Neuropeptide receptors NPR-1 and NPR-2 regulate C. 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's 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 the 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 inter neurons. Genetic rescue experiments suggest that NPR-2 expressed in the AIZ interneurons is required for the 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's behavior and that NPR-1 and NPR-2 act on distinct interneurons to affect C. elegans avoidance response to MeSa.
Introduction

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 (DE BOER and DICKE 2004; JAMES 2003; 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.

*C. elegans* has been an efficient model for studying the molecular and neural mechanisms underlying odorant-elicited behaviors (BARGMANN 2006; BARGMANN 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. 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 supplementary 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 H2O 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 (less than 3 mm in diameter and 0.5 cm from the periphery) at opposite ends. 1 μl NaN3 (1.0 M) was spotted at these sites to paralyze animals that locomote nearby. The plate was loosely sealed with paraffin membrane and kept in a 20 °C incubator for four hours (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. 30 to 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* gDNA together with an *npr-1* promoter (2 kb upstream the *npr-1* start codon) was subcloned to the pPD95.79 vector in-frame with *GFP* using Xmal/AgeI restriction sites.

To construct the *flp-18p::flp-18* transgene, a PCR-amplified full-length *flp-18* gDNA with a *flp-18* promoter (1.7kb upstream the *flp-18* start codon) and a 3’UTR fragment...
(0.8kb downstream 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 BamHI/AgeI restriction sites. An npr-2 promoter (2 kb upstream the start codon of npr-2) was subcloned to the pPD95.79::npr-2 backbone using PstI/BamHI restriction sites.

We inserted a PCR-amplified full-length npr-1 gDNA fragment to pPD95.79 in-frame with GFP using Xmal/AgeI 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 100X DIC/fluorescent objective of a Leica 5000B inverted microscope (Fig. S2A) or a Leica TCS
SP5 II laser confocal microscope (Fig. 4C) and compared to anatomical and morphological characteristics described in Wormatlas (www.wormatlas.org). We used Dil-stained sensory neurons (TONG and BURGLIN 2010) as landmarks to facilitate the verification the identities of npr-2-expressing neurons (Fig. S2A).

**Statistical analysis**

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’s *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. 1993). After eight hours exposure to MeSa, wild-type animals exhibit a dose-dependent avoidance response, reaching a maximal response at 2 μl MeSa or higher doses (Fig. 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 hours (Fig. 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 (Fig. S1A). We found that wild-type males have a slightly stronger MeSa avoidance response compared to hermaphrodites (Fig. 1C), implying that sex might affect 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 the MeSa avoidance, we screened 32 *C. elegans* mutants with previously described or putative behavioral defects, in which 30 distinct genes were affected (Fig. 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 (Fig. 1D). Nine genes with strong effects on the 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 (Chen et al. 2014; Dwyer et al. 1998), 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 (Fujiwara et al. 1999; Lewis and Hodgkin 1977), the HSP90 chaperone gene daf-21 (Birnby et al. 2000; Vowels and Thomas 1994), the neuropeptide gene flp-18 (Rogers et al. 2003) and the receptor guanylyl cyclase gene daf-11 (Birnby et al. 2000; Vowels and Thomas 1994).

We also identified six genes with moderate effects on the MeSa avoidance response (Fig. 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; Macsko et al. 2009) and is a key regulatory gene for ethanol adaptation (Davies et al. 2004), pathogen susceptibility (Reddy et al. 2009; Styer et al. 2008) 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) and npr-1(ok1447) (STAWICKI et al. 2013). We found that all strains exhibited apparent MeSa avoidance defects (Fig. 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 (Fig. 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 (Fig. 2B), while transgene expression in npr-1-expressing neurons other than RMGs failed to rescue (Fig. 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 (Fig. 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 (Fig. 3A), a deletion mutation of flp-18 caused a strong MeSa avoidance defect (Fig. 1D and 3B). This defect could be rescued by a flp-18 transgene (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 1D) is a deletion mutant (*ok419*) (Fig. 4A) of the neuropeptide receptor gene *npr-2* (DE BONO and BARGMANN 1998). *npr-2*(ok419) mutants were previously found to have 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 (Fig. 4B), suggesting that *npr-2* is required for the behavior.

To identify *npr-2*-expressing cells, we generated transgenic animals expressing GFP (Fig. 4C) under control of the rescuing *npr-2* promoter and identified a total of 15 to 17 GFP-positive neurons that likely express *npr-2* (Fig. 4C and Fig. S2A). These neurons include sensory neurons OLQs (2 to 4), ASHL/R, ADFL/R, FLPL/R and PVDL/R and interneurons AIZL/R, SABD and PVQL/R (Fig. 4C, Fig. S2A and 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 (Fig. 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 (Fig. 4D), while other promoters (Table S3) that do not drive transgene expression in AIZs had no apparent rescuing effects (Fig. 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 (Fig. 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 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 (Fig. 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 (Fig. 5B).
Such a wiring diagram implies a neural pathway that consists of AWBs, AlZs 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 (Fig. 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.
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 (De Boer and Dicke 2004; James 2003; 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; Chan 1996b; Higashi *et al.* 2010). Excess intake of MeSa could be life threatening probably due to severe, rapid-onset salicylate poisoning (Chan 1996a; Chan 1996b; 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 (Fig. 1D). Therefore TRPV channels might not be the MeSa
receptors in AWBs. Recently EpOR1, a 7-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 over 1000 G protein-coupled receptors, among which over 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 (REDDY et al. 2009; STYER et al. 2008) 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 (CHOI et al. 2013; MACOSKO et al. 2009). 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 (Fig. 4). AIZs also have a well-defined function in mediating cryophilic migration (KIMATA et al. 2012; MORI and OSHIMA 1995), and are involved in chemotaxis to NaCl (INO 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 (Fig. 1D and Fig. 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*) (CHEN et al. 2009).
2014; Dwyer et al. 1998), 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) (Bargmann 2006; Birnby et al. 2000; Coburn and Bargmann 1996; Ferkey et al. 2007; Komatsu et al. 1996; Watts and Browse 2002). 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 as daf-11 in the dauer pathway (Birnby et al. 2000; Vowels and Thomas 1994). 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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Author Contributions

JL and LM designed the experiments. JL, ZX, ZT and LM performed the experiments. JL and LM analyzed the data. JL, ZZ and LM wrote the manuscript. The authors declare no conflict of interests.

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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 hrs 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 hrs exposure to 2 μl MeSa. Statistics: different from the index at 4 hrs. 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).
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).
Figure 3

(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. A: anterior; P: posterior; D: dorsal; V: ventral.

(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).
Figure 5

Panel A: Graph showing MeSa avoidance index for different genotypes.

Panel B: Diagram illustrating the interaction between MeSa, AIZ, and RMG neurons, with symbols indicating sensory, interneuron, and motor neurons, and connections showing gap junctions and chemical synapses.

Panel C: Bar chart depicting MeSa avoidance index for various npr mutations.
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.
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