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Regulation of olfactory behavior by RNAi pathways in Caenorhabditis elegans

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Regulation of olfactory behavior by RNAi pathways in *Caenorhabditis elegans*

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Abstract

Responding to environmental cues is extremely important for the survival of animals. One pathway that plays a relevant role in the detection and response to environmental cues is the olfaction pathway. Past research has shown that a form of gene regulation known as RNA interference (RNAi) is required in sensory neurons for the detection of unfavorable environmental cues. To further explore this, we hypothesized that RNAi is also required in sensory neurons for the detection of attractive cues. To test our prediction, we examined whether RNAi would be required in AWA and AWC sensory neurons for the detection of the favorable environmental odorants, diacetyl and benzaldehyde, respectively. We found that mutants without a completed RNAi pathway were not able to sense attractive odorants to the same degree as wild type animals. Knowing that mutant animals deficient in the RNAi pathway were unable to sense attractive volatile odorants as effectively as wild type animals, we tested our hypothesis by using rescue strains which restored the RNAi pathway in specific neurons. We performed population chemotaxis assays to determine the probability of a worm being attracted to a specific odorant. Our results showed that the rescue strains which should have restored the phenotype of RNAi mutants to that of wildtype were unable to restore gene function. We hypothesize that the RNAi pathway is active downstream of the sensory neurons in an interneuron, and we know that RNAi is active somewhere in the olfaction pathway.

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Executive Summary

How animals detect and respond to environmental cues, such as food availability, are extremely important factors in determining the survival of an organism. If olfaction is disrupted, animals cannot detect environmental stimuli, including food and mates, and can potentially endanger themselves. An exemplary model organism with which to investigate the mechanism of how animals detect and respond to environmental cues is the microscopic soil nematode, *Caenorhabditis elegans*. In mammalian systems, each olfactory neuron typically responds to only one stimulus, so detection of complex odorants requires the use of multiple neurons (Chess et al., 1994). *C. elegans* has a more multi-modal integrated nervous system in which each sensory neuron can detect multiple environmental stimuli, and olfactory neurons are able to work independently (Bargmann, 2006). This allows neurons to be investigated individually without needing to consider the entire neuronal network.

Sensation of environmental cues relies on the gene expression of protein components in the sensory signaling pathway. Previous research done by the Hall Lab showed that the RNA interference pathway, which regulates the levels of endogenous gene expression, was necessary in sensory neurons for the response to environmental stressors, such as starvation and high temperature (Bharadwaj and Hall, 2017). The RNAi pathway functions when a protein complex binds to double stranded RNA to create small interfering RNA, or siRNA. The siRNAs can then target the homologous sequence in messenger RNA, or mRNA, to destroy the fragments or act in the nucleus. This is a pathway for gene silencing.

Based on this information, we hypothesized that the RNAi pathway was also necessary for chemosensation of attractive odorants in the corresponding sensory neurons. Two volatile odorants whose detection pathways have already been explored are diacetyl and benzaldehyde.

Both are compounds which are produced as a metabolic byproduct of *E. coli*, which is the food source of lab grown *C. elegans*. Diacetyl is sensed through the AWA neuron and benzaldehyde is sensed through the AWC neuron.

In order to test our hypothesis, we performed chemotaxis assays to measure the ability of the worms to move toward a volatile attractive odorant. These assays require a drop of an odorant and a control on a petri dish to determine which side of the plate, or odorant, the worms would travel toward. We then used an equation to determine the chemotaxis index, or amount of the population that was attracted to each section of the plate. Using these assays, we tested the chemotaxis behavior of wild type animals, strains that have mutations in RNAi genes, and several genetically modified lines created to restore the function of RNAi in specific neurons. Preliminary data from our lab indicated that the RDE-1 and MUT-7 RNAi proteins are required for chemotaxis to attractive stimuli (Hager and Hall). We proposed that if we restored gene function of *rde-1* and *mut-7* in each of the two neurons of interest, we will be able to restore the chemotaxis behavior. These results would show that the RNAi pathway is necessary for chemotaxis toward volatile attractive odorants by acting in the sensory neurons.

We performed one type of experiment for each of the odorants, diacetyl and benzaldehyde. Each odorant was used for AWA *rde-1* or *mut-7* rescues and for AWC *rde-1* rescues. The corresponding null mutant was used as a negative control, and wild type animals as a positive control. First, we found that mutants without a completed RNAi pathway were not able to sense attractive odorants to the same degree as wild type animal. When we tested AWA *mut-7* rescue lines in the presence of diacetyl, their chemotaxis index was not significantly different from the null mutants, suggesting that MUT-7 is not required in AWA for attractive response to diacetyl. The AWA *rde-1* rescues and AWC *rde-1* rescues in the presence of

diacetyl also were not significantly different than the negative control, but in addition they were not different from the wildtype. This makes sense for the AWC *rde-1* rescues, as diacetyl is not sensed in the AWC neuron. The AWA *rde-1* rescues were trending upward toward being significantly higher than the null mutants. In benzaldehyde, the AWA *mut-7* rescues and AWA *rde-1* rescues did not appear to rescue and were not significantly different from the negative controls which was expected because the AWA neuron does not sense benzaldehyde. The AWC *rde-1* rescues should have restored the RNAi function in chemosensation of benzaldehyde, but the chemotaxis index was significantly lower than that of wild type. However, the AWC rde-1 rescue strains appeared to have a chemotaxis index which was trending upward toward being significantly different from that of the corresponding null mutant.

The results of the assays showed that restoring RNAi function by the gene reinsertion into either AWA or AWC neurons did not restore chemotaxis behavior in the transgenic strains. We believe that there are other targets located within the olfaction pathway that require RNAi in order to function properly. In addition to the sensory neurons, attractive behavior toward volatile odorants requires several layers of interneurons, or intermediary steps that help carry signals from the environment to the motor neurons. We believe that RNAi functions downstream of the AWA and AWC neurons somewhere in these interneurons. While the rescue strains did not show restored chemosensation, the fact that knocking out RNAi function inhibited the ability of the mutant strains to chemotax toward the attractive odorants means that the RNAi pathway is involved in volatile odorant chemotaxis.

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Introduction

How animals detect and respond to environmental cues, such as food availability, are extremely important factors in determining the survival of an organism. If olfaction is disrupted, animals cannot detect environmental stimuli, including food and mates, and can potentially endanger themselves. An exemplary model organism with which to investigate the mechanism of how animals detect and respond to environmental cues is the microscopic soil nematode, *Caenorhabditis elegans*. In mammalian systems, each olfactory neuron typically responds to only one stimulus, so detection of complex odorants requires the use of multiple neurons (Chess et al., 1994). *C. elegans* has a more multimodal integrated nervous system in which each sensory neuron can detect multiple environmental stimuli, and olfactory neurons are able to work independently (Bargmann, 2006). These features allow neurons to be investigated individually without needing to consider the entire neuronal network.

C. elegans contains odor sensing mechanisms which are dependent on odorant concentration. These mechanisms occur at the sensory neuron and olfactory receptor levels (Taniguchi et al., 2014). The entire receptor and sensory neuron complex can be called an olfactory receptor neuron and reflects how odor molecules bind to receptors in the form of G protein coupled receptors, which transmit information from the receptors down the axons to the nerve net by intracellular signaling (Taniguchi et al., 2014). G protein coupled receptors (GPCRs) localized to cilia in the "nose" of worms detect environmental stimuli and signal to promote an appropriate behavioral response (Bargmann, 2006). Mutations that disrupt G-protein signaling result in the animals' inability to detect and respond to specific environmental stimuli (Bargmann, 2006).

During chemotaxis, sensory neurons function to detect volatile attractant molecules that allow worms to detect favorable environments. Two neurons documented to sense attractive volatile odorants are the AWA and AWC neurons (Taniguchi et al., 2014). Both AWA and AWC neurons have ciliated ends within amphid sheath cells, meaning the ends are not exposed to the outside of the body (Figure 1) (Bargmann, 1993; Hart and Chao, 2010). The ciliated ends are involved in signal transduction and both ends contain GPCRs that allow for attractive odorant detection (Hart and Chao, 2010). The AWA neuron is responsible for detection of the volatile odorant diacetyl at low concentrations, which is an attractive volatile odorant produced as a metabolic byproduct of *Escherichia coli*, which is what the worms eat in a laboratory setting (Chuang and Collins, 1968). The AWC neuron is responsible for detection of the volatile odorant benzaldehyde (Bargmann, 1993). Within these neurons several genes exist that regulate the functioning of AWA and AWC, and a mutation in just one gene can cause the entire pathway to be defective.

The *odr* genes are a class of genes that, if mis-expressed, can lead to defective phenotypes in the volatile odorant response pathway (Riddle et al., 1997). ODR-10 is a low concentration diacetyl receptor in the AWA neuron that couples to the G-protein α subunit, ODR-3 (Sengupta et al., 1996; Roayaie et al. 1998). The binding of diacetyl causes a signal transduction event that results in the OCR-2 and OSM-9 ion channel opening and neuron firing (Figure 2; Hoffmann et al., 2010). The proper localization of ODR-10 to the sensory cilia is dependent on the protein ODR-4 (Dwyer et al., 1998). The absence of *odr-10* results in the failure of animals to show attractive behavior toward diacetyl (Hart and Chao, 2010). Mutations to the *odr-10* gene effect only chemosensation of diacetyl (Sengupta et al., 1996).

Benzaldehyde is a volatile odorant that requires multiple different pathways to determine a behavioral response. Within the AWC sensory neurons, ODR-3 mutants have been shown to be defective for benzaldehyde sensation (Roayaie et al. 1998; Bargmann et al. 1993). Unfortunately, due to the expression of ODR-3 in several odorant signaling pathways, it is difficult to isolate and attribute the effect to any one odorant pathway (Figure 3) (Hart and Chao, 2010). One protein known to be required for AWC signaling is ODR-1 (Hart and Chao, 2010). ODR-1 is required for olfactory learning and downregulation of chemotaxis processes in the presence of an odorant over a long period of time (Krzyzanowski et al., 2013). Furthermore, benzaldehyde detection is context dependent. For instance, adult animals in starved conditions that are immediately exposed to benzaldehyde have an enhanced adaptation pathway, meaning that they are less inclined to chemotax toward the volatile odorant in chemotaxis assays (Hart and Chao, 2010). The benzaldehyde chemotaxis pathway is not characterized as thoroughly as that of the diacetyl pathway, however, benzaldehyde is becoming more popular as an experimental odorant.

Besides the *odr* genes, there are other classes of genes that, when knocked down, are defective for chemotaxis. For example, one class of molecules known as PUFAs, or polyunsaturated fatty acids, are known to be necessary in the neurons of *C. elegans*, and if the enzymes that metabolize these PUFAs are not present, serious issues in neuronal function can arise (Lesa et al., 2003). PUFA mutants have defective odorant sensing pathways for the AWA neurons, but not for the AWC neurons (Kahn-Kirby et al. 2004). Additionally, GPA proteins are a class of G protein alpha subunits which are involved in creating a stimulatory signal in both the AWA and AWC neurons (Janson et al., 1999; Lans et al., 2004).

There are many neurons that connect to both the AWA and AWC neuron in order to carry signals throughout the worm (Figure 4). The sensory neurons are not connected to a central "brain" as in humans, but they are also not connected directly to motor neurons. There is a complicated pathway of neurons that forms the nerve ring and carries signals from reception to the corresponding destination. In the AWA and AWC neurons, there is a common pathway that spans from the sensory neurons to the motor neurons (Figure 2). This neuron network contains many interneurons, which carry signals between sensory and motor neurons (Tsalik and Hobert, 2003). Figure 2 demonstrates the connectivity of these neurons and shows that there are many neurons active in the volatile attractant olfaction pathway in *C. elegans*.

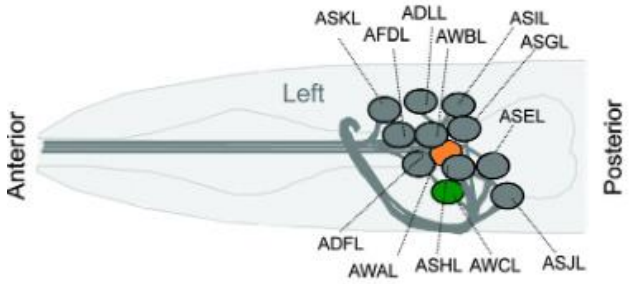

Figure 1. Diagram showing the location of the AWA (orange) and AWC (green) neurons. Adapted from Ortiz et al., 2006.

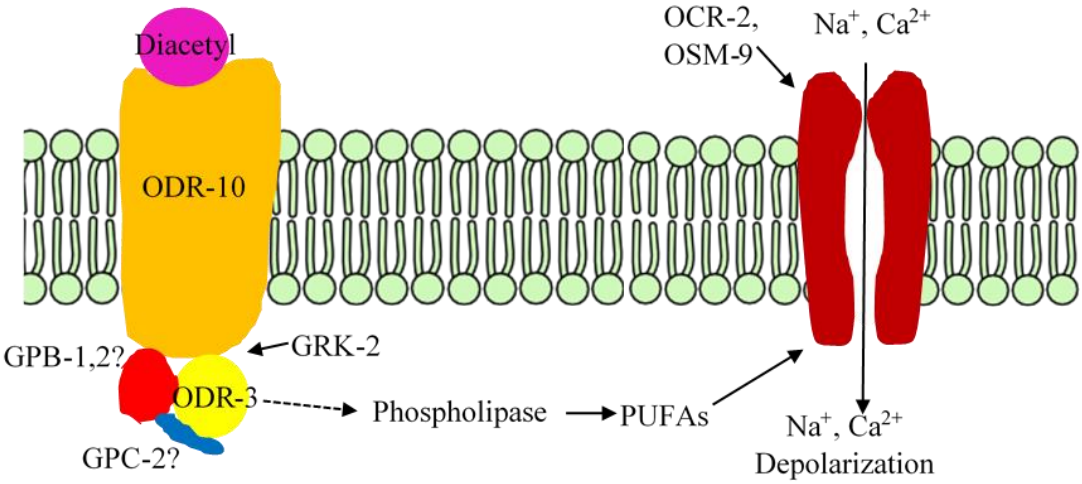

Figure 2. Simplified AWA signaling pathway for diacetyl. Diacetyl binds to ODR-10 and proceeds through the pathway following the arrows in the diagram. Solid arrows represent a known step. Dotted arrows represent that an unknown amount of proteins or other molecules are present between the connected molecules. A question mark after the molecule represents that the position in the diagram is questionable, but the molecule is present somewhere in the pathway. Figure adapted from Hart and Chao, 2010.

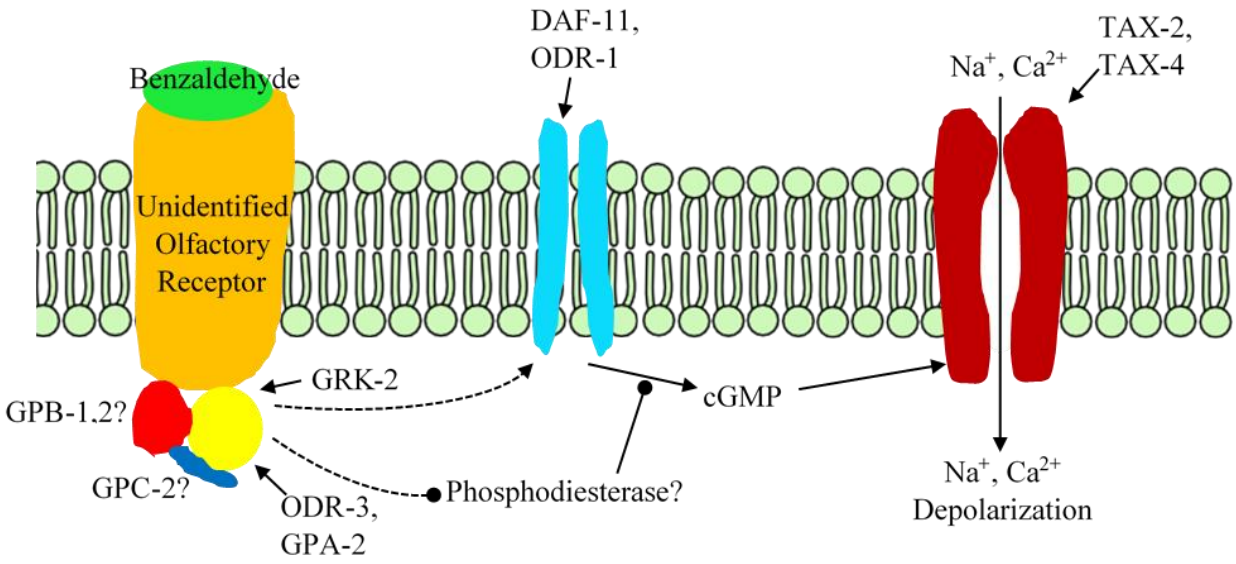

Figure 3. AWC signaling pathway for benzaldehyde. Benzaldehyde binds to an unidentified olfactory receptor. The known pathway is more elaborate than that of the AWA neuron, however, this pathway is the less studied of the two. Solid arrows represent a known step. Dotted arrows represent that an unknown amount of proteins or other molecules are present between the connected molecules. A question mark after the molecule represents that the position in the diagram is questionable, but the molecule is present somewhere in the pathway. Figure adapted from Hart and Chao, 2010.

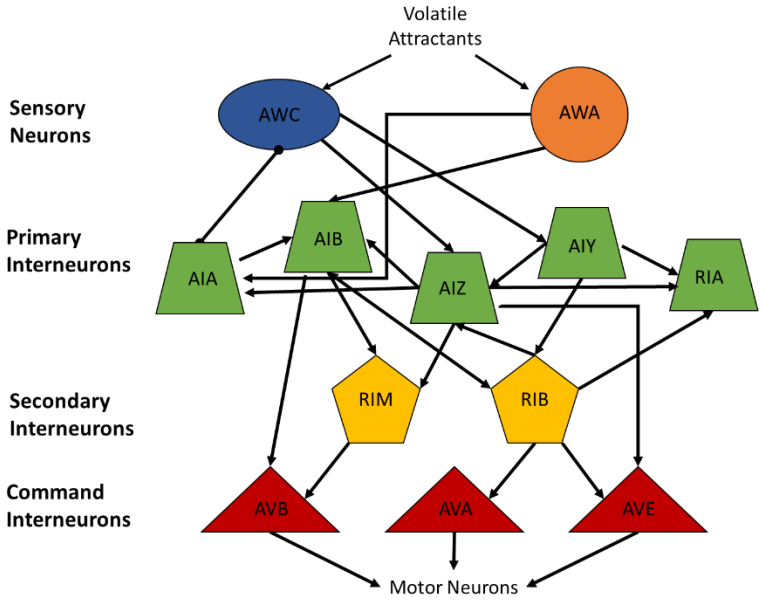

Figure 4. This figure shows a simplified model of how volatile odorant signals are carried from the AWA and AWC neurons through the nervous system to the motor neurons. Adapted from Tsalik and Hobert, 2003.

Regulation of olfaction by RNA interference pathways

Previous work by the Hall lab showed that G protein gene expression in neurons is positively regulated by RNA interference (RNAi) (Bharadwaj and Hall 2017). RNAi is an endogenous method of gene regulation that works by small interfering RNA (siRNA) mediated recruitment of effector complexes to homologous sequences in the cytoplasm or nucleus (Grishok, 2005). The RNAi pathway has been highly studied in *C. elegans*, as the first insight into RNAi was discovered using *C. elegans* to determine that double-stranded RNA (dsRNA) triggers RNAi activity (Fire et al., 1998). This work also showed that dsRNA that is introduced into the *C. elegans* body causes the mRNA with the identical sequence to be destroyed, resulting in gene silencing (Fire et al., 1998). In *C. elegans*, RNAi occurs when a Dicer complex cleaves noncoding double stranded RNA into 26 nucleotide siRNAs (Figure 5). These siRNAs are bound to an argonaute, such as RDE-1. Mutator proteins, such as MUT-7, enter the pathway to promote the amplification of 22G siRNAs in the presence of 22G siRNAs. The 22G siRNAs associate with a worm specific argonaute (WAGO) to regulate target genes (Billi, 2014). Argonautes (AGO) have many functions within gene silencing and one that is important in this context is its ability to act as a guide to the siRNAs (Azlan et al., 2016). The AGO proteins guide the siRNAs through the process of gene regulation, starting from when a protein complex containing AGO cleaves the dsRNA, and ending with recognition of the complementary target gene sequence (Azlan et al., 2016). The dsRNA leaves the nucleus to bind to dicer. This cuts up the dsRNA into 26G siRNAs. These siRNAs interact with MUTator proteins to amplify the process of creating 22G siRNAs, which bind to WAGO proteins. These complexes can then bind to target sequences to either silence or amplify specific gene sequences.

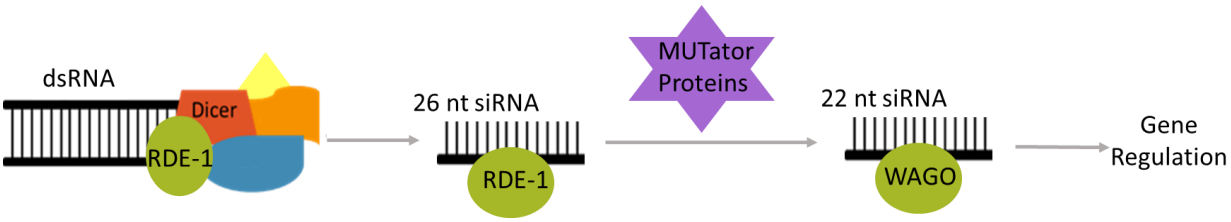

Figure 5. This simplified diagram depicts the RNAi pathway in *C. elegans* chemosensation. "MUTator proteins" includes MUT-7. Figure adapted from Billi, 2014.

RNAi has been shown to be required in odor adaptation and response to unfavorable environmental stimuli. Previous work done in our lab showed that RNAi is necessary in sensory neurons for the detection of unfavorable environmental conditions (Bharadwaj and Hall, 2017). In populations where the RNAi pathway was disrupted, the worms were unable to sense high pheromone, starvation, or high temperature conditions. When the RNAi pathway was rescued in sensory neurons by the reintegration of a mutator protein, the rescue strain was able to sense high pheromone conditions again (Bharadwaj and Hall, 2017). The ability of the sensory neuron rescue strains to have a functioning RNAi pathway indicated that RNAi was required in the sensory neurons to detect negative stimuli. Additionally, RNAi has been linked previously to olfactory adaptive behaviors in *C. elegans* (Juang et al., 2013). Olfactory adaptation occurs when prolonged exposure to a specific odorant causes an individual to temporarily be unable to sense the odorant. This phenomenon has been shown to be regulated by RNAi in the AWC sensory neuron, based on similar null mutant and neuronal rescue experiments as described by Bharadwaj and Hall (2017; Juang et al., 2013). However, the role of RNAi pathways in the detection and response to favorable conditions has not been well characterized.

For my thesis, I hypothesized that RNAi is required to respond to favorable conditions by acting in attractive stimuli sensory neurons to regulate gene expression. We suspect that RNAi mutant animals are unable to sense attractive odorants due to a downregulation of genes in the

olfaction pathway, similar to what was observed for unfavorable stimuli. The goal of this project was to determine whether RNAi is necessary for proper functioning of chemosensation by performing behavioral assays using strains expressing RNAi genes in specific olfactory neurons.

Methods

Strains

Caenorhabditis elegans strains utilized during the experiments within this study are included in Table 1. Strains were maintained in a 15˚C incubator environment on nematode growth media plates seeded with *Escherichia coli* OP50 using standard protocols (Stiernagle et al., 1999).

Strain Name	**Genotype**
N2	Wild type N2 Bristol
WM27	*rde-1(ne219)* V
NL1820	*mut-7(pk720)* III
SH299	*rde-1*; pdrEx77(*odr-10::rde-1::gfp*)#1
SH300	*rde-1*; pdrEx78(*odr-10::rde-1::gfp*)#2
SH297	*mut-7*(*pk710*); pdrEx85(*odr-10*::*mut-7*::gfp; *unc-122p*::dsRed)
SH298	*mut-7*(*pk710*); pdrEx86(*odr-10*::*mut-7*::gfp; *unc-122p*::dsRed)
SH347	*rde-1*(*ne219*); pdrEx103[*Psrd-17*::*rde-1*::*gfp*; *unc-122p*::dsRed]
SH349	*rde-1*(*ne219*); pdrEx105[*Psrd-17*::*rde-1*::*gfp*; *unc-122p*::dsRed]

Table 1. The table above lists the identifiers used to separate the strains as well as the actual full name of each strain.

Chemotaxis Behavioral Assays

The development of strains was coordinated so that all worms being tested would reach day two of adulthood at the same time. Worms were synchronized by picking 20 larval L4 worms from each strain to 10 cm plates. SH299 was picked first because this strain took the longest to develop to adulthood. Next, SH300 and WM27 were picked two days after SH299, and the N2 wildtype strain was picked four days after SH299. Two days after the N2 wild type strain are picked, 10 cm plates were poured with 10 mL of agar. The agar used contained 500 mL milliQ dH_2O, 8 g agar, 2.5 mL 1 M phosphate buffer, 500 uL 1 M $CaCl_2$, and 500 uL 1 M

$MgSO_4$. Approximately 24 hours later when the plates are dried, the chemotaxis assays were performed. For each experiment, two plates were used per odorant for wild type, a null mutant, and the two corresponding rescue strains.

The day of the assay the plates were marked with three sections as shown in figure 6. The three sections were labeled as control, odorant, and center. The odorants diacetyl and benzaldehyde were diluted in ethanol to 1:1000 and 1:200 concentrations, respectively, to reach the concentration necessary for use in the chemotaxis behavioral assays. 1 uL of 1 M sodium azide was placed on the odorant spots in the two side sections of each plate in order to stop the worms from moving once they picked a side to chemotax toward. 1 uL of the odorants was placed on the corresponding odorant mark on the experimental side of the plates, while 1 uL of of 200 proof ethanol was placed on the control side of the plates (Figure 6). Worms were removed from the growth plates using an S-basal (NaCl, K_2HPO_4, KH_2PO_4, cholesterol, and H_2O) wash. Approximately 100-200 worms were placed in the center and each plate, and gently blotted dry with a KimWipe to remove excess liquid. The plates were then left to sit for 75 minutes at room temperature, after which the number of worms in each section was counted. The assays performed using the wild-type, WM27, SH347, and SH349 strains had a slightly modified protocol where dead or unmoving worms from the origin spot were censored from the analysis.

The transgenic worms must be observed using a fluorescent dissecting microscope because not all progeny of the transgenic worms will carry the transgene of interest. For the chemotaxis assays the plates containing transgenic lines need to be counted twice, once with the fluorescent dissecting microscope and once with a regular dissecting microscope.

The formula shown in figure 7 was used to calculate the chemotaxis index (CI), also called the attraction index (AI).

Cloning and Injection Methods

The strains SH347 and SH349 had to be created before they could be utilized in chemotaxis assays. These strains consist of *rde-1(ne219)* worms, or *rde-1* null worms, with the construct *srd-17p*::*rde-1*::*gfp* coinjected with *unc-122p*::dsRed. The transgene construct was made using overlapping PCR according to the manufacturer's instructions using Phusion High Fidelity DNA Polymerase (New England Bio Labs Inc). The PCR product was then gel purified using the E.Z.N.A. Gel Purification Kit (Omega Bio-tek). The gel purified product was cloned using the Zero Blunt TOPO PCR Cloning Kit and then transformed into DH5α *E. coli* competent cells (Thermo Fisher Scientific). After getting colonies on LB + Kan plates, the bacteria were grown, and the DNA plasmids extracted using the E.Z.N.A. plasmid DNA mini kit protocol (Omega Bio-tek). The construct was verified with Sanger sequencing (Genewiz). The plasmid was then mixed with another plasmid containing the marker gene, *unc-122p*::dsRed, and injected into 1 day old hermaphrodites of *rde-1* null mutants (strain WM27). DsRed fluoresces red when observed under a fluorescent dissecting microscope and was used as a coinjection marker to determine whether the construct of interest was present in the worms. Progeny were then screened approximately one week later to determine which were expressing the coinjection marker dsRED gene, and presumably the rescue transgene since they are injected together.

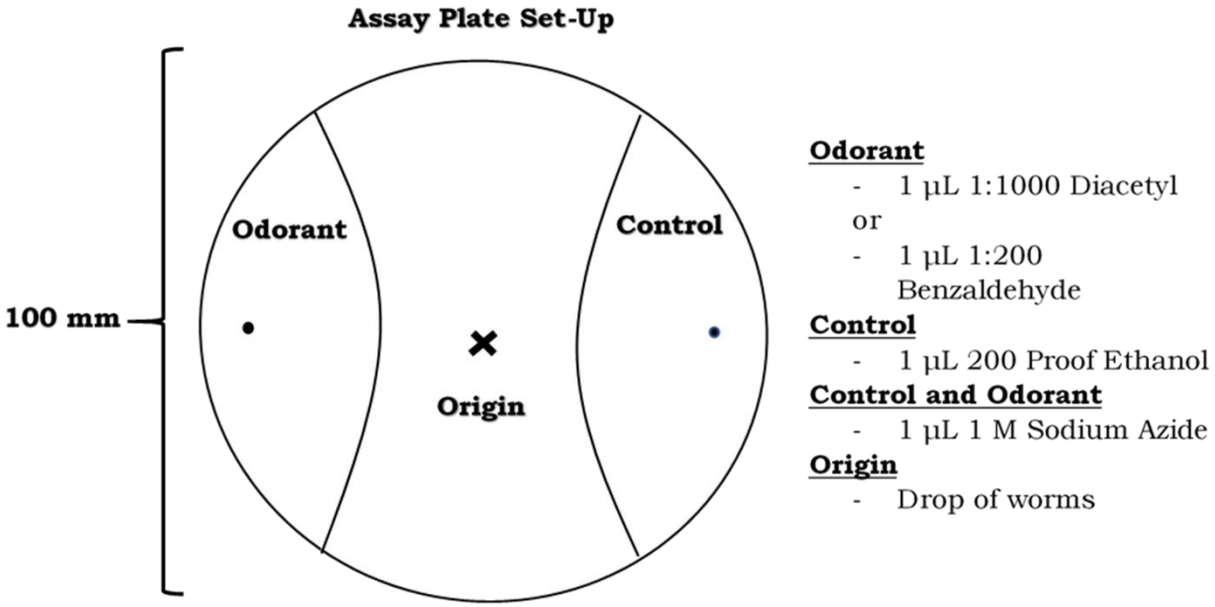

Figure 6. Above is the template for the assay plate set up. Each X marks the exact spot in the experimental and control zones where 1 µL of sodium azide is placed to paralyze the animals when close enough. The control zone gets an additional 1 µL of ethanol while the experimental zone gets an additional 1 µL of either the odorant diacetyl or benzaldehyde (Bargmann et al., 1993).

Chemotaxis Index Equation

$$\text{Chemotaxis Index (C.I.)} = \frac{\text{odorant total \# of animals} - \text{control total \# of animals}}{\text{total \# of animals on plate}}$$

Figure 7. The above equation uses the number of animals in the experimental zone, control zone, and both zones combined to determine the chemotaxis attraction index (Bargmann et al., 1993).

Results

Before any experiments could be performed, rescue strains needed to be created. Both the *mut-7* AWA and *rde-1* AWA rescue strain had been previously made in our lab. In order to make the *rde-1* AWC rescue, we performed overlapping PCR to create *srd-17p::rde-1::gfp* as described in the methods sections. This rescue would allow us to activate the RNAi pathway specifically in the AWC neuron. Once the rescue strain was created, it was dye filled and observed under a compound microscope to see that the GFP was localized to the AWC neuron (data not shown). This result showed that RNAi had been rescued in the neuron of interest.

To test our hypothesis that RNAi is necessary in olfactory neurons for chemosensation, we utilized chemotaxis assays which quantified attraction behaviors of adult worms in the presence of an odorant. This chemotaxis assay had previously been utilized by our lab to determine that RNAi proteins RDE-1 and MUT-7 were required for olfaction behaviors towards attractive stimuli (Hager and Hall unpublished, 2017). However, the requirement for functional RNAi proteins in neurons had not been tested. First, we investigated the role of RNAi in diacetyl attractive behaviors. If worms are chemotaxing toward an odorant, we expect a higher chemotaxis index (CI), as a higher CI indicates that more worms are able to sense and move toward the odorant. We expect a low CI to be indicative of an inability in the worms to sense and chemotax toward the odorant. To verify our assay was working, we tested the ability of N2 wild type to chemotax toward diacetyl and obtained a chemotaxis index, or CI, of 0.65 which is consistent with what has been previously reported (Bargmann et al., 1993).

Next, we tested the chemotaxis behavior of animals mutant for a gene required for the RNAi pathway, either *rde-1* or *mut-7*, which had been previously identified as required for chemotaxis. For the *rde-1* mutant strain we obtained an experimental CI of 0.42, which was

decreased, but not significantly, compared to wild type. Regardless, we tested whether RDE-1 was required in AWA neurons for chemotaxis towards diacetyl. It was expected to see the AWA rescue strains in the presence of diacetyl have CIs similar to those of wild type. The wild type, *rde-1* mutants, and two independent AWA *rde-1* rescue strains did not exhibit significant differences in CI for diacetyl (Figure 8). However, the *rde-1* null strain appeared to be trending toward having a significantly lower CI than that of wild type. Additionally, the rescue strains did not have significantly different CIs compared to the *rde-1* null strain.

Next, we performed assays using two independent lines of the AWC *rde-1* transgenic *C. elegans*, *rde-1* null mutants, and wild type. Since the AWC neuron is not involved in diacetyl chemosensation, the AWC *rde-1* rescue should have displayed a decreased CI similar to that of the *rde-1* mutant strain in the presence of the odorant diacetyl. The AWC *rde-1* rescue strains show a CI that is not significantly different than that of wild type, which is what we expected to see based on our hypothesis (Figure 8). The AWC *rde-1* rescue strains did not exhibit CIs that were significantly different than those of the *rde-1* null mutant strain.

For the *mut-7* strain, we obtained a CI of 0.06 using diacetyl, which was significantly lower than that of the wildtype (Figure 8). Additionally, the *mut-7* AWA rescue strains had a CI that was significantly different than that of the wildtype (Figure 8). This showed that the rescue strains were trending toward a CI that would indicate a partial rescue. Overall, the diacetyl experiments showed that the AWA *mut-7* rescues did not rescue the RNAi phenotype while the AWA and AWC *rde-1* rescues both did not exhibit significant differences in CI when compared to that of the wild type.

Next, we performed the same experiments with the odorant benzaldehyde. Using benzaldehyde as the odorant, we obtained a chemotaxis index of 0.76 for the wild type strain

(Figure 9). Based on previous data obtained in the Hall Lab, we expected the *rde-1* null strain and the *mut-7* null strain to have significantly lower chemotaxis indices than that of the wildtype. We obtained an experimental CI of 0.38 for the *rde-1* null mutant and 0.08 for the *mut-7* null mutant. Both CIs for the null mutants were significantly lower than the corresponding wildtype CI (Figure 9).

In parallel to the *rde-1* null mutant, we tested whether RDE-1 was required specifically in AWA neurons for chemotaxis towards benzaldehyde. We expected to see no significant difference between the AWA *rde-1* rescue lines and the *rde-1* null mutant but expected a significantly lower CI than that of the wild type because benzaldehyde chemosensation takes place in the AWC neuron, not the AWA which is where these rescue strains were expressing RDE-1. The *rde-1* null mutant and the AWA rescued strain all exhibited significantly lower chemotaxis indices than the corresponding wild type (Figure 9). The rescue strains did not show significant differences in chemotaxis index compared to the *rde-1* null strain.

We performed similar benzaldehyde chemotaxis assays using two independent lines of transgenic AWA *mut-7* rescues with the corresponding controls. We expected that the rescue strains in the presence of benzaldehyde would have CIs similar to those of the *mut-7* strain, since AWA is not the sensory neuron that detects benzaldehyde. As expect, the rescue strains did not exhibit significantly different chemotaxis indices when compared to the *mut-7* null mutant strain.

Finally, we performed assays using two independent lines of the *rde-1* AWC rescues, *rde-1* null mutants, and wildtype. We expected to see that the chemotaxis index would be rescued to wild type levels when *rde-1* was expressed in the AWC neuron. According to our results, both rescue strains are significantly different from the wildtype, which is the opposite of what we expected to see (Figure 9). The AWC *rde-1* rescue strains appear to be trending

towards having a CI that is significantly greater than that of the mutant, but the difference is not statistically significant. Overall, the benzaldehyde experiments show that both of the AWA rescue strain types seem to have CIs that are significantly lower than that of the wild type (excluding *mut-7* AWA line 2 which needs more replicates), and the AWC rescue strains also had CIs that were significantly lower than that of wild type. The AWC rescues appeared to trend toward a statistical rescue, and it is possible that a full rescue could be observed with more replicates.

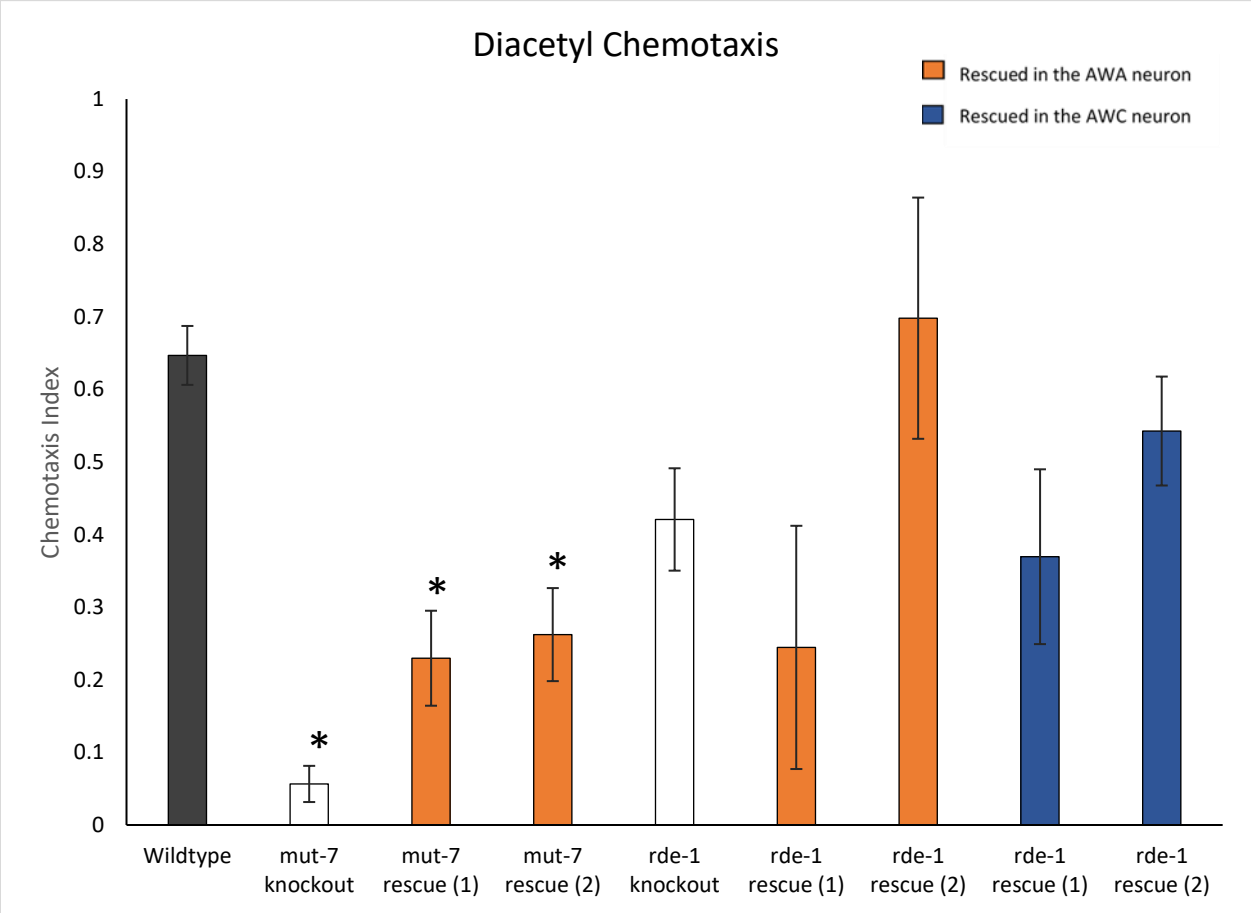

Figure 8. The graph shows the diacetyl attraction index for all tested strains. *Mut-7* null mutants show significant difference from wild type, but r*de-1* null mutants do not show significant difference from wild type. The data were analyzed using an ANOVA with tukey post hoc correction. (* $p \leq 0.05$) where N=14 trials for wildtype and *rde-1* and N=6, N=4, N=4, N=4, N=6, and N=8 for the rescue strains in the order that they are located from left to right on the graph. The average number of worms used per trial for each strain is as follows: 109.857, 84.34, 12.17, 32, 136.67, 120.25, 15.25, 29.14, and 25.875. A total of 7,300 worms were used.

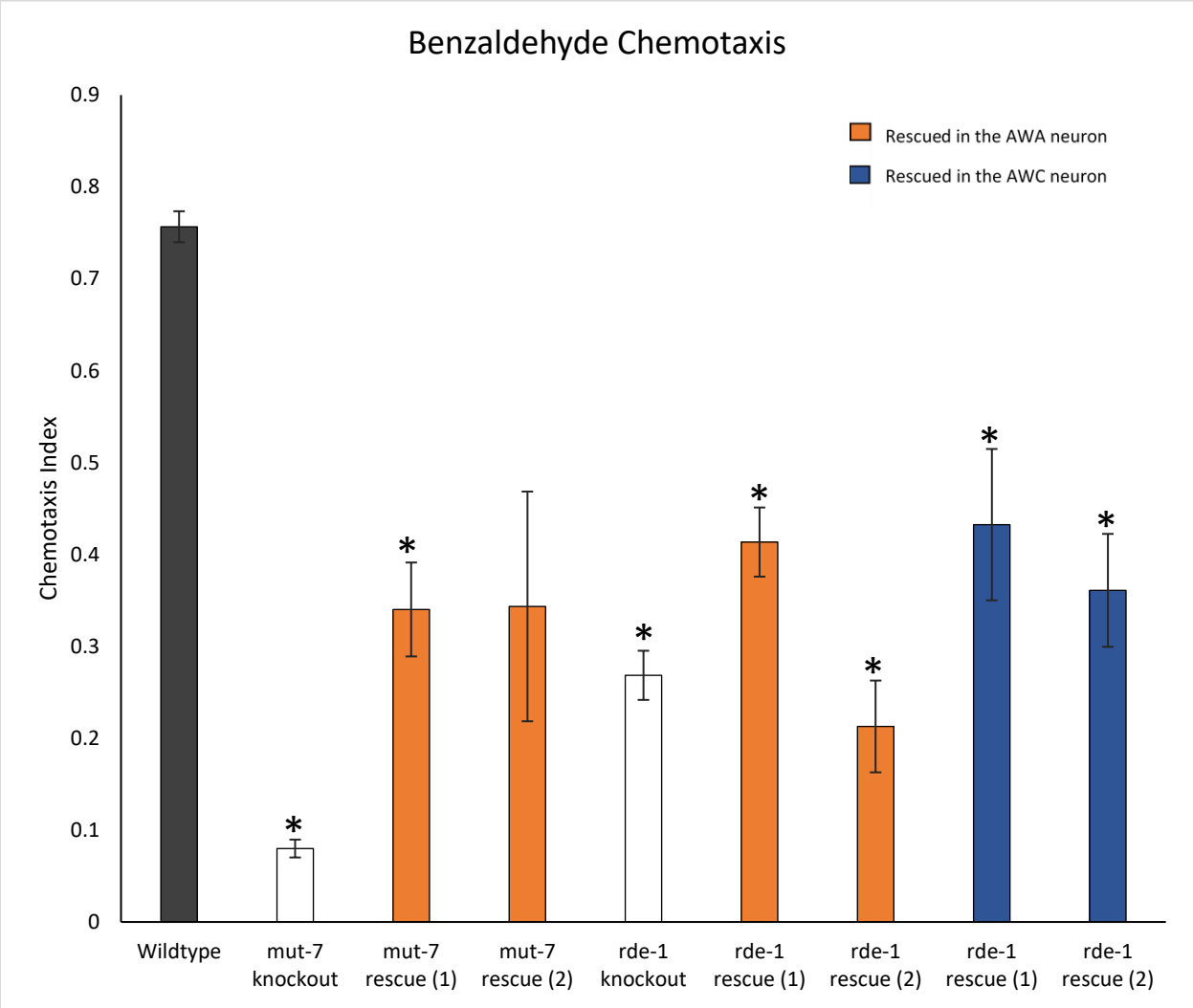

Figure 9. The graph shows the benzaldehyde attraction index for all tested strains. Both *mut-7* null mutants and *rde-1* null mutants show significant difference from wild type. The data were analyzed using an ANOVA with tukey post hoc correction. (* $p \leq 0.05$) where N=28 for wildtype and *rde-1* and N=6, N=4, N=16, N=18, N=6, and N=8 for the rescue strains in the order that they are located from left to right on the graph. The average number of worms used per trial for each strain is as follows: 134.68, 64, 16.34, 27, 29.93, 52.875, 56.278, 22.86, and 25. A total of 15,462 worms were used.

Discussion

Preliminary results from the Hall Lab showed that a subset of RNAi proteins, including RDE-1 and MUT-7, are required for attractive chemotaxis toward diacetyl and benzaldehyde in adult animals (Hager and Hall). Thus, we postulated that the MUT-7 and RDE-1 proteins are required in AWA and AWC sensory neurons for worms to respond to the attractants diacetyl and benzaldehyde. My results showed that RNAi deficient mutants were not able to sense the volatile odorants diacetyl or benzaldehyde when comparing the CIs of the strains to the wild type. Additionally, isolating RNAi function to either the AWA or AWC neuron did not produce the same odorant sensing phenotype that we observed in wild type.

Throughout all experiments performed, we observed that the neuron specific rescue lines failed to exhibit significantly different chemotaxis behaviors compared to the corresponding null mutants. Since we know that the wildtype transgenes were expressing in the correct neurons, we believe that there are other places in the volatile odorant sensation pathway where RNAi could be functioning. It is possible that RNAi functions in an interneuron downstream of the primary detection neuron (Figure 2), which would explain why rescuing the RNAi pathway in the AWA or AWC neurons did not rescue the behavior. Diacetyl and benzaldehyde are first detected in the AWA and AWC neurons, but there are a variety of other neurons that carry the detection signal before the signal reaches a motor neuron. AWA and AWC send signals first to primary interneurons, then to secondary interneurons, then to command interneurons, which finally carry the signal to the motor neurons (Figure 2; Tsalik and Hobert, 2003). There are several interneurons at each stage that could receive or send the signals, and these interneurons rely heavily on each other. Because of the interconnectedness of the olfactory pathway, there are

many places were RNAi could be required either in addition to or in place of the RNAi located in the sensory neurons.

It does not appear that RNAi is required in the two individual neurons tested based solely on the assay data; however, we recommend that two pan neuronal rescues be created, for both *rde-1* and *mut-7*. It is possible that the RNAi pathway is operating downstream of the initial responding neuron. The pan neuronal rescues would allow us to see whether or not either *rde-1* or *mut-7* are acting in any neuron as part of the chemosensation pathway. If it is possible to rescue gene function using the pan neuronal rescues, that means RNAi functions in chemosensation and chemotaxis, just not where it was initially thought to function. This result would help us to determine the neuron where RNAi is required for chemosensation, RNAi is required in any neurons for attractive chemosensation.

The results presented in this thesis are different than what our lab has previously found regarding RNAi and environmental cues. Bharadwaj and Hall found that RNAi was required in sensory neurons to detect unfavorable environmental conditions (2017). Our results suggest that while RNA is required specifically in sensory neurons to detect unfavorable conditions, RNAi is required in other, non-sensory, neurons in order to detect favorable environmental conditions. It is possible that RNAi is required in the sensory neurons as well as other interneurons of the olfaction pathway, or that RNAi is required only in another, untested interneuron or interneuron type in order to complete the olfaction pathway.

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