)

VM396 *ocr-2(ak47) IV* (TOBIN *et al.* 2002)

MT1073 *egl-4(n478) IV* (TRENT *et al.* 1983)

PR675 *tax-6(p675) IV* (KUHARA *et al.* 2002)

XA3702 *npr-2(ok419) IV* (COHEN *et al.* 2009)

CSM415 *npr-2(ok419) IV* (COHEN *et al.* 2009) (backcrossed 5 times in this study)

CX10 *osm-9(ky10) IV* (COLBERT and BARGMANN 1995)

CSM111 *npr-3(tm1583) IV* (COHEN *et al.* 2009) (backcrossed 5 times in this study)

MT4810 *odr-3(n2046) V* (ROAYAIE *et al.* 1998)

CX2205 *odr-3(n2150) V* (ROAYAIE *et al.* 1998)

DR47 *daf-11(m47) V* (THOMAS *et al.* 1993)

CB3330 *che-11(e1810) V* (PERKINS *et al.* 1986)

PR673 *daf-21(p673) V* (BIRNBY *et al.* 2000)

CSM123 *flp-21(ok889) V* (this study, backcrossed 5 times)

IK105 *pkc-1(nj1) V* (OKOCHI *et al.* 2005)

CX14394 *npr-5(ok1583) X* (FLAVELL *et al.* 2013)

CB1033 *che-2(e1033) X* (LEWIS and HODGKIN 1977)

MT4583 *odr-1(n1936) X* (L'ETOILE and BARGMANN 2000)

NL792 *gpc-1(pk298) X* (JANSEN *et al.* 2002)

CX4 *odr-7(ky4) X* (SENGUPTA *et al.* 1994)

PY1479 *kin-29(oy38) X* (LANJUIN and SENGUPTA 2002)

CSM125 *flp-18(gk3063) X* (this study, backcrossed 5 times)

CX4148 *npr-1(ky13) X* (DE BONO and BARGMANN 1998)

RB1330 *npr-1(ok1447) X* (STAWICKI *et al.* 2013)

CX3937 *lim-4(ky403) X* (SAGASTI *et al.* 1999)

FK311 *ceh-36(ks86) X* (KOGA and OHSHIMA 2004)

FK134 *ttx-3(ks5) X* (HOBERT *et al.* 1997)

RB761 *npr-7(ok527) X* (COHEN *et al.* 2009; STYER *et al.* 2008)

IC683 *npr-9(tm1652) X* (BENDENA *et al.* 2008)

RB1365 *npr-16(ok1541) X* (STYER *et al.* 2008)

CB4856 (Hawaiian) (WICKS *et al.* 2001)

Transgenic animals used in this study include:

CSM320-CSM322 *npr-1(ky13) X; macEx205-207[npr-1p::npr-1::GFP, myo-3p::GFP]*

CSM352-CSM354 *npr-1(ky13) X; macEx234-236[ncs-1p::npr-1::GFP, myo-3p::GFP]*

CSM453-CSM455 *npr-1(ky13) X; macEx291-293[flp-21p::npr-1::GFP, myo-3p::GFP]*

CSM456-CSM458 *npr-1(ky13) X; macEx294-296[flp-8p::npr-1::GFP, myo-3p::GFP]*

CSM190 *npr-1(ky13) X; macEx136[myo-3p::GFP]*

CSM211 *npr-1(ky13) X; macEx153[myo-3p::GFP]*

CSM319 *npr-1(ky13) X; macEx204[myo-3p::GFP]*

CSM38 *npr-1(ky13) X; macEx83[npr-1p::GFP, myo-3p::GFP]*

CSM241 *npr-1(ky13) X; macEx178[npr-1p:: GFP, myo-3p::GFP]*

CSM287 *npr-1(ky13) X; macEx201[npr-1p:: GFP, myo-3p::GFP]*

CSM349-CSM351 *npr-1(ky13) X; macEx231-233[gcy-32p::npr-1::GFP, myo-3p::GFP]*

CSM468-CSM470 *npr-1(ky13) X; macEx306-308[ocr-2p::npr-1::GFP, myo-3p::GFP]*

CSM471-CSM473 *npr-1(ky13) X; macEx309-311[ocr-4p::npr-1::GFP, myo-3p::GFP]*

CSM474-CSM476 *npr-1(ky13) X; macEx312-314[odr-2p::npr-1::GFP, myo-3p::GFP]*

CSM480-CSM482 *npr-1(ky13) X; macEx318-320[lin-12p::npr-1::GFP, sra-6p::npr-1::GFP, flp-12p::npr-1::GFP, myo-3p::GFP]*

CSM332-CSM334 *npr-2(ok419) IV; macEx217-219[npr-2p::npr-2::GFP, myo-3p::GFP]*

CSM459-CSM461 *npr-2(ok419) IV; macEx297-299[ser-2p::npr-2::GFP, myo-3p::GFP]*

CSM494-CSM496 *npr-2(ok419) IV; macEx332-334[npr-2p::GFP, myo-3p::GFP]*

CSM335-CSM337 *npr-2(ok419) IV; macEx220-222[myo-3p::GFP]*

CSM355-CSM357 *npr-2(ok419) IV; macEx237-239[sra-6p::npr-2::GFP, myo-3p::GFP]*

CSM483-CSM485 *npr-2(ok419) IV; macEx321-323[unc-4p::npr-2::GFP, ggr-1p::npr-2::GFP, myo-3p::GFP]*

CSM486-CSM488 *npr-2(ok419) IV; macEx324-326[ocr-2p::npr-2::GFP, myo-3p::GFP]*

CSM489-CSM491 *npr-2(ok419) IV; macEx327-329[mec-3p::npr-2::GFP, myo-3p::GFP]*

CSM477-CSM479 *npr-2(ok419) IV; macEx315-317[ocr-4p::npr-2::GFP, myo-3p::GFP]*

CSM492-CSM493 *npr-2(ok419) IV; macEx330-331[mgl-3p::npr-2::GFP, myo-3p::GFP]*

CSM462-CSM464 *flp-18(gk3063) X; macEx300-302[myo-3p::GFP]*

CSM465-CSM467 *flp-18(gk3063) X; macEx303-305[flp-18p::flp-18, myo-3p::GFP]*

CSM508-CSM510 *flp-18(gk3063) X; macEx342-344[ser-2p::flp-18, myo-3p::GFP]*

CSM511-CSM513 *flp-18(gk3063) X; macEx345-347[lin-12p::flp-18, myo-3p::GFP]*

CSM514-CSM516 *flp-18(gk3063) X; macEx348-350[flp-1p::flp-18, myo-3p::GFP]*

CSM517-CSM519 *flp-18(gk3063) X; macEx351-353[glr-8p::flp-18, myo-3p::GFP]*

CSM520-CSM522 *flp-18(gk3063) X; macEx354-356[nmr-1p::flp-18, myo-3p::GFP]*

CSM523-CSM525 *flp-18(gk3063) X; macEx357-359[trxr-1p::flp-18, myo-3p::GFP]*

CSM526-CSM528 *flp-18(gk3063) X; macEx360-362[ttx-3p::flp-18, myo-3p::GFP]*

CSM213 *macEx154[npr-2p::GFP]*

CSM232 *macEx171[npr-2p::GFP]*

References

BENDENA, W. G., J. R. BOUDREAU, T. PAPANICOLAOU, M. MALTBY, S. S. TOBE *et al.*, 2008 A *Caenorhabditis elegans* allatostatin/galanin-like receptor NPR-9 inhibits local search behavior in response to feeding cues. Proc Natl Acad Sci U S A **105:** 1339-1342.

BIRNBY, D. A., E. M. LINK, J. J. VOWELS, H. TIAN, P. L. COLACURCIO *et al.*, 2000 A transmembrane guanylyl cyclase (DAF-11) and Hsp90 (DAF-21) regulate a common set of chemosensory behaviors in *Caenorhabditis elegans*. Genetics **155:** 85-104.

BRENNER, S., 1974 The genetics of *Caenorhabditis elegans*. Genetics **77:** 71-94.

BROCKIE, P. J., D. M. MADSEN, Y. ZHENG, J. MELLEM and A. V. MARICQ, 2001 Differential expression of glutamate receptor subunits in the nervous system of *Caenorhabditis elegans* and their regulation by the homeodomain protein UNC-42. J Neurosci **21:** 1510-1522.

CHALASANI, S. H., S. KATO, D. R. ALBRECHT, T. NAKAGAWA, L. F. ABBOTT *et al.*, 2010 Neuropeptide feedback modifies odor-evoked dynamics in *Caenorhabditis elegans* olfactory neurons. Nat Neurosci **13:** 615-621.

CHAO, M. Y., J. LARKINS-FORD, T. M. TUCEY and A. C. HART, 2005 *lin-12* Notch functions in the adult nervous system of *C. elegans*. BMC Neurosci **6:** 45.

CHOU, J. H., C. I. BARGMANN and P. SENGUPTA, 2001 The *Caenorhabditis elegans odr-2* gene encodes a novel Ly-6-related protein required for olfaction. Genetics **157:** 211-224.

COBURN, C. M., and C. I. BARGMANN, 1996 A putative cyclic nucleotide-gated channel is required for sensory development and function in *C. elegans*. Neuron **17:** 695-706.

COHEN, M., V. REALE, B. OLOFSSON, A. KNIGHTS, P. EVANS *et al.*, 2009 Coordinated regulation of foraging and metabolism in *C. elegans* by RFamide neuropeptide signaling. Cell Metab **9:** 375-385.

COLBERT, H. A., and C. I. BARGMANN, 1995 Odorant-specific adaptation pathways generate olfactory plasticity in *C. elegans*. Neuron **14:** 803-812.

DE BONO, M., and C. I. BARGMANN, 1998 Natural variation in a neuropeptide Y receptor homolog modifies social behavior and food response in *C. elegans*. Cell **94:** 679-689.

DWYER, N. D., E. R. TROEMEL, P. SENGUPTA and C. I. BARGMANN, 1998 Odorant receptor localization to olfactory cilia is mediated by ODR-4, a novel membrane-associated protein. Cell **93:** 455-466.

FLAVELL, S. W., N. POKALA, E. Z. MACOSKO, D. R. ALBRECHT, J. LARSCH *et al.*, 2013 Serotonin and the neuropeptide PDF initiate and extend opposing behavioral states in *C. elegans*. Cell **154:** 1023-1035.

GOLDEN, J. W., and D. L. RIDDLE, 1985 A gene affecting production of the *Caenorhabditis elegans* dauer-inducing pheromone. Mol Gen Genet **198:** 534-536.

GREER, E. R., C. L. PEREZ, M. R. VAN GILST, B. H. LEE and K. ASHRAFI, 2008 Neural and molecular dissection of a *C. elegans* sensory circuit that regulates fat and feeding. Cell Metab **8:** 118-131.

HOBERT, O., I. MORI, Y. YAMASHITA, H. HONDA, Y. OHSHIMA *et al.*, 1997 Regulation of interneuron function in the *C. elegans* thermoregulatory pathway by the *ttx-3* LIM homeobox gene. Neuron **19:** 345-357.

JANSEN, G., D. WEINKOVE and R. H. PLASTERK, 2002 The G-protein gamma subunit *gpc-1* of the nematode *C.elegans* is involved in taste adaptation. EMBO J **21:** 986-994.

KIM, K., and C. LI, 2004 Expression and regulation of an FMRFamide-related neuropeptide gene family in *Caenorhabditis elegans*. J Comp Neurol **475:** 540-550.

KOGA, M., and Y. OHSHIMA, 2004 The *C. elegans ceh-36* gene encodes a putative homemodomain transcription factor involved in chemosensory functions of ASE and AWC neurons. J Mol Biol **336:** 579-587.

KOMATSU, H., I. MORI, J. S. RHEE, N. AKAIKE and Y. OHSHIMA, 1996 Mutations in a cyclic nucleotide-gated channel lead to abnormal thermosensation and chemosensation in *C. elegans*. Neuron **17:** 707-718.

KUHARA, A., H. INADA, I. KATSURA and I. MORI, 2002 Negative regulation and gain control of sensory neurons by the *C. elegans* calcineurin TAX-6. Neuron **33:** 751-763.

L'ETOILE, N. D., and C. I. BARGMANN, 2000 Olfaction and odor discrimination are mediated by the *C. elegans* guanylyl cyclase ODR-1. Neuron **25:** 575-586.

LANJUIN, A., and P. SENGUPTA, 2002 Regulation of chemosensory receptor expression and sensory signaling by the KIN-29 Ser/Thr kinase. Neuron **33:** 369-381.

LEWIS, J. A., and J. A. HODGKIN, 1977 Specific neuroanatomical changes in chemosensory mutants of the nematode *Caenorhabditis elegans*. J Comp Neurol **172:** 489-510.

LI, W., J. BANDYOPADHYAY, H. S. HWAANG, B. J. PARK, J. H. CHO *et al.*, 2012 Two thioredoxin reductases, *trxr-1* and *trxr-2*, have differential physiological roles in *Caenorhabditis elegans*. Mol Cells **34:** 209-218.

MACOSKO, E. Z., N. POKALA, E. H. FEINBERG, S. H. CHALASANI, R. A. BUTCHER *et al.*, 2009 A hub-and-spoke circuit drives pheromone attraction and social behaviour in *C. elegans*. Nature **458:** 1171-1175.

MANSER, J., and W. B. WOOD, 1990 Mutations affecting embryonic cell migrations in *Caenorhabditis elegans*. Dev Genet **11:** 49-64.

MILLER, D. M., 3RD, and C. J. NIEMEYER, 1995 Expression of the *unc-4* homeoprotein in *Caenorhabditis elegans* motor neurons specifies presynaptic input. Development **121:** 2877-2886.

NELSON, L. S., M. L. ROSOFF and C. LI, 1998 Disruption of a neuropeptide gene, *flp-1*, causes multiple behavioral defects in *Caenorhabditis elegans*. Science **281:** 1686-1690.

OKOCHI, Y., K. D. KIMURA, A. OHTA and I. MORI, 2005 Diverse regulation of sensory signaling by *C. elegans* nPKC-epsilon/eta TTX-4. EMBO J **24:** 2127-2137.

PERKINS, L. A., E. M. HEDGECOCK, J. N. THOMSON and J. G. CULOTTI, 1986 Mutant sensory cilia in the nematode *Caenorhabditis elegans*. Dev Biol **117:** 456-487.

ROAYAIE, K., J. G. CRUMP, A. SAGASTI and C. I. BARGMANN, 1998 The G alpha protein ODR-3 mediates olfactory and nociceptive function and controls cilium morphogenesis in *C. elegans* olfactory neurons. Neuron **20:** 55-67.

ROGERS, C., V. REALE, K. KIM, H. CHATWIN, C. LI *et al.*, 2003 Inhibition of *Caenorhabditis elegans* social feeding by FMRFamide-related peptide activation of NPR-1. Nat Neurosci **6:** 1178-1185.

SAGASTI, A., O. HOBERT, E. R. TROEMEL, G. RUVKUN and C. I. BARGMANN, 1999 Alternative olfactory neuron fates are specified by the LIM homeobox gene *lim-4*. Genes Dev **13:** 1794-1806.

SCHACKWITZ, W. S., T. INOUE and J. H. THOMAS, 1996 Chemosensory neurons function in parallel to mediate a pheromone response in *C. elegans*. Neuron **17:** 719-728.

SEGALAT, L., D. A. ELKES and J. M. KAPLAN, 1995 Modulation of serotonin-controlled behaviors by Go in *Caenorhabditis elegans*. Science **267:** 1648-1651.

SENGUPTA, P., H. A. COLBERT and C. I. BARGMANN, 1994 The *C. elegans* gene *odr-7* encodes an olfactory-specific member of the nuclear receptor superfamily. Cell **79:** 971-980.

STAWICKI, T. M., S. TAKAYANAGI-KIYA, K. ZHOU and Y. JIN, 2013 Neuropeptides function in a homeostatic manner to modulate excitation-inhibition imbalance in *C. elegans*. PLoS Genet **9:** e1003472.

STYER, K. L., V. SINGH, E. MACOSKO, S. E. STEELE, C. I. BARGMANN *et al.*, 2008 Innate immunity in *Caenorhabditis elegans* is regulated by neurons expressing NPR-1/GPCR. Science **322:** 460-464.

THOMAS, J. H., D. A. BIRNBY and J. J. VOWELS, 1993 Evidence for parallel processing of sensory information controlling dauer formation in *Caenorhabditis elegans*. Genetics **134:** 1105-1117.

TOBIN, D., D. MADSEN, A. KAHN-KIRBY, E. PECKOL, G. MOULDER *et al.*, 2002 Combinatorial expression of TRPV channel proteins defines their sensory functions and subcellular localization in *C. elegans* neurons. Neuron **35:** 307-318.

TRENT, C., N. TSUING and H. R. HORVITZ, 1983 Egg-laying defective mutants of the nematode *Caenorhabditis elegans*. Genetics **104:** 619-647.

TROEMEL, E. R., J. H. CHOU, N. D. DWYER, H. A. COLBERT and C. I. BARGMANN, 1995 Divergent seven transmembrane receptors are candidate chemosensory receptors in *C. elegans*. Cell **83:** 207-218.

TSALIK, E. L., T. NIACARIS, A. S. WENICK, K. PAU, L. AVERY *et al.*, 2003 LIM homeobox gene-dependent expression of biogenic amine receptors in restricted regions of the *C. elegans* nervous system. Dev Biol **263:** 81-102.

UCHIDA, O., H. NAKANO, M. KOGA and Y. OHSHIMA, 2003 The *C. elegans che-1* gene encodes a zinc finger transcription factor required for specification of the ASE chemosensory neurons. Development **130:** 1215-1224.

WATTS, J. L., and J. BROWSE, 2002 Genetic dissection of polyunsaturated fatty acid synthesis in *Caenorhabditis elegans*. Proc Natl Acad Sci U S A **99:** 5854-5859.

WAY, J. C., and M. CHALFIE, 1989 The *mec-3* gene of *Caenorhabditis elegans* requires its own product for maintained expression and is expressed in three neuronal cell types. Genes Dev **3:** 1823-1833.

WICKS, S. R., R. T. YEH, W. R. GISH, R. H. WATERSTON and R. H. PLASTERK, 2001 Rapid gene mapping in *Caenorhabditis elegans* using a high density polymorphism map. Nat Genet **28:** 160-164.