The NALCN Channel Regulator UNC-80 Functions in a Subset of Interneurons To Regulate Caenorhabditis elegans Reversal Behavior

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ABSTRACT NALCN (Na+ leak channel, non-selective) is a conserved, voltage-insensitive cation channel that regulates resting membrane potential and neuronal excitability. UNC79 and UNC80 are key regulators of the channel function. However, the behavioral effects of the channel complex are not entirely clear and the neurons in which the channel functions remain to be identified. In a forward genetic screen for C. elegans mutants with defective avoidance response to the plant hormone methyl salicylate (MeSa), we isolated multiple loss-of-function mutations in unc-80 and unc-79. C. elegans NALCN mutants exhibited similarly defective MeSa avoidance. Interestingly, NALCN, unc-80 and unc-79 mutants all showed wild-type-like responses to other attractive or repelling odorants, suggesting that NALCN does not broadly affect odor detection or related forward and reversal behaviors. To understand in which neurons the channel functions, we determined the identities of a subset of unc-80-expressing neurons. We found that unc-79 and unc-80 are expressed and function in overlapping neurons, which verified previous assumptions. Neuron-specific transgene rescue and knockdown experiments suggest that the command interneurons AVA and AVE and the anterior guidepost neuron AVG can play a sufficient role in mediating unc-80 regulation of the MeSa avoidance. Though primarily based on genetic analyses, our results further imply that MeSa might activate NALCN by direct or indirect actions. Altogether, we provide an initial look into the key neurons in which the NALCN channel complex functions and identify a novel function of the channel in regulating C. elegans reversal behavior through command interneurons.

KEYWORDS UNC-80 UNC-79 NCA NALCN MeSa avoidance

The NALCN (Na+ leak channel, non-selective) channel is a non-selective, voltage-independent cation channel broadly expressed in the animal kingdom (Ren 2011; Liebeskind et al. 2012). NALCN functions in neurons to balance the K+ leak, set the resting membrane potential, regulate spontaneous firing of neurons and modulate membrane potential in response to environmental stimuli (Ren 2011).

NALCN can affect a variety of biological processes in mammals. NALCN mutant mice die within 24 hr after birth due to disrupted respiratory rhythm (Lu et al. 2007). The channel has been implicated in the regulation of pacemaking activity in mouse gastrointestinal tract (Kim et al. 2012), clock neuron rhythms (Flourakis et al. 2015), firing and glycolytic sensitivity of substantia nigra pars reticulata neurons (Lutas et al. 2016), excitability of the retrotrapezoid nucleus neurons (Shi et al. 2016), rapid eye movement sleep (Funato et al. 2016), and rhythmic stability within the respiratory network (Yeh et al. 2017). Mutations in NALCN and its regulatory protein UNC80 are the causes of human diseases (Köroğlu et al. 2013; Al-Sayed et al. 2013; Perez et al. 2016; Stray-Pedersen et al. 2016; Fukai et al. 2016;
Bramswig et al. (2018) that are collectively called NALCN channelopathies (Bramswig et al. 2018).

NALCN also regulates neuronal activities and behaviors in invertebrates. Drosophila NALCN mutants exhibit the narrow abdomen (na) phenotype, disrupted circadian rhythm and resistance to the volatile anesthetics halothane (Krishnan and Nash 1990; Nash et al. 2002; Lear et al. 2005). C. elegans has two NALCN homologs, nca-1 and nca-2, that function redundantly to regulate the response to volatile anesthetics (Humphrey et al. 2007), recycling of synaptic vesicles (Jospin et al. 2007), neural circuit activity (Gao et al. 2015), motor behavior (Pierce-Shimomura et al. 2008), the propagation of neuronal activity from cell bodies to synapses (Yeh et al. 2008), ethanolic responses (Speca et al. 2010) and developmentally timed sleep (Huang et al. 2018).

In mammalian cells, NALCN channel can be regulated by G protein-coupled receptor TACR1, Src kinases (Lu et al. 2009), a [Ca\textsuperscript{2+}]-sensing G protein-coupled receptor (Lu et al. 2010) and the M3 muscarinic receptors (M3R) (Swayne et al. 2009). In C. elegans, NCA channels function downstream of the Gq-Rho pathway (Topaliou et al. 2017a), can be negatively regulated by dopamine through the D2-like dopamine receptor DOP-3 (Topaliou et al. 2017b) and may interact with the SEK-1 p38 MAPK pathway (Hoyt et al. 2017).

Studies in C. elegans, Drosophila and mice identified the conserved proteins UNC79 and UNC80 (orthologs of C. elegans UNC79 and UNC-80, respectively) as key regulators of the NALCN channel (Humphrey et al. 2007; Jospin et al. 2007; Yeh et al. 2008; Pierce-Shimomura et al. 2008; Lu et al. 2009, 2010; Wang and Ren 2009; Speca et al. 2010; Lear et al. 2013; Moose et al. 2017). In C. elegans, loss-of-function (lf) mutants of unc-79 or unc-80 phenocopy nca-2(lf); nca-1(lf) double mutants (Humphrey et al. 2007; Jospin et al. 2007; Yeh et al. 2008; Pierce-Shimomura et al. 2008; Huang et al. 2018). At the molecular level, UNC-79 and UNC-80 are required for the proper expression and axonal localization of NCA channels (Humphrey et al. 2007; Jospin et al. 2007; Yeh et al. 2008). In Drosophila, loss-of-function mutations in unc79, unc80 or NALCN cause indistinguishable defects in circadian locomotion rhythmicity (Lear et al. 2013) and similarly abnormal responses to halothane (Humphrey et al. 2007). In mice, UNC79, UNC80 and NALCN form a complex to execute the channel function (Lu et al. 2009, 2010). An ER-associated protein, NLF-1, can promote axonal localization of NCA channels in C. elegans (Xie et al. 2013). The Drosophila ortholog of NLF-1 is required for the regulation of circadian neuron excitability (Flourakis et al. 2015).

To date, many questions remain to be answered about the function and regulation of the NALCN channel complex, including but not limited to how the channel impacts different behaviors, what neurons mediate the channel functions, how the channel interacts with other neuronal molecules and how the channel is gated. The efficient genetics and well-described neurons of C. elegans can be utilized to address some of the questions.

Methyl salicylate (MeSa) is the volatile methyl ester of salicylic acid produced by many plants, and is widely used in medicated oils or mouthwash (Chan 1996; Davis 2007; Lachenmeier et al. 2013). In plants, MeSa is required for systemic acquired resistance as a defense to a broad spectrum of pathogens (Park et al. 2007, 2009; Tripathi et al. 2010; Liu et al. 2011b, 2011a). Interestingly, behavioral studies found that MeSa can repel herbivores (Hardie et al. 1994; Ulland et al. 2008; Snoeren et al. 2010) and attract beneficial carnivorous insects (James 2003; De Boer and Dicke 2004; James and Price 2004; Zhu and Park 2005; Lee 2010; Mallinger et al. 2011). The molecular and neuronal mechanisms underlying the behavioral effects of MeSa remain unclear.

We previously found that C. elegans exhibits a strong avoidance response to MeSa and can be useful for understanding the neuronal effects of MeSa (Luo et al. 2015). To identify new genes affecting this behavior, we screened for mutants with defective MeSa avoidance. Surprisingly, the screen isolated novel loss-of-function mutations in unc-80 and unc-79. In this study, we examined how these genes affect C. elegans avoidance to MeSa and locomotion. We identified a subset of interneurons that express unc-80 and analyzed the roles of these neurons in mediating the MeSa avoidance. Our findings suggest that the NALCN complex functions in command interneurons to regulate C. elegans reversal behavior.

**MATERIALS AND METHODS**

**Strains**

See supplemental Materials and Methods.

**MeSa avoidance assay**

C. elegans MeSa avoidance assay was performed as previously described (Luo et al. 2015). 30 to 200 animals were examined in each experiment.

**Locomotion assay**

Synchronized L4 animals were picked into new plates seeded with OP50 bacteria one night before the assay. Body bends were measured by touching an animal on the tail with a worm pick to help initiate locomotion, followed by counting the number of body bends (one head turn) for 1 min.

**Genetic screen for and identification of mutants with defective avoidance response to MeSa**

Synchronized L4 wild-type animals (P0) were mutagenized with EMS (ethyl methanesulfonate) as described (Brenner 1974). F1 progeny were allowed to grow to young adults and bleached to generate synchronized F2 progeny for the MeSa avoidance assay. Adult F1 animals that failed to avoid MeSa were collected from each individual plate and bleached to generate synchronized adult F2 progeny for a new round of MeSa assay. After six rounds of selection, an individual F6 progeny that failed to avoid MeSa was picked from each plate, allowed to propagate and retested in the MeSa avoidance assay. From ~60,000 F1 animals, we isolated 15 independent mutants (Figure 1A).

Genetic complementation tests using the response of F1 males to MeSa as readout identified three distinct groups, with group one containing 11 mutants (mac379, mac380, mac381, mac382, mac384, mac385, mac386, mac388, mac390, mac391, mac394), group two containing three mutants (mac383, mac389, mac393) and group three containing one mutant (mac387) (Table S1). We selected mac379, mac382 and mac388 from group one, and mac383 from group two for genomically sequencing as described (Zhou et al. 2018). Sequencing results indicated that mac379, mac382 and mac388 carried distinct nonsense mutations in the unc-80 gene and mac383 carried a nonsense mutation in unc-79. The mac387 mutation in the third complementation group was not further analyzed, as it appeared to affect general locomotion, followed by counting the number of body turns (one head turn) for 1 min.

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**Molecular biology**

See supplemental Materials and Methods. PCR primers are listed in Table S5.

**Transgene experiments**

Germline transgene experiments were performed as described (Mello et al. 1991).
Identiﬁer.

We used DiI-labeled sensory neurons (Tong and Burglin 2010) as gene mixtures contained 20 to 50 ng/µl as co-injection marker.

For neuron-speciﬁc rescue experiments, transgenic mixtures containing 10 to 20 ng/µl reporter construct were injected to wild type.

For neuron-speciﬁc knockdown experiments, we used a previously described method with minor modiﬁcations (Shen et al. 2014). The transgene mixtures contained 20 to 50 ng/µl (Promoter::Cas9::NLS::3‘UTR, 25 ng/µl PU6::unc-79_sgRNA#1 and #2 each (or PU6::unc-80_sgRNA#1 and #2 each), and 10 to 20 ng/µl pPD95_86 (Promoter::GFP) or 2.5 ng/µl pCFJ90 (Promoter::mCherry) as co-injection marker.

For neuron-speciﬁc rescue experiments, transgenic mixtures contained 10 to 20 ng/µl Promoter::unc-80_cDNA and 20 ng/µl pPD95_86 (Promoter::GFP) or 2.5 ng/µl pCFJ90 (Promoter::mCherry) as co-injection marker.

Identification of unc-79- and unc-80-expressing neurons

We used DiI-labeled sensory neurons (Tong and Burglin 2010) as landmarks to facilitate the identiﬁcation of Punc-79-expressing neurons. Images of ﬂuorescence-positive neurons in transgenic animals expressing GFP and/or mCherry reporters were captured using a 63X DIC/ﬂuorescent Leica TCS SP5 II laser confocal microscope and neuronal identities were inferred by overlapping ﬂuorescence signals and by comparing to the anatomical and morphological characteristics of neurons described in Wormatlas (www.wormatlas.org).

Statistical analysis

P values were determined by Paired two-tailed Student’s t-test or Bonferroni’s multiple comparison using GraphPad Prism 7.0 software.

Data availability

Strains and plasmids are available upon request. Supplemental Materials and Methods, supplemental Figures and Tables, and raw data for the behavioral experiments (File S1) and raw images for the neuronal labeling (File S2) can be accessed at ﬁgshare: https://doi.org/10.25387/g3.10060007.

RESULTS

A screen identiﬁed mutants with defective MeSa avoidance response

To identify novel genes affecting the MeSa avoidance behavior, we performed a genetic screen for mutants that failed to avoid MeSa, from which 15 strains were isolated (Figure 1A). Genetic complementation tests divided the mutations to three groups (Table S1).

To investigate whether the mutants had defects in responding to other odorants, we examined a representative mutant in each complementation group (Table S1, mac379, mac383 and mac387, respectively) for chemotaxis. mac379 and mac383 mutants exhibited wild type-like responses (Figure 1B) to repelling odorants 1-octanol and 2-nonanone, and attractive odorants diacetyl and benzaldehyde (Bargmann et al. 1993), suggesting that these mutants have largely normal odorant responses, including odorant detection and odorant-triggered forward or reversal movement. The third mutant, mac387, was defective in detecting each odorant (Figure 1B). We postulate that mac387 might cause broad defects in chemotaxis and did not analyze it further.

Loss-of-function mutations in unc-80 and unc-79 caused MeSa avoidance defect

To identify the causal mutations leading to the defective MeSa avoidance, we determined the genomic sequences of mac379, mac382 and mac388 mutants from group 1 and mac383 from group 2 (Table S1). A comparison of candidate genes found that unc-80 was the only affected gene shared by mac379, mac382 and mac388, with W1524stop, W220stop and W1967stop as the respective mutations (Table S1). Besides W1524stop, mac379 also carried a
transgenic lines and their respective loss-of-function mutants. (C) The locomotion (body bends/min, blue columns, 40 animals per strain) and MeSa avoidance indexes (red columns, three to six biological replicates) of single and double mutants. Genotypes are labeled at bottom.

missense mutation (G927R) in unc-80 (Fig. S1A and Table S1). Sanger sequencing identified distinct nonsense or splice site mutations in unc-80 from other mutants of group 1 (Table S1).

Meanwhile, we identified an R885stop mutation in unc-79 among the candidate genes for mac383 (group 2) (Table S1 and Fig. S1B). Considering that unc-80 and unc-79 interact with nca to affect C. elegans behavior (Humphrey et al. 2007; Jospin et al. 2007; Yeh et al. 2008), we speculated that unc-79 might be the causal gene of mac383. Indeed, Sanger sequencing identified distinct deletion/frameshift mutations in unc-79 from mac389 and mac393 mutants of group 2 (Table S1).

To verify that unc-79 is required for the MeSa avoidance behavior, we performed transgene rescue experiments. Driven by a long endogenous promoter (Figure 2A, Punc-79a, 5.0 kb), an unc-79a gDNA transgene (Figure 2A) strongly rescued the defective locomotion and MeSa avoidance of unc-79(mac383) mutants (Figure 2B). Driven by a short promoter (Figure 2A, Punc-79a, 2.0 kb), the unc-79a gDNA transgene only rescued the defective MeSa avoidance but not the defective locomotion (Figure 2B). We also examined a transgene that covers the shorter unc-79b isoforms (Fig. S1B and 2A, Punc-79b) and found that it failed to rescue either defective behavior (Figure 2B).

Similarly, an unc-80 gDNA transgene that covers all annotated unc-80 isoforms (Fig. S1A) driven by an unc-80 endogenous promoter (Figure 2A, Punc-80, 3.6 kb) can significantly rescue the defective locomotion and MeSa avoidance of unc-80(mac379) mutants (Figure 2B). Compared to the fully rescued MeSa avoidance, the defective locomotion was only partially rescued (Figure 2B).

unc-79 and unc-80 loss-of-function mutants are hypersensitive to the anesthetic halothane and exhibit the “fainter” phenotype (Morgan and Casperbi 1985; Sedensky and Menecely 1987; Morgan et al. 1988, 2007; Humphrey et al. 2007; Jospin et al. 2007; Yeh et al. 2008). Similar “fainter” phenotype was observed in all unc-80 and unc-79 mutants isolated in this study. In addition, unc-79(e1291) and unc-80(e1272) animals, two previously described loss-of-function mutants (Jospin et al. 2007), exhibited defective MeSa avoidance resembling that of unc-79(mac383) or unc-80(mac379) mutants (File S1, additional raw data). Together, these results suggest that unc-79 and unc-80 are specifically required for C. elegans MeSa avoidance behavior and we isolated loss-of-function mutations in unc-79 and unc-80.

nca-1 and nca-2 were redundantly required for the MeSa avoidance behavior

C. elegans nca-1 and nca-2 encode functionally redundant NALCN channels (Humphrey et al. 2007; Morgan et al. 2007; Jospin et al. 2007; Yeh et al. 2008). To investigate whether nca is required for the MeSa avoidance behavior, we examined nca single and double mutants. We found that either nca-1(gk9lf) or nca-2(gk5lf) single mutants exhibited wild type-like MeSa avoidance (Figure 2C), while nca-2(one); nca-1(f0) double mutants were strongly defective in avoiding MeSa (Figure 2B and 2C). An nca-1 gDNA transgene (Figure 2A, Pnca-1), an nca-2 gDNA transgene (Figure 2A, Pnca-2), or both transgenes together can strongly rescue the defective MeSa avoidance of the double mutants (Figure 2B). Though the locomotion defect of the double mutants was significantly rescued by either transgene (body bends/min, see Materials and Methods), the two transgenes together appeared to have a stronger effect (Figure 2B). Similar to unc-79(f0) or unc-80(f0) mutants, nca(f0) double mutants exhibited normal responses to other attractants and repellents (Figure 1B).
A gain-of-function (gf) mutation in nca-1, e625, causes the “coiler” phenotype (Yeh et al. 2008). To understand how unc-79 or unc-80 interacts with nca-1(e625gf) in affecting the MeSa avoidance, we generated double mutants. Consistent with previous findings (Yeh et al. 2008), we found that unc-79(lf) or unc-80(lf) completely suppressed the “coiler” phenotype of the nca-1(gf) mutants. The double mutants also exhibited defective MeSa avoidance (Figure 2C).

The ER protein NCA localization factor-1 (NLF-1) is required for axonal localization of NCA proteins (Xie et al. 2013). To examine whether nlf-1 is required for the MeSa avoidance, we generated three nlf-1 deletion lines (Table S2) using a CRISPR/Cas9 method (Friedland et al. 2013). Taking nlf-1(mac409) as the representative loss-of-function allele (Figure 2C), we found that nlf-1(mac409) itself, or together with nca-1(lf), nca-2(lf) or nca-1(gf), did not cause obviously defective MeSa avoidance (Figure 2C). We observed that nlf-1(lf) can suppress the “coiler” phenotype of nca-1(gf) mutants, consistent with previous findings (Xie et al. 2013).

To understand how these genes affect other behaviors, we examined the locomotion of the mutants. unc-79(lf) and unc-80(lf) single mutants, or double mutants carrying either unc-79(lf) or unc-80(lf), all exhibited severely defective locomotion (Figure 2C).

Interestingly, nca-1(lf) single mutants exhibited weakly defective locomotion, while nca-2(lf) single mutants were moderately defective (Figure 2C). ncalc(lf) double mutants had severely defective locomotion similar to that of unc-79(lf) or unc-80(lf) mutants (Figure 2C). We found that nlf-1(lf) mutants exhibited a moderately defective locomotion, which can be enhanced by nca-1(lf) or nca-2(lf) (Figure 2C). However, the defective locomotion of nlf-1(lf) mutants appeared to be partially improved by nca-1(gf) (Figure 2C).

The identification of a subset of unc-80-expressing neurons

Previous studies found that unc-80, unc-79 and nca are broadly expressed in C. elegans neurons (Jospin et al. 2007; Yeh et al. 2008). However, the classes of individual neurons remain to be identified. To understand the neuronal mechanism underlying the function of the NCA channel complex, we generated transgenic animals expressing GFP driven by Punc-80 (Figure 2A). In these animals, GFP was observed in multiple neurons, ventral nerve cord and vulval muscles (Fig. S2A), consistent with previous findings (Jospin et al. 2007; Yeh et al. 2008).

We next used transgene double-labeling to identify individual neurons that express unc-80. In animals co-expressing Punc-80::GFP (Figure 3A, left panel) and Pmnr-1:Cherry (Figure 3A, middle panel) (neurons labeled by Pmnr-1 are listed in Table S3) (Brockie et al. 2001b), we observed visible GFP expression in AVA, AVE and AVG neurons (Figure 3A, right panel) but no obvious expression in AVD and RIM neurons, two classes of neurons also reported to be labeled by Pmnr-1. In addition, a neuron similar to RII appeared to be consistently labeled by GFP and mCherry (Figure 3A, right panel). Additional double-labeling using Punc-80::GFP (Figure 3B, 3C and 3D, left panels) with mCherry driven by the mgl-1 promoter (Table S3) (Wenick and Hobert 2004; Greer et al. 2008) (Figure 3B, 3C and 3D, middle panels) identified four other classes of GFP-expressing neurons, including RMD and I3 (Figure 3B, right panel), I4 (Figure 3C, right panel) and NSM (Figure 3D, right panel). Except for these neurons, multiple other GFP-expressing neurons remain to be identified.

Using a previously described nlf-1 promoter (Xie et al. 2013) to drive the mCherry transgene, we confirmed the expression of nlf-1 in head neurons, ventral nerve cord and tail neurons (Fig. S2B) (Xie et al. 2013). Neuronal double-labeling found that AVA and AVE neurons, among other unidentified neurons, were co-labeled by Punc-80:GFP and Pmgl-1:mCherry (Fig. S3A). The expression of nlf-1 in AVA and AVE was also described previously (Xie et al. 2013).

Neuron-specific transgene rescued the defective MeSa avoidance of unc-80(lf) mutants

To understand the function of unc-80 in different neurons, we performed neuron-specific transgene rescue experiments. An unc-80 cDNA transgene driven by Punc-80 can significantly rescue the defective locomotion and MeSa avoidance of unc-80(lf) mutants (Figure 4A, Punc-80). Still, the locomotion was partially rescued while the MeSa avoidance was more strongly rescued. The nlf-1 promoter had a similar rescuing effect as the unc-80 promoter (Figure 4A, Pmgl-1), consistent with the result that unc-80 and nlf-1 were co-expressed in multiple neurons (Fig. S3A).

To examine whether the unc-80 transgene might be effective in a subset of unc-80-expressing neurons, we tested the nmr-1 promoter (Table S3). The Pnmr-1::unc-80 transgene significantly rescued the defective MeSa avoidance but not the defective locomotion of unc-80(lf) mutants (Figure 4A, Pnmr-1). The glr-1 promoter (Table S3), which also drives transgene expression in AVA, AVE and AVG neurons (Brockie et al. 2001a), similarly rescued the defective MeSa avoidance but not the defective locomotion (Figure 4A, Pglr-1).

To determine whether the unc-80 transgene can be effective in a smaller set of neurons, we tested three other promoters, Pfl-1, Pfl-18 and unc-7b (Table S3) (Nelson et al. 1998; Rogers et al. 2003; Altun et al. 2009), which label unc-80-expressing AVA/AVE, AVA, and AVG neurons, respectively. Each transgene by itself failed to rescue either defective behavior (Figure 4A, Pfl-1, Pfl-18 or Punc-7b). However, when injected together, the three transgenes can significantly rescue the defective MeSa avoidance of unc-80(lf) mutants (Figure 4A, Pfl-1/Pfl-18/Punc-7b). Similar to the nmr-1 or the glr-1 promoter, the defective locomotion was not rescued (Figure 4A, Pfl-1/Pfl-18/Punc-7b).

To understand whether other neurons might be involved in the MeSa avoidance behavior, we tested the mgl-1 promoter, which drives overlapping expression with Punc-80 in RMD, I3, I4 and NSM neurons (Figure 3B, 3C and 3D). However, the Pmgl-1::unc-80 transgene failed to rescue either the defective MeSa avoidance or the defective locomotion of unc-80(lf) mutants (Figure 4A, Pmgl-1). The sla-11 promoter (Table S3) (Altun-Gultekin et al. 2001) did not rescue the defective behaviors either (Figure 4A, Psla-11), consistent with our finding that its expression did not obviously overlap that of Punc-80 (Fig. S3B).

Neuron-specific knockout of unc-80 caused defective locomotion

To investigate whether unc-80 expression in specific neurons is essential for its function, we used a CRISPR/Cas9-based method (Shen et al. 2014) to examine whether neuron-specific knockout of unc-80 would phenocopy the behavioral defects of unc-80(lf) mutants.

Transgenic animals expressing two unc-80-targeting sgRNAs (Table S4) and Cas9 driven by the pan-neuronal unc-119 promoter (Table S3) (Maduro and Pilgrim 1995) exhibited obviously defective locomotion (Figure 4B, Punc-119). Limiting the expression of Cas9 to a subset of interneurons, e.g., AVA, AVE and AVG, using the nmr-1 promoter also caused defective locomotion (Figure 4B, Pnmr-1). However, Punc-80::Cas9 failed to cause obviously defective locomotion (Figure 4B, Punc-80). We postulate that the expression of Punc-80::Cas9 might not be sufficient in disrupting the unc-80 locus in these animals.
Interestingly, different from the locomotion, the MeSa avoidance response was not affected in any of the knockdown lines (Figure 4B, red columns).

**unc-79 and unc-80 functioned in overlapping neurons**

Though it is widely assumed that UNC-79 and UNC-80 function together in the same neurons, there is limited genetic evidence supporting this notion. To validate this assumption, we generated transgenic lines expressing GFP driven by the PLunc-79a promoter (Figure 2A). In these animals, GFP was expressed in multiple head and tail neurons, ventral nerve cord and vulval muscles (Fig. S2C), a pattern similar to that of unc-80 (Fig. S2A). Using Punc-79a to drive an unc-79a_cDNA::GFP transgene (Fig. S4A), we found similar expression of the UNC-79a::GFP fusion protein in head neurons (Fig. S4B), ventral nerve cord (Fig. S4B), vulval muscles (Fig. S4C), motor neurons (Fig. S4C) and tail neurons (Fig. S4D). This transgene also significantly rescued the defective MeSa avoidance and locomotion of unc-79(lf) mutants (File S1, additional raw data), similar to that of the unc-79a_gDNA transgene (Figure 2B).

We next examined the expression of the Punc-79a promoter. Interestingly, GFP driven by Punc-79a was only detected in several pairs of head neurons (Fig. S2D), a pattern similar to that described by Humphrey et al. (Humphrey et al. 2007). The expression of Punc-79a::GFP in anterior and posterior portions of the intestine (Fig. S2D) was likely non-specific as Punc-79a::GFP was not detected there (Fig. S2C).

To investigate whether unc-79 and unc-80 were expressed in the same neurons, we generated transgenic animals co-expressing Punc-79a::GFP and Punc-80::mCherry. In these animals, GFP and mCherry co-labeled a large number of head neurons (Figure 5A), the vulval muscles (Figure 5B) and a few tail neurons (Figure 5C). One or more head neurons (Figure 5A, right panel, arrow heads), a motor neuron (Figure 5B, right panel, arrow head) and a tail neuron (Figure 5C, right panel, arrow head) appeared to be labeled by only GFP or mCherry but not both, which might be caused by mosaicism or variable expression of the transgenes. A Punc-80::unc-80 cDNA transgene could significantly rescue the defective locomotion and MeSa avoidance of unc-80(lf) mutants (Figure 4A, PLunc-79a), consistent with the overlapping expression of the two genes.

The limited expression pattern of the PSunc-79a promoter (Fig. S2D) and its partial rescuing strength (Figure 2B) suggest that it might define a subset of functioning unc-79 neurons. We combined Dil tracing and neuronal double-labeling to identify the neurons labeled by this promoter.

In Punc-79a::GFP transgenic animals, we found that GFP co-labeled ASH and ASJ that were also stained with Dil (Figure 6A). The expression of Punc-79a::GFP (Figure 6B and 6C, left panels) in ASJ (Figure 6B, right panel) and ASH (Figure 6C, right panel) was

![Figure 3](https://example.com/image.png) Neuronal double-labeling identified a subset of unc-80-expressing neurons. (A) GFP expression driven by the unc-80 promoter (left panel, Punc-80), mCherry driven by the nmr-1 promoter (middle panel, Pnmr-1) and the merged image (right panel) showing GFP expression in AVA, AVE and AVG neurons and probably in the RIH neuron. (B, C, D) GFP driven by Punc-80 (left panels), mCherry driven by Pmgl-1 (middle panels) and the merged images showing GFP expression in RMD and I3 (B, right panel), I4 (C, right panel) and NSM (D, right panel) neurons. VNC: ventral nerve cord. NR: nerve ring. A: anterior. V: ventral. Results were based on three independent transgenic lines. Pictures were taken from a line with the most robust expression of reporters.
verified by co-labeling with Pssu-1::mCherry (Table S3) (Carroll
et al. 2006) (Figure 6B, middle panel) and Psra-6::mCherry (Table
S3) (Troemel et al. 1995) (Figure 6C, middle panel), respectively.
Pssu-1::GFP (Figure 6D, left panel) also appeared to label the
RIA neurons (Figure 6D, right panel), which were identified by
co-labeling with Pglr-3::mCherry (Table S3) (Brockie et al. 2001a)
(Figure 6D, middle panel). The fourth pair of neurons labeled by P
unc-79a::GFP was similar to either of the closely positioned motor
neurons RMF or RMH (Fig. S3C). Unlike the Punc-79a promoter, a P
unc-79a::unc-80 transgene failed to rescue the defective locomotion
or MeSa avoidance of unc-80(lf) mutants (Figure 4A, Punc-79a), consis-
tent with the finding that Punc-79a::mCherry and Punc-80::GFP did
not appear to co-label neurons (Fig. S3D).

We next used the CRISPR/Cas9 knockdown to investigate the
function of unc-79 in neurons. Transgenic animals expressing two
unc-79-targeting sgRNAs (Table S4) and the pan neuronally expressed
Punc-119::Cas9 exhibited significantly defective locomotion (Figure 4B,
Punc-119). Cas9 driven by the long or short unc-79 promoter also
cause defective locomotion (Figure 4B, Punc-79a and Punc-79a).
However, none of the unc-79 knockdown lines exhibited obviously
defective MeSa avoidance (Figure 4B, red columns), which is similar to
unc-80 knockdown animals.

**DISCUSSION**

In this study, we found that unc-79, unc-80 and the nca genes are
specifically required for C. elegans avoidance response to methyl salicy-
late. We verified that unc-79 and unc-80 are expressed and function in
overlapping neurons. We identified and examined the functional
relevance of a subset of unc-80-expressing neurons. Our findings suggest a
novel role of the NALCN-related genes in the command interneurons
AVA and AVE to regulate C. elegans reversal behavior.

Genetic, cellular, biochemical and electrophysiological studies sug-
gest that UNC79, UNC80 and NALCN form a channel complex to
regulate resting membrane potential and neuronal excitability
(Humphrey et al. 2007; Lu et al. 2007, 2009, 2010; Jospin et al. 2007;
Xie et al. 2013; Perez et al. 2016, 2018; Stray-Pedersen et al. 2016; Fujita
et al. 2017; Bramswig et al. 2018) that are collectively called NALCN channelopathies (Bramswig et al. 2018). A notion derived from these studies is that NALCN, UNC80 and UNC79 should be expressed in the same neurons to perform their functions. However, a closer look at their co-expression in neurons
has been insufficient. Here, we provide both neuronal identification and transgene rescue results to verify the co-expression and function of
unc-80 and unc-79 in the same neurons. Though not surprising, our
work could be useful for analyzing the neuronal functions of these
genes in C. elegans.

unc-79 and unc-80 loss-of-function mutants exhibited indistin-
guishable MeSa-specific avoidance defects, a phenotype that is also
shared by the NALCN mutants (nca). Interestingly, all these mutants
displayed wild type-like responses to other attractants or repellents,
suggesting they had largely normal odorant sensation and forward/re-
versal behaviors. Therefore, MeSa might trigger an avoidance response
that is specifically mediated by the NALCN channel complex.

Our genetic and neuronal analyses suggest that unc-80 expression in a
subset of unc-80-expressing neurons, e.g., in the command interneu-
rons AVA and AVE and the anterior guidepost neuron AVG defined
Figure 5 Neuronal double-labeling found that unc-79 and unc-80 were expressed in largely overlapping neurons. (A) GFP expression driven by the long unc-79a promoter (left panel, \( P_{unc-79a} \)) and mCherry driven by the unc-80 promoter (middle panel, \( P_{unc-80} \)) and the merged image (right panel) showing multiple head neurons co-expressing GFP and mCherry. (B) Same as (A), showing vulval muscles co-labeled by GFP and mCherry. (C) Same as (A, B), showing a few tail neurons co-labeled by GFP and mCherry. Arrow heads point to neurons that appear to express only one fluorescent reporter. A: anterior. V: ventral. Results were based on three independent transgenic lines. Pictures were taken from a line with the most robust expression of reporters.

by the \( nmr-1 \) or \( glr-1 \) promoter, is sufficient for eliciting the MeSa avoidance. However, unlike the endogenous \( unc-79 \), \( unc-80 \) or \( nlf-1 \) promoters, the \( nmr-1 \) or \( glr-1 \) promoter did not rescue the defective locomotion. This result is consistent with the study of \( nlf-1 \), in which Xie et al. (Xie et al. 2013) found that an \( P_{nmr-1::nlf-1} \) transgene alone was not sufficient in rescuing the defective body bending frequency of \( nlf-1 \) mutants, while a combination of \( P_{nmr-1::nlf-1} \) and \( Psra-11::nlf-1 \) transgenes would do. Therefore, broader expression of \( unc-80 \) in more interneurons might be necessary for generating a normal locomotion.

The key role of AVA in mediating NALCN functions was also suggested by two recent studies, in which the authors found that \( nlf-1 \) was expressed in AVA and AVE to regulate \( C. elegans \) locomotion (Xie et al. 2013) and NCA can activate the AVA neurons to potentiate persistent motor circuit activity in \( C. elegans \) (Gao et al. 2015). Together, our findings suggest a neural network containing at least AVA, AVE and AVG in regulating \( C. elegans \) behaviors.

AVA neurons are key regulators of a variety of \( C. elegans \) behaviors, e.g., touch-induced movement (Chalfie et al. 1985; White et al. 1986), reversal locomotion (Pokala et al. 2014), mechanosensory habituation (Sugi et al. 2014), variability in reversal response to odor stimuli (Gordus et al. 2015) and repetitive reversals caused by glutamate spillover (Katz et al. 2019). The expression of \( unc-80 \) and presumably \( unc-79 \) and \( nca \) in AVA neurons implicate the NALCN channel complex as a regulator of these behaviors.

MeSa was first isolated in the 19th century and has been used as a natural flavoring agent and topical pain relief for decades (https://pubchem.ncbi.nlm.nih.gov/compound/methyl_salicylate). Recent studies suggest that MeSa is synthesized by many plants to signal the systemic acquired resistance to multiple pathogens (Liu et al. 2011a). Surprisingly, MeSa can attract beneficial carnivorous insects and also repel herbivores (Hardie et al. 1994; James 2003; De Boer and Dicke 2004; James and Price 2004; Zhu and Park 2005; Ulland et al. 2008; Lee 2010; Snoeren et al. 2010; Mallinger et al. 2011). We were particularly intrigued by the latter findings and established the \( C. elegans \) MeSa avoidance assay to study the genetics underlying the behavioral effects of MeSa (Luo et al. 2015). In our previous study, neuron-specific rescue experiments suggest that the \( npr-1 \)-expressing inter/motor neurons RMG and the \( npr-2 \)-expressing interneurons AIZ might be involved in the MeSa avoidance behavior (Luo et al. 2015). Genetic mutants lacking sensory neurons imply that AWB might play a major role in detecting MeSa, while AWC might play a minor role (Luo et al. 2015). Together, the involvement of AWC, AIZ and AVA neurons in mediating the MeSa avoidance is consistent with the placement of these neurons in a core circuit that regulates \( C. elegans \) chemotaxis (Gordus et al. 2015).

Previously, the human TRPV1 channel was shown to be inhibited by MeSa (Ohta et al. 2009). However, the wild type-like response to MeSa by two TRPV channel mutants (\( osm-9 \) and \( ocr-2 \)) (Luo et al. 2015) and the finding that all five TRPV channel expression was not detected in AWB neurons (Colbert et al. 1997; Tobin et al. 2002) do not support a role of a TRPV channel as the MeSa receptor in \( C. elegans \). Alternatively, a GPCR expressed in antenna sensory neurons of the tortricid moth was found to exhibit high sensitivity to MeSa in the insect s9 cells (Jordan et al. 2009). This result is consistent with the findings that
GPCRs can regulate NALCN activities in mammals (Lu et al. 2009, 2010; Swayne et al. 2009; Yeh et al. 2017; Philippart and Khaliq 2018), NCA are downstream targets of neuronal G-protein signals in C. elegans (Topalidou et al. 2017a, 2017b) and GPCR signals are involved in C. elegans avoidance response to MeSa (Luo et al. 2015). Therefore, a GPCR might act as the MeSa receptor in certain C. elegans sensory neurons.

We made a few intriguing findings in this study. First, restoring unc-80 expression specifically in interneurons using Pnmr-1, Pglr-1 or Pflp-1/Pflp-18/Punc-7b significantly rescued the defective MeSa avoidance of unc-80(lf) mutants, suggesting that NALCN expression in interneurons shared by these promoters but not in other neurons might be sufficient for MeSa to elicit the reversal behavior. Second, unc-79 transgene expression driven by the Punc-79a promoter could strongly rescue the defective MeSa avoidance of unc-79(lf) mutants, suggesting that a limited restoration of NALCN function, potentially only in sensory neurons ASH and ASJ and two pairs of interneurons (RIA and RMF/RMH) but not in other classes of neurons, might be sufficient for MeSa to trigger the reversal behavior. Third, only the locomotion but not the MeSa avoidance behavior was significantly affected by neuron-specific knockdown of unc-80 or unc-79, suggesting that partial expression of the genes due to incomplete knockdown is sufficient for MeSa to elicit a strong avoidance response.

There results prompt us to postulate that MeSa might cause the avoidance behavior by acting on more than one component, e.g., a group of neurons, of a multi-component neural network controlling the reversal behavior. In this network, the expression of the NALCN complex in any MeSa-responding components would be sufficient for triggering the avoidance behavior. Only when the NALCN complex is absent in all MeSa-responding components, the animals would exhibit a defective avoidance response. In addition, a remote possibility derived from these findings is that MeSa might act on the NALCN complex as an agonist. Therefore, a continuing investigation on the molecular mechanism underlying the MeSa avoidance behavior is warranted for further understanding of the function of the NALCN complex.

Finally, that MeSa might activate the NALCN channel complex provides a potential molecular explanation on why MeSa can repel herbivores and attract beneficial insects. For example, herbivores might express NALCN on neurons that promote avoidance behavior, while beneficial insects might express NALCN on neurons that promote attraction behavior.

CONCLUSION

In short, we found that unc-79, unc-80 and nca genes are specifically required for C. elegans avoidance response to the plant hormone MeSa. We verified that unc-79 and unc-80 are co-expressed and function in overlapping neurons. The command interneurons AVA, AVE and the guidepost neuron AVG can be sufficient for unc-80 regulation of the MeSa avoidance. Our results suggest a novel function of the NALCN complex in the molecular basis of plant hormone-evoked reversal behavior in C. elegans.
complex expressed in command interneurons as a regulator of C. elegans reversal behavior.

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