Drew University

College of Liberal Arts

Worms on the Brain:

The Role of Autism-Related Genes *nlg-1* and *nrx-1*

in Thermotactic and Chemotactic Behavior of *C. elegans*

A Thesis in Biochemistry and Molecular Biology

by

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Abstract

Neuroligin (NLG) and neurexin (NRX) are molecules involved in synaptic function. Previous studies have reported that *Caenorhabditis elegans* (*C. elegans*) neurexin directs neuronal connectivity through action at synapses with partnering neurons and muscles. This study employs *C. elegans* as a model system to study behavioral defects associated with mutations in the neuroligin and neurexin genes, which in humans have been found to be linked to various autism spectrum disorders (ASDs). Carrying a single neuroligin and neurexin gene (*nlg-1* and *nrx-1* respectively), *C. elegans* can be mutated in such genes and the resulting phenotypic responses can be observed. The focus of this study is to observe the thermotactic and chemotactic behavior of *nlg-* and *nrx-*disrupted strains of *C. elegans* versus the wildtype N2 strain in order to obtain insight into the implications of these mutations and the role that these genes play in neurodevelopment. Using adapted thermotaxis and chemotaxis assays, wildtype N2 and mutants VC228 (*nlg-1*, ok259), SG1(*nrx-1*, dx1), and MLB1316 (*nlg-1*, ok259; *nrx-1*, dx1) were observed for thermotactic and chemotactic behavioral differences. There was found to be a significant difference between the thermotactic behavior of both single and double mutant *nlg-1* and *nrx-1* worms compared to that of wildtype N2 worms ($P<0.05$). Additionally, there appeared to be a difference in thermosensory ability between worms raised at 16°C and those raised at 25°C. All control and mutant strains were repelled by the chemorepellent 1-octanol, but *nrx-1* mutants were notably less repelled than other strains. The conclusions of this study allow increased understanding of the neurodevelopmental processes occurring within *C. elegans*, implying that thermosensory

and chemosensory behavior are neuronally connected. Our findings also support *C. elegans* as a good model to study neurodevelopment and human neurological disorders like ASD.

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Introduction

C. elegans as a model system for human diseases

Many research endeavors aim to understand the mechanisms and moving parts of human diseases. In 1963, Sydney Brenner proposed using the free-living, transparent roundworm *C. elegans* as a model for studying human diseases (Brenner 1973). These worms are naturally found in rotting vegetation, feeding on bacteria. With a lifespan of 14 days and a life cycle of 3 days, these 1mm long nematodes are easy to cultivate and use in research. Life cycle stages are shown in Figure 1. As the first eukaryote to have its genome completely sequenced, *C. elegans* have 959 somatic cells, 6393 chemical synapses, 302 neurons, and 8 dopamine neurons. Additionally, because of their transparency, internal proteins and neurons can be tagged with fluorescent proteins like Green Fluorescent Protein (GFP) to visualize expression in vivo, making them useful research organisms. Recent research has utilized these worms in numerous ways.

A study by Benbow et al. used *C. elegans* as a model of Alzheimer's disease. The researchers were able to generate a transgenic worm model expressing both human Aβ1-42 peptide and human tau protein. They observed behavioral dysfunction and age-dependent neurodegenerative changes in the Aβ:tau transgenic animals (Benbow et al. 2020). Another study by Cho et al. utilized *C. elegans* in a biotechnological microfluidic platform. The researchers developed a device that can deliver spatially and temporally controlled multi-modal stimuli to *C. elegans* to study multisensory integration in the brain (Cho et al. 2020). Additionally, the worms are being used in the lab to study Parkinson's disease (PD). In addition to studying worm homologues of human genes, *C.*

elegans can express human PD-related genes that result in age-dependent degeneration of dopaminergic neurons (Cooper et al. 2018). Researchers can track this degeneration by tagging the dopamine neurons with green fluorescent protein.

As 60-80% of *C. elegans* genes have human counterparts and 42% of human disease genes have *C. elegans* counterparts, we can easily translate findings from experiments completed with this model (Brenner 1973). In this study we utilize *C. elegans* to uncover the importance of NLG and NRX, two genes that have often been linked to human ASD.

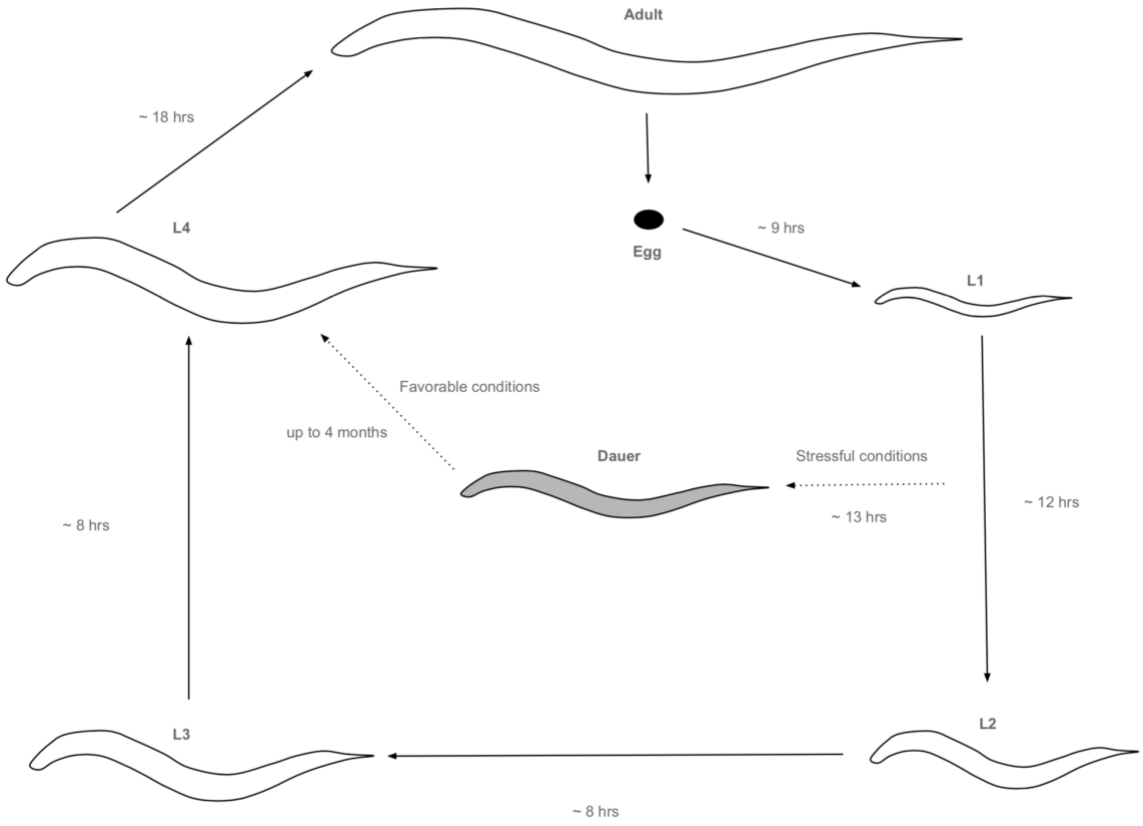


Figure 1. *C. elegans* life cycle chart adapted from WormAtlas and Bustos and Partridge 2017. Adult worms form and lay eggs that proceed through a cycle of larval stages to adulthood. Under stressful conditions, *C. elegans* have the ability to enter into a dauer stage during which a dauer-specific cuticle forms around the worm. Temperature of cultivation can speed up or slow down the larval development process. The behavioral assays in this study raised worms to larval stage 4 or adulthood to observe consistent results.

ASD as a Pervasive Developmental Disorder

Pervasive developmental disorders (PDDs) refer to a group of disorders characterized by delays in the development of socialization and communication skills. Autism spectrum disorders (ASDs) are grouped into this category (Muhle et al 2004). ASDs comprise classical idiopathic autism, Asperger's syndrome, Rett syndrome, and PDDs not otherwise specified. In these disorders, mental retardation (~70% of cases) and epilepsy (~30% of cases) are frequently observed. This data has led to speculation that autism may be caused by an imbalance of excitatory vs. inhibitory synaptic transmission (Südhof 2008). ASDs have also been characterized by aberrant neurodevelopment. This abnormal development results in early overgrowth followed by abnormally slowed growth during childhood and adolescence, a period in which brain growth in non-ASD subjects increases. The childhood and adolescence period is a critical stage of development. Individuals with ASD may fail to interact with the environment during this period, prompting selective synapse elimination during a time when normal synaptogenesis should be occurring rapidly (Cheng et al. 2011).

Most PDD subtypes, including autism, are not linked to a particular genetic or non-genetic cause. Many studies have determined that there are likely multiple genetic factors interacting to form the main causes of autism (Muhle et al 2004, Südhof 2008). The exact identity and number of genes involved in autism are still being studied, although there exist distinct genes and gene combinations conserved among those affected that have been discovered. The interaction of multiple genes within an individual's genome is reflected by the wide phenotypic variability of ASDs and the

spectrum they exist upon. It has been suggested that epidemiologic factors such as toxic exposures, teratogens, and prenatal infections account for a small number of cases of autism. For the majority of cases, data from whole-genome screens has suggested interactions of at least 10 genes in the causation of autism (Muhle et al 2004). Recent studies have identified mutations in the genes encoding neurexins and neuroligins as a cause for ASDs (Südhof 2008).

In 2014, the Centers for Disease Control and Prevention issued an autism prevalence report. The prevalence of autism has risen to 1 in every 59 births in the US, twice as great as the 2004 rate of 1 in 125. As noted by the CDC, autism is the fastest-growing developmental disability in the US (CDC 2014). Due to such effects, ASD is of interest to many researchers not in the hopes of finding a "cure," but in the thought of learning more about the general pathology of the disorder. The research in this study focuses on specific mutations in the neuroligin and neurexin genes that have been linked in humans to various ASDs. Using *C. elegans* as a model system to study behavioral defects associated with these mutations, we hope to advance what is currently known in the literature about the nature of ASDs and how neurodevelopment plays a role. Even with a nervous system composed of only 302 neurons, *C. elegans* can perceive a number of environmental stimuli, in the form of chemicals, light, or temperature, producing specific behavioral outputs by utilizing different sets of behavioral strategies (Kimata et al 2012). Thermotaxis and chemotaxis assays have been widely used in *C. elegans* research to observe such outputs. In this study, we are utilizing such assays to look at the function of NLG and NRX specifically.

Neuroligins and Neurexins in ASD

Mutations in the human neuroligin and neurexin genes have been associated with synaptic dysfunction and cognitive disease (Daoud et al. 2009; Lawson-Yuen et al. 2008; Sudhof 2008; Tabuchi et al. 2007; Ylisaukko-oja et al. 2005; Zhang et al. 2009). In humans, there are three neurexin genes and five neuroligin genes. All mammals express five neuroligin genes, with neuroligin 3 and 4 localized to the human X-chromosome, and a neuroligin 5 gene on the Y-chromosome that is complementary to neuroligin 4. Two X-chromosomal neuroligin genes in particular, *NLG3* and *NLG4*, were shown to be mutated in patients with ASDs. Interestingly enough, ASD is seen to be more prevalent in males. Two males with autism in a Swedish family had an insertion in *NLG4*, which led to premature truncation of the protein D396X. In another Swedish family with autism, an R451C substitution in *NLG3* was identified (Jamain et al. 2003 and Ylisaukko-oja et al. 2005). In a study done by Ylisaukko-oja et al., the protein coding sequence and splice sites of *NLG1, NLG3, NLG4, and NLG4Y* genes of a sample of 30 males and females with ASD was analyzed by direct sequencing. Finding no extreme alterations of protein structure, the authors labelled these mutations as silent, concluding that there were no obvious functional mutations identified in any of the genes in question (Ylisaukko-oja 2005). However, further studies have looked more into the connection of the *NLG4* and X-linked neuroligin 4 (*NLG4X*) genes specifically to ASDs. Daoud et al. found that a mutation in *NLG4X* increases gene expression and is most likely caused by altered binding of transcription factors in the 1 base pair (G335A) substitution mutated promoter sequence. Another study by Lawson-Yuen et al. examined mutations in the *NLG3* and

NLG4 genes. The researchers found that these specific genes have been reported in families with members having mental retardation and/or pervasive developmental disorders ranging across the spectrum. The study also identified a family with a novel *NLG4* gene deletion encompassing exons 4, 5, and 6. They predicted this mutation to result in a significantly truncated protein. The family in the study showed a wide spectrum of different neuropsychiatric illnesses associated with the same *NLG4* mutation (Lawson-Yuen et al. 2008). Family members within the study showed varied phenotypes, all associated with the same mutation. This suggests that epigenetic factors play a role in determining disease presentation. It might also suggest that mutations affecting synapse function may be associated with multiple neurological conditions (Lawson-Yuen 2008). Similarly, two brothers with classical ASD were described in a study by Zhang et al. in which the *NLG4* gene contained a single amino-acid substitution (R87W). It was concluded that the substitution inactivated the synapse formation activity of *NLG4* and subsequently decreased synapse strength. These observations suggest that just a point mutation in *NLG4* can cause ASD by a loss-of-function mechanism (Zhang 2009). Such conclusions point out the interest and importance in experimenting with NLG in terms of ASD and neurodevelopment and function. NRX has yet to be studied in this capacity, but as it has proven to be an important synaptic partner, we decided to investigate it further alongside NLG.

Neuroligin, Neurexin, and Synaptic Function

All information processed in the brain involves synapses and abnormalities in neurological function affect these synapses. Defined as specialized intercellular junctions dedicated to transfer information from a neuron to a target cell, synapses can transmit information quickly and efficiently. As such, synaptic function is tightly regulated (Craig and Kang 2007). In general, synapse formation and specification involves three distinct steps:

1. Recognition of the target cell by the neural growth cone
2. Formation of synaptic junctions and recruitment of synaptic components
3. Maturation of synaptic junctions and circuit specification

Synapse formation and synaptic diversity are intricately linked and depend on the actions of synaptic cell-adhesion molecules. In neural circuit function, the input and output depend on both the circuit's synaptic connectivity and the properties of individual synapses in the circuit pattern. Neurexins and neuroligins are two well-studied synaptic cell-adhesion molecules. These two molecules are not only required for synapse function, but their dysfunction impairs the properties of synapses and disrupts neural networks. (Südhof 2008). The molecules exist in numerous isoforms, which are mRNAs that are produced from the same locus but differ in structure and function. While neuroligins are associated with postsynaptic function, neurexins, grouped into α-neurexins and β-neurexins, are linked to presynaptic function.

Neuroligins, similar to neurexins, are type-I membrane proteins. However, neuroligins have a much simpler domain and are less structurally diverse. Previous data suggest that the binding of these two synaptic partner molecules depends on numerous factors, such as which isoforms are expressed and which splice variants are being used (Südhof 2008).

Neuroligin and neurexin in other animal models

Previous literature has examined the neuroligin and neurexin genes and gene mutations in animal models. Through analysis of neuroligin knockout (KO) mice, a study by Südhof suggests that neuroligins function in the maturation of synaptic junctions, but not in the initial recognition and formation of synaptic junctions. In both KO neuroligin and KO α-neurexin mice, synapse number and ultrastructure is relatively normal. However, in both strains, synapse function is severely impaired. These findings characterize neuroligins and neurexins as synaptic cell-adhesion molecules essential for proper assembly of synapses, but not necessarily essential for the initial formation of synapses (Südhof 2008).

Another study by Tabuchi et al. introduced the R451C substitution (arginine 451 to cysteine) in the neuroligin 3 gene (*NLG3*) in mice (Tabuchi et al. 2007). This mutation has been previously linked to autism spectrum disorders (ASDs) in humans (R451C) (Comoletti et al. 2004). The mutant mice showed impaired social interactions but enhanced spatial learning abilities. These observed behavioral changes were accompanied by an increase in inhibitory synaptic transmission, but had no effect on excitatory

synapses. Deletion of *NLG3* did not act in the same way, indicating that the R451C amino acid substitution represents a gain-of-function mutation. These data suggest that increased inhibitory synaptic transmission may contribute to human ASDs and that the R451C KI mice may be a useful model for studying neurological behaviors. However, another more simplistic animal model has also proven useful in the exploration of neuroligin and neurexin mutations in the context of neurodevelopment and ASDs.

Neuroligins and Neurexins in C. elegans

The nematode *C. elegans* has singular homologs of the neuroligin and neurexin genes, *nlg-1* and *nrx-1* respectively. Previous studies have reported that in C. elegans, the neurexin gene directs neuronal connectivity through action at synapses with partnering neurons and muscles. Many of these studies support neuroligin as a primary trans-synaptic binding partner with neurexin (Philbrook et al. 2018; Boucard et al. 2005; Comoletti et al. 2006; Ichtchenko et al. 1995; Ichtchenko et al. 1996; Nguyen and Südhof 1997). It has been found that *nlg-1* and *nrx-1* mediate a retrograde synaptic signal that inhibits neurotransmitter release in neuromuscular junctions (Hu et al. 2012). These junctions are the areas of contact between a motor neuron and a muscle fiber where a muscle contraction can be caused when the neuron transmits a signal to the fiber. In *C. elegans* lacking the *nlg-1* and *nrx-1* genes, this retrograde signalling was blocked. In addition, these mutations were associated with prolonged acetylcholine release. In a study done by Hu et al., *nlg-1* and *nrx-1* mutant *C. elegans* (KP5330 *nlg-1*(ok259); SG1 *nrx-1*(ds1)) were analyzed for altered synapse development. This study's results support a

mechanism in which mutations in NLG and NRX prolong post-synaptic responses. Mutations inactivating *C. elegans nlg-1* and *nrx-1* were all associated with prolonged ACh release. Mutations in the corresponding human genes are all linked to ASD (Hu et al. 2012).

A study by Calahorro and Ruiz-Rubio has shown that *nlg-1* and *nrx-1* mutant-specific effects in *C. elegans* are due to impaired acetylcholine and/or GABA inputs (Calahorro and Ruiz-Rubio 2012). Although these mutants have been observed to be phenotypically similar to wildtype N2, they display a variety of more subtle phenotypes that have potential to be studied in relation to autism spectrum disorders. A deficit in chemosensation of 1-octanol and a lack of thermal response are two behavioral abnormalities. Other deficits include a hyporeversal phenotype, increased sensitivity to oxidative stress, decreased lifespan, and defective basal and enhanced slowing response (Schmeisser and Parker 2017). Perturbations of synapse development and function followed by altered cognition have been shown to be a hallmark of ASDs, and genetic mutations in neuroligin and neurexin as discussed above have often been shown to be partially responsible (Glessner et al 2009; Schmeisser and Parker 2017; Sudhof 2008).

Neurodevelopment of C. elegans

The simplicity and consistency of structure of the *C. elegans* nervous system makes it an excellent model to study the complexity of the nervous system. The cell divisions taking place during the organism's development are highly reproducible. This has allowed the nematode's complete cell lineage to be determined from fertilized zygote

to mature adult. While the human nervous system contains 10^{10} neurons in 5 classes, *C. elegans* have 302 neurons in 118 different classes, providing extreme variety (White 1986). The bulk of the *C. elegans* nervous system is situated in the head and around the pharynx, making the upper portion of the worm richly endowed with sensory receptors and neurons (Figure 2). The neurons of the nervous system of *C. elegans* have simple, unbranched morphologies. Most neurons have only a single process, and chemical synapses in *C. elegans* occur *en passant*, in which synaptic axon terminals are formed along the axon shaft between neighbouring parallel processes (White 1986). Synaptogenesis is a process involving the formation of a neurotransmitter release site in the presynaptic neuron, a receptive field at the postsynaptic neuron, and the precise alignment of pre- and post-synaptic junctions.

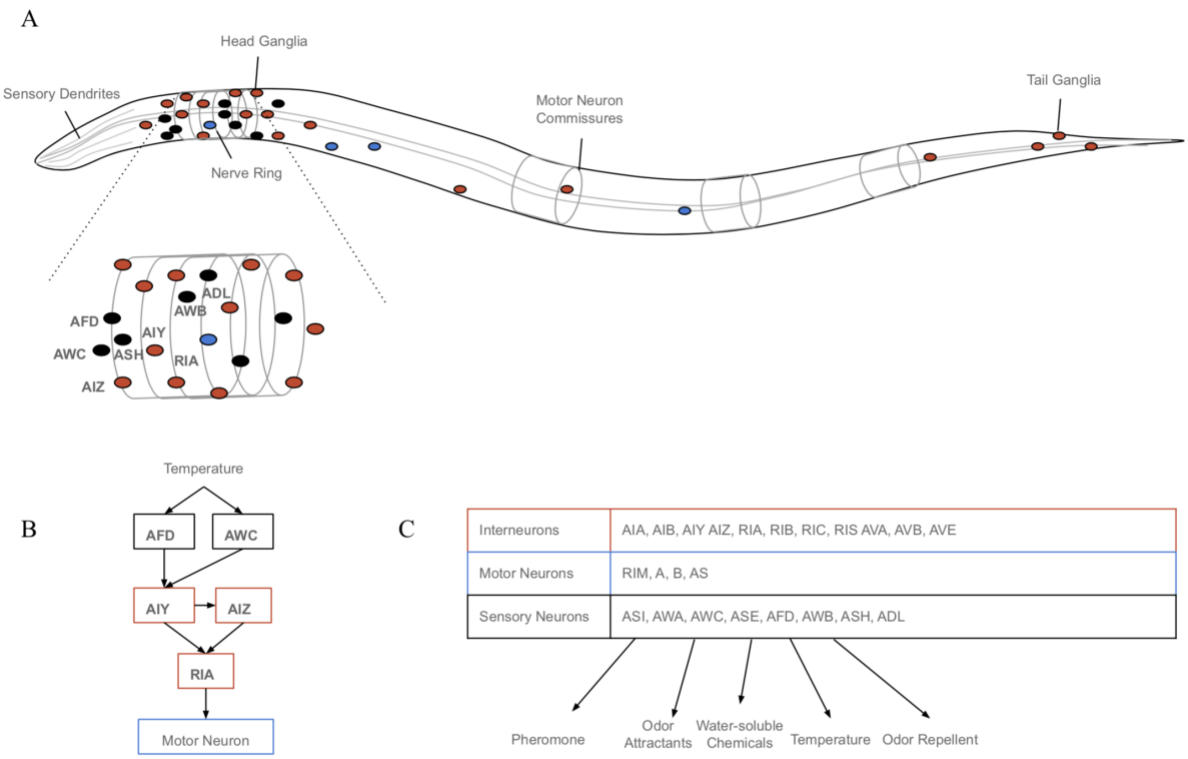


Figure 2. Notable neurons in *C. elegans* adapted from Aoki and Mori 2015, Bargmann 2006, Mori and Ohshima 1995, and WormBook. (A) Map of *C. elegans* neuronal system with close-up of nerve ring depicting specific neurons involved in thermotactic behavior. (B) Proposed mechanism of neuronal action upon thermal stimulation. (C) Table of notable inter- motor and sensory neurons of *C. elegans*.

Each nerve process makes a variety of synapses (White et al., 1986). Designated synaptic partners often make contact through fasciculation for a long distance, but form synapses only at specific sites (Jin 2005). While neuroligins are known to trigger presynaptic differentiation and promote glutamatergic synaptic function, neurexins

trigger postsynaptic differentiation and promote GABAergic synaptic function (Craig 2007). There have been numerous behavioral assays detailed in the literature that have been used to further investigate the effects of mutations in NLG and NRX in *C. elegans*.

Chemotaxis

In *C. elegans*, chemosensory and thermosensory responses share neuronal connections (Kimata et al 2012). *C. elegans* use chemosensation for many life processes including finding food, avoiding harmful toxins, and mating. The worms sense chemicals with chemosensory neurons that penetrate the cuticle to expose their sensory cilia to the environment: the amphid in the head, phasmid in the tail, and inner labial neurons (Bargmann 2006; Ward et al. 1975; Ware et al. 1975; see Figure 2). There are 32 chemosensory neurons that are either directly or indirectly exposed to the environment through openings generated by glial cells. An additional pair of neurons in the amphid (AFD) is thermosensory and is not exposed.

Previous literature has shown that *nlg-1* mutants have specific deficits in chemosensation and the processing of chemosensory cues (Hunter et al 2010). In the study completed by Hunter et al., *nlg-1* mutant worms (*ok259*) showed a specific lack of response to 1-octanol. These worms responded similarly to wildtype in their response to the attractant diacetyl and even repellants cupric acetate and nonanone. The repulsive odor 1-octanol is detected by a combination of ASH, ADL, and AWB neurons.

The study by Bargmann also notes the act of imprinting. Imprinting is a specialized learning process in which early developmental experiences affect preferences

of mature animals. Olfactory (chemoreception that forms the sense of smell) imprinting is best known in salmon, which follow cues to return to its river of origin for spawning. *C. elegans* also exhibits a form of olfactory imprinting. Exposure of young animals to benzaldehyde or other odors sensed by AWC results in enhanced chemotaxis to the odor in adults (Bargmann 2006; Remy and Hobert 2005). It is possible that this imprinting also occurs within and/or influences the thermosensory behavior of *C. elegans*.

Thermotaxis and Thermal Stimuli

The thermotaxis assay stands as a model to elucidate how nervous systems sense and memorize environmental conditions to regulate behavioral strategies in *C. elegans*. It represents a relatively simple way to compare the behavior of a wildtype versus mutant strain. Thermotactic behavior was first reported in 1975 by Hedgecock and Russel who reported that the worms have the ability to remember the temperature at which they were cultivated (Tc), and migrate toward it when exposed to a temperature gradient (Kimata et al 2012). This specific behavior could be considered a form of imprinting.

A study completed by Hedgecock and Russel in 1975 indicated that *nlg-1* mutant *C. elegans* lack a thermal response. Such research inspired the following study by Hunter et al. examining the thermotactic behavior of *nlg-1* disrupted worm strain (ok259). The researchers in this study also noted a difference between the mutant strain and the wildtype N2 strain. In the project conducted by Hunter, experimental set-up was inspired by conditions seen in the Hedgecock and Russel study. Thermal response assays were performed after establishing a thermal gradient on an unseeded 100-mm plate by placing

a vial of frozen glacial acetic acid (16.7°C) in the center of an inverted plate in a 25°C incubator. Approximately 50 worms were transferred to the thermal gradient plate and allowed to move freely for 30 minutes; their positions were then scored on an overlay of concentric circles demarking eight equal areas (Hunter et al. 2010). A similar thermal response assay was conducted by Ramot et al. in which a thermal gradient was generated across an aluminum plate by two thermoelectric Peletier devices on either end. The agar plate on which the worms were put was placed on top of this established aluminum gradient plate (Ramot et al. 2008). While the Ramot study focused on the mechanism and robustness of the thermotaxis assay, the scientists in the Hunter study found that *nlg-1* mutants did not accumulate at a specific temperature, but instead moved independently of temperature. It was also concluded that this behavior was independent of the temperature at which the animals were grown or their feeding state and that a normal thermal response was restored by transgenic expression of an NLG1 fusion protein. Hunter et al. thus concluded that *nlg-1* mutants were either unable to sense temperature, or were indifferent to changes in ambient temperature (Hunter et al. 2010).

Interestingly, thermal perceptual deficits have been observed in ASD patients as well as mutant *C. elegans* (Duerden et al. 2015). A study by Duerden et al. examined 20 high-functioning adolescents with ASD and 55 typically developing adolescents on their thermal and pain sensitivity thresholds. Adolescents with ASD were less sensitive to warm and cool stimuli compared to those without ASD. Warm temperature detection thresholds were higher and cool detection temperature thresholds lower in participants with ASD. A study by Glauser et al. overlayed a thermal sensitivity assay and a chemical

sensitivity assay, exposing *C. elegans* to both a temperature gradient as well as a chemoattractant. This study found that heat-induced repulsion overcame chemical attraction, as it was observed that heat-induced repulsion was observed with and without the addition of the chemoattractant (Glauser et al. 2011).

Our study focused on the thermosensory and chemosensory behavior of *C. elegans*. Based on conclusions made in previous studies, we hypothesized that worms with mutations in neuroligin and neurexin genes would behave differently from wildtype in both thermosensory and chemosensory abilities. Observing the behaviors of *nlg-1* and *nrx-1* single mutant strains VC228 and SG1 respectively, and double mutant strain MLB1316, we were able to compare them to the wildtype N2 strain in both migration toward cultivation temperature when placed on a gradient and repulsion from 1-octanol. As these responses are maintained by various sets of neurons and synaptic connections, results found in this study can be used to gain further knowledge of the pathway of ASD and other neurological disorders.

Methods

General worm husbandry

Standard laboratory methods *for C. elegans* were described by Sydney Brenner (Brenner 1974). *C. elegans* were grown on NGM agar plates seeded with bacterial strain OP50. A group of worms were grown at 25℃, another group at 16℃, and a third group at room temperature, 20℃, until they reached L4 stage.

M9 Buffer

A buffer to transfer the worms was made up of KH_2PO_4 (3g), Na_2HPO_4 (6g), NaCl (5g), $MgSO_4$ (1M, 1mL), and water to 1L. This mixture was autoclaved for 15 minutes and allowed to cool before using.

Larval stage synchronization

Worms were synchronized following a protocol adapted from Cold Spring Harbor Laboratory's Synchronization via Bleaching (Egg Prep) protocol (Cold Spring Harbor Laboratories 2007). In this protocol, a healthy plate of multiple generations of worms was washed off with M9 buffer and scraped to include the eggs sticking to the agar. The worms were then transferred into a 15mL conical tube and pelleted. The supernatant M9 was removed and a 20% alkaline hypochlorite solution (4.0mL non-germicidal bleach, 4.75mL 1M NaOH, 6.25mL ddH_2O) was added. The tube was rocked on a rocking tube shaker until worm bodies began to break down, then centrifuged at max speed (4000 rpm) for 1 minute. The supernatant was removed without disturbing the pellet, and roughly 7mL of M9 added, agitated, and spun again. The washing steps were repeated at least one more time before letting the tube rock overnight for the eggs to hatch. The next day, worms were plated onto round agar plates at the L1 stage at either 25℃, 16℃, or 20℃. This respective temperature serves as the temperature of cultivation (Tc).

C. elegans strains

A total of six strains of worms were used in this study, obtained from the *C. elegans* Genetic Stock Center (CGC) at the University of Minnesota in Minneapolis, MN. The N2 strain was used as wildtype, IK589 (*ttx-7*, nj50) as an athermotactic control, IK130 (*pkc-1*, nj3) as thermophilic control, OH8 (*ttx-3*, mg158) as cryophilic control, VC228 (*nlg-1*, ok259) as a neuroligin mutant, and SG1(*nrx-1*, dx1) as a neurexin mutant. The MLB1316 double mutant strain (*nlg-1*, ok259; *nrx-1*, dx1) was created in the lab by mating the VC228 and SG1 strains.

Generating double mutants

N2 males were obtained from the *C. elegans* Genetic Stock Center (CGC) at the University of Minnesota. Five N2 males were picked onto a small agar mating plate with one VC228 hermaphrodite to generate VC228 males. Two subsequent mating plates were set up with 5 VC228 males and 1 SG1 hermaphrodite. Seven offspring from each of these matings were plated out on separate agar plates. Progeny was collected from these seven plates, specific *nrx* and *nlg* genomic regions were amplified by PCR and analyzed by agarose gel electrophoresis. Progeny yielding mutant PCR bands for both *nlg* and *nrx* were collected. Forty of these worms were individually plated out and the PCR process was repeated. Four of these worms gave a homozygous wildtype *nrx* and heterozygous mutant *nlg* banding pattern. The worms from those four plates were allowed to self-fertilize expecting that 1/4 would be double mutants, 1/2 would be homozygous wildtype *nrx* and heterozygous mutant *nlg*, and 1/4 would be homozygous mutant *nrx* and

homozygous wildtype *nlg*. Ten individual worms were picked and let to self-produce on a new plate. The progeny were once again pooled and analyzed by PCR and gel electrophoresis.

Genomic DNA Preparation

DNA isolation for all of the strains followed the same protocol. Worms were washed with M9 (1.5mL) from NGM 1% agarose plates seeded with OP50, then centrifuged at full speed for 1 minute to allow a pellet to form. The supernatant was aspirated and the washing and centrifugation steps were repeated. The resulting pellet was flash frozen in liquid nitrogen. Five volumes of worm genomic DNA lysis buffer (100mM Tris, 50mM EDTA, 200mM Nacl, 0.5% SDS, 0.1 mg/mL proteinase K) was added and the tube was incubated at 65℃ for 1-2 hours. A second incubation occurred at 95℃ for 20-30 minutes to deactivate the proteinase K. RNAse A (0.1mg/mL) was added and the tube was incubated at 37℃ for 1 hour. To collect, purify, and elude the DNA, the Zymo quick DNA Microprep Kit was used (catalog # D3020 & D3021). The protocol for solid tissue samples was followed and the product concentration was measured on a Thermo Scientific Nanodrop.

PCR and agarose gel electrophoresis

Specific *nlg* and *nrx* mutations of the VC228 and SG1 strains were analyzed by amplification of specific genomic regions, followed by sequencing of the purified PCR product. The same steps were followed for the wildtype N2 strain, and mutant sequences

were compared. The following PCR master mix was conserved throughout all strains. The volume of gDNA added might have fluctuated based on concentration after elution.

NEB HotStart PCR MasterMix	**25uL**
5uM Forward Primer	**5uL**
5uM Reverse Primer	**5uL**
gDNA	**1uL**
Nuclease-free Water	**14uL**

PCR primers were designed using Primer-BLAST tool by NCBI (Primer3) and the appropriate Tm was calculated by NEB Tm Calculator (v 1.12.0). Below are the respective primer sequences and Tm used for the PCR reaction of *nlg-1* mutant VC228 and *nrx-1* mutant SG1.

nlg-1

F: 5’ GTTCCCACGTTTTTGTCGGG 3’ *Tm*: 68℃

R: 3’ ACACGTGCCTGTTCCTGAAA 5’

nrx-1

F: 5’ GTATCCAAAATGGGCGCACTC 3’ *Tm*: 58℃

R: 3’ CTGGCAGTCACAATGTGGTC 5’

To check DNA fragment lengths for each strain, agarose gel electrophoresis was set up using 1X TAE and agarose to make a 1% gel. Mixtures containing PCR sample (5uL), TE (5uL), and loading dye (NEB, 6X) (5uL) were loaded into the wells. The gel was run for ~1.5 hours at 80V and was imaged directly after. The 1kb Plus DNA Ladder from New England Biolabs was used for a marker (gel loading dye, purple 6X, 1,000 ug/mL).

Strain Sequencing

Genomic DNA from N2 and SG1 was isolated and used as template for PCR to amplify the deleted regions of *nrx-1* and the same was done with N2 and VC228 for *nlg-1*. The PCR products were then sequenced using the Sanger chain termination procedure. The strains were sequenced using a SeqStudio Genetic Analyzer from Applied Biosystems by Thermo Fisher Scientific (serial # 232001046). The sequencing reaction and purification protocol from the *BigDye ™ Terminator v3 Cycle Sequencing Kit User Guide* was followed. The analyzed and raw sequences for mutant strains SG1 and VC228 are depicted in the results (Figure 7).

Thermotaxis assay

NGM lite agar was made according to an adapted recipe containing Sigma-Aldrich Bacteriological agar (9.6g in 600 mL water). The agar mixture was autoclaved for 30 minutes, and cooled. $CaCl_2$ (1M, 0.6mL), $MgSO_4$ (1M, 0.6mL), and KPO_4 (1M, 3mL) was added. This was poured (60mL each) into rectangular petri plates from Fisher

Scientific (catalog # 267060). Two vertical lines were drawn in black permanent marker on the back plastic of each plate to mark the middle of the plate (Figure 4). The plate was placed on a temperature gradient, described below. L4 worms were washed off of their respective NGM agar plates using a pasteur pipet with M9 buffer (1.5mL). The worms were transferred to a microcentrifuge tube and washed twice with M9 (1.5mL). The worms were then transferred using a pasteur pipet to the middle of the thermotaxis plate and blotted dry with a Kimwipe. The plate was then put back into the Mastercycler gradient Thermal Cycler PCR machine programmed with a temperature gradient based on Tc. For a Tc of 25℃, the gradient spanned from 19℃ to 25℃. For a Tc of 16℃, the gradient spanned from 16℃ to 22℃. The temperature of the machine and the agar plate was confirmed with an AstroAI digital laser infrared thermometer, 380 non-contact temperature gun with range of -50℃ ~ 380℃. After 30 minutes, the plate was examined under a Leica Zoom 2000 microscope. The number of worms on the plate were counted and their positions were recorded.

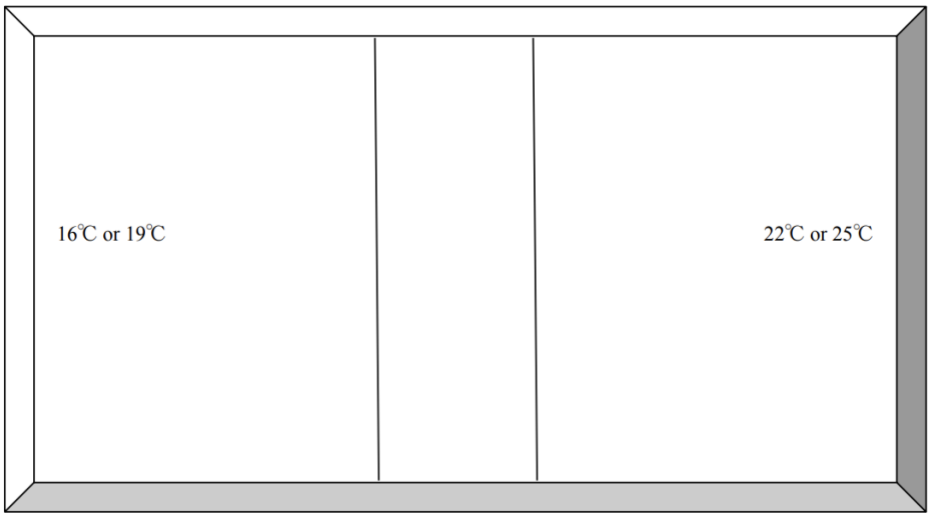


Figure 4. Thermotaxis plate diagram. Temperature gradient dependent on Tc.

Chemotaxis assay

NGM lite agar was made and poured into round 16cm petri dishes. A line was drawn down the middle of the back of the plate in black permanent marker, with a 5cm circle in the center (Figure 5). L4 worms were washed off of their respective NGM agar plates with M9 buffer (1.5mL) and transferred to a microcentrifuge tube. After washing twice with M9 (1.5mL each wash), worms were transferred to the center circle of the plate using a pasteur pipet and blotted dry with a Kimwipe. Ethanol (2uL, 100%) was added to one side of the plate and 1-octanol (2uL, 1%) was added to the other. After 30 minutes, the plate was examined and the number of worms on the plate were counted and their positions recorded.

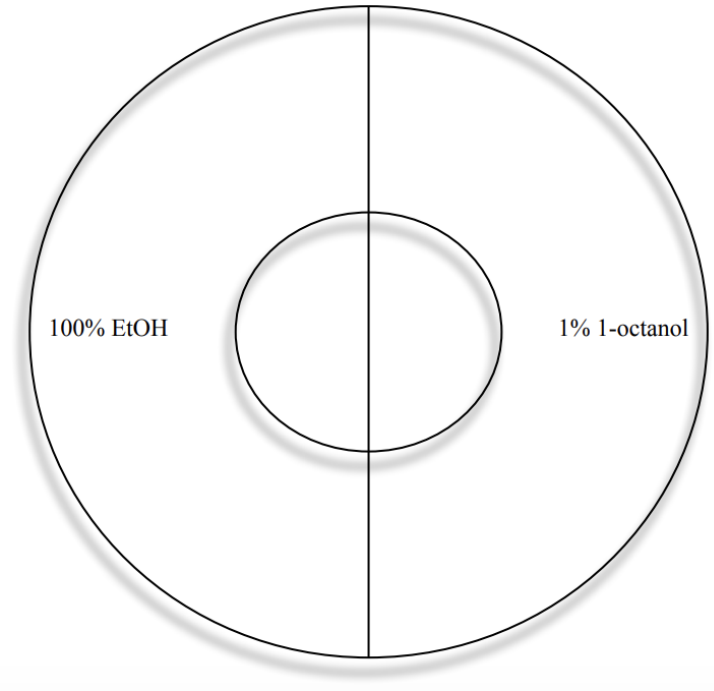


Figure 5. Chemotaxis plate diagram. Test side contains 2uL 1% 1-octanol and control side contains 2uL 100% ethanol.

Calculating Thermotactic and Chemotactic Indices

The thermotactic index (TI) was calculated for each worm strain during each thermotaxis assay. Using the raw counts of worms in the left, middle, and right sections of the plate after 30 minutes, the index was calculated by

$$TI = \frac{(Tc - \text{other temperature})}{(Tc + \text{other temperature})}$$

Thermotactic averages for each strain at both cultivation temperatures were taken and SD between assay repetitions was recorded (Table 1A, B). Similarly, chemotaxis data

was recorded by counting worms on the test (1% 1-octanol) side of the agar plate and on the control (100% ethanol) side. The chemotactic index (CI) was calculated by

$$CI = \frac{(Test - control)}{(Test + control)}$$

Chemotactic averages were taken for each strain as well as SD between assay repetitions (Table 1C, D).

Results

Molecular characterization of mutant strains

Through thermotaxis and chemotaxis behavioral assays, we were able to characterize neuroligin and neurexin mutant *C. elegans* strains and compare them to the wildtype. The resulting thermotactic and chemotactic indices collected reveal trends similar to those previously seen in the literature by Bargmann and Hunter et al. Distinct and significant differences were seen between our control strains, the wildtype strain, and our single and double mutants.

Genetic sequencing of *nlg-1* mutant VC228 revealed that compared to N2, the mutant strain has a 2,341 base pair (bp) deletion including exons 8-13 and a 334 bp insertion, together forming a 2,007bp overall deletion (Figure 6B). This specific strain has been previously reported as a functional null mutant with defects in synaptic transmission (Rawsthorne et al. 2020). The *nrx-1* mutant SG1 was also confirmed via

genetic sequencing by a member of the lab, Stephanie Wang. The regions of deletion were located by PCR from strain-specific genomic DNA. These PCR products were then sequenced to show specific deletions. The sequences of VC228 and SG1 deletions were subsequently blasted against N2 genomic DNA to identify the size of the deletions and determine the exact exons and amino acids removed. SG1 was found to have a 1,498 bp deletion with exons 3-6 deleted and 135 amino acids out of frame (Figure 6A). The mutant sequences were compared to the known wildtype N2 sequence (WormBase Strain00000001) that has 16 exons and 845 amino acids. Portions of the analyzed and raw sequences for mutant strains SG1 and VC228 are depicted below (Figure 7).

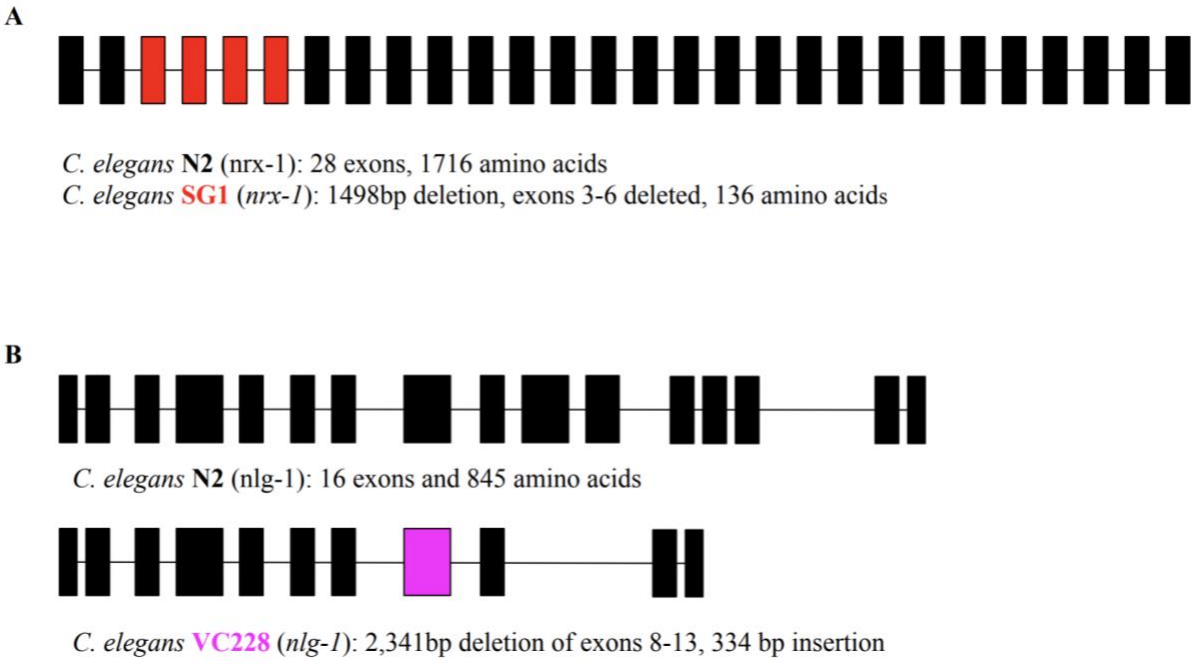
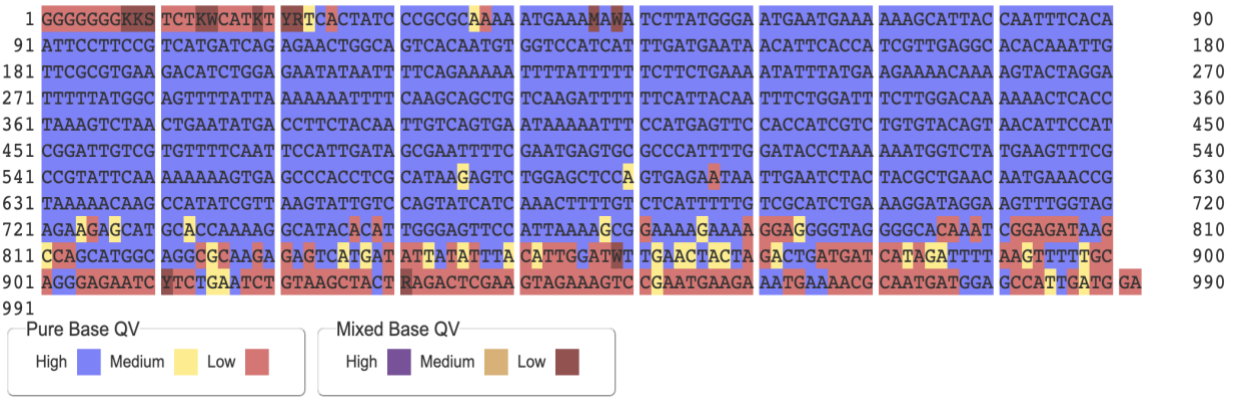


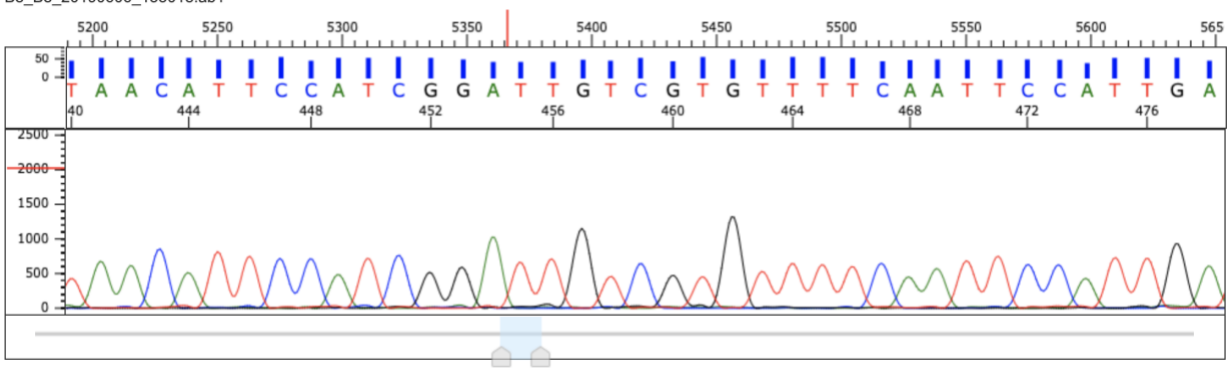
Figure 6. Visual representation of *nlg-1* and *nrx-1* mutations compared to wildtype. (A) N2 and SG1 exon map. (B) *C. elegans* nlg-1 gene compared to the mutant form found in the VC228 strain.

SG1

B8_B8_20190606_155018.ab1

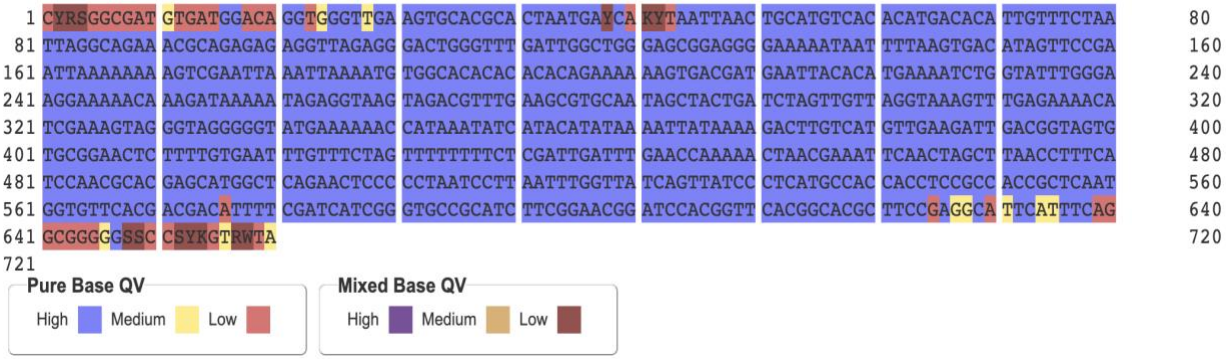


B8_B8_20190606_155018.ab1



VC228

E3_E3_20191016_101544.ab1



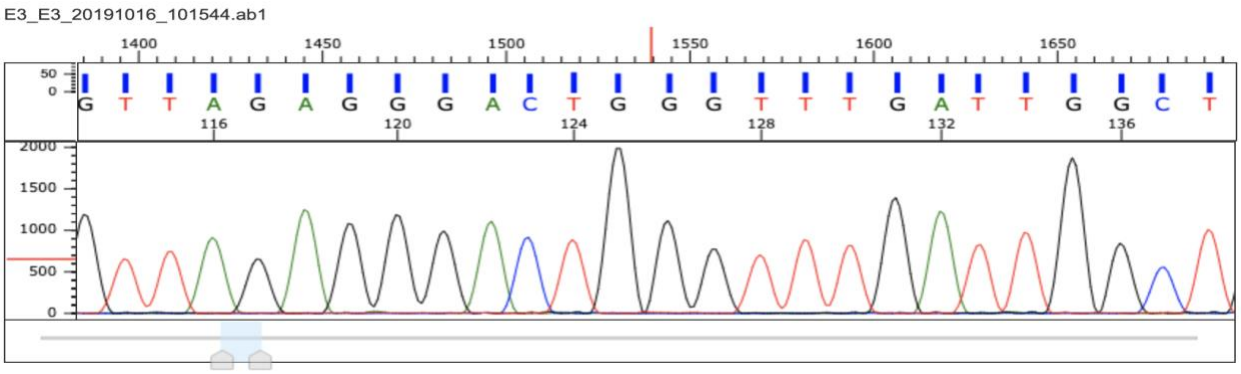


Figure 7 . Sanger sequencing reaction results of mutant *nlg-1* (VC228) and *nrx-1* (SG1) strains. Raw sequence is shown on top with a portion of the analyzed sequence on the bottom.

The double mutant strain, MLB1316, was also analyzed against the wildtype and single mutants. Genomic DNA was isolated from all strains and amplification of the genes in question was completed by PCR. When comparing the single and double mutant strains to the wildtype on agarose gel electrophoresis, the genetic difference was apparent. The *nrx-1* mutant strain SG1 shows a NLG band (2,786 bp) matching up with that of wildtype N2 and a mutant *nrx-1* band (402 bp). The *nlg-1* mutant strain VC228 shows a NRX band (1900 bp) identical to that of N2 and a mutant *nlg-1* band (779 bp). Additionally, two clones (MLB1316:4 and MLB1316:7) of the double mutant strain show mutant *nlg-1* and *nrx-1* bands that match up with those of VC228 and SG1 (Figure 8). These results confirm that single mutants VC228 and SG1 along with double mutant MLB1316 had the expected sequence deletions.

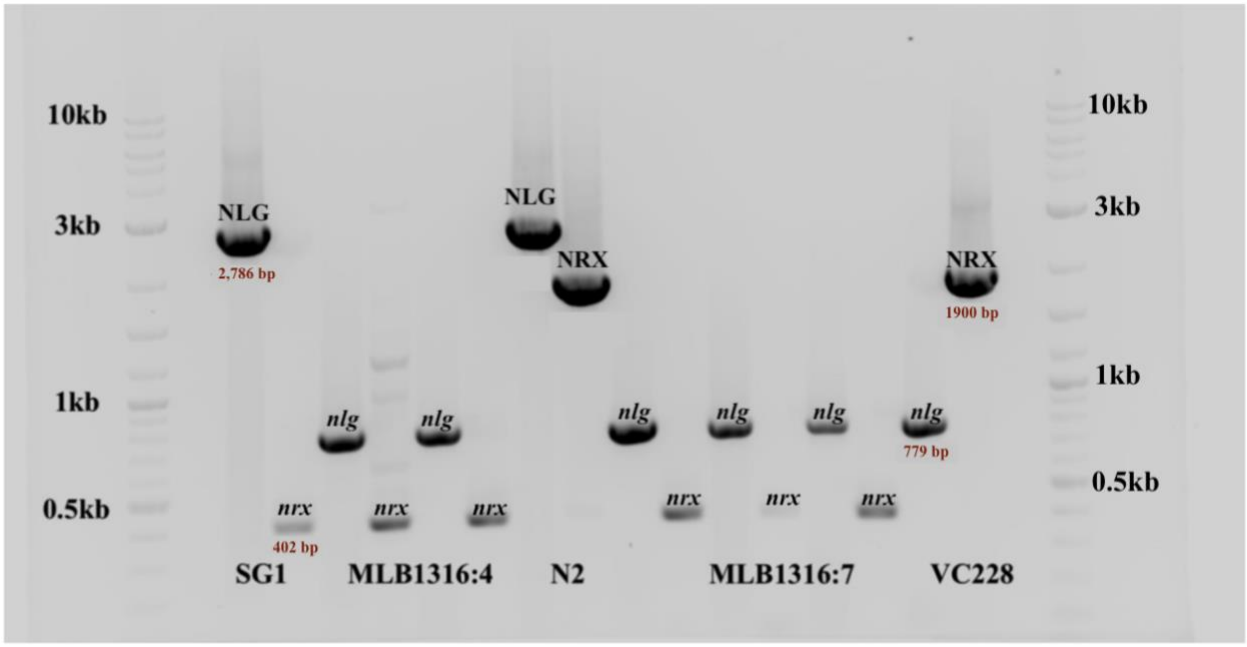


Figure 8. NLG and NRX PCR product bands of SG1, MLB1316 clone 4, N2, MLB1316 clone 7, and VC228. NLG bands of each strain are shown on the left with NRX bands on the right. Base pair length of wildtype and mutant PCR products are shown in red.

Thermotaxis data

There was found to be a significant difference between the thermotactic behavior of both single and double mutant *nlg-1* and *nrx-1* worms compared to that of wildtype N2 worms. Additionally there appeared to be a difference in thermosensory ability between worms raised at 16°C and those raised at 25°C (Figure 9). Average thermotactic indices were calculated for each strain and each temperature (Table 1 A,B). Although there existed some variance between the thermotactic behavior of worms raised at 16°C and worms raised at 25°C, both wildtype N2 (TI= 0.9034 at Tc 25°C, 0.9445 at Tc 16°C), *nlg-1* mutant VC228 (TI= 0.2034 at Tc 25°C, 0.8108 at Tc 16°C), and *nrx-1* mutant SG-1

(TI= 0.6364 at Tc 25°C, 0.8971 at Tc 16°C) all moved in the direction of their Tc when placed on a gradient. This is concluded by a positive TI value. However, worms raised at 25°C did seem to move less toward their Tc than those raised at 16°C. This can be seen by a TI closer to 0 than to 1, as a TI of 1 signifies that all worms moved toward their Tc. *Nlg-1* mutant VC228 worms raised at 25°C in particular had a notably low TI (0.2034) but also had a very high standard deviation between assay repetitions (0.6400). Athermotactic control strain IK589 acted indifferent to the temperature gradient, as raised at 25°C (TI= -0.1766) these worms migrated more toward a temperature opposite their Tc, and at 16°C (TI= 0.1744), they moved more toward their Tc. Thermophilic control strain IK130 moved toward higher temperatures no matter the Tc (TI= 0.7908 at Tc 25°C, -0.4592 at Tc 16°C), and cryophilic control strain OH8 moved toward lower temperatures (TI= -0.9045 at Tc 25°C, 0.8810 at Tc 16°C). Interestingly, the double mutant strain MLB1316 showed almost opposite results. Raised at 16°C, the strain did not move toward its Tc with a very low TI of 0.0893. Conversely, raised at 25°C, the strain had a TI of 0.5490, indicating that more worms moved toward the Tc, although the large number of worms that did not move from the starting point at all complicates interpretation of this result (Table 1B). Most of the double mutant strain also seemed to remain in the middle of the assay plate, around 19°C or 22°C, after the 30 minutes (Table 1B,D). This likely has to do with the varying number of worms added to the middle of plate for each assay repetition.

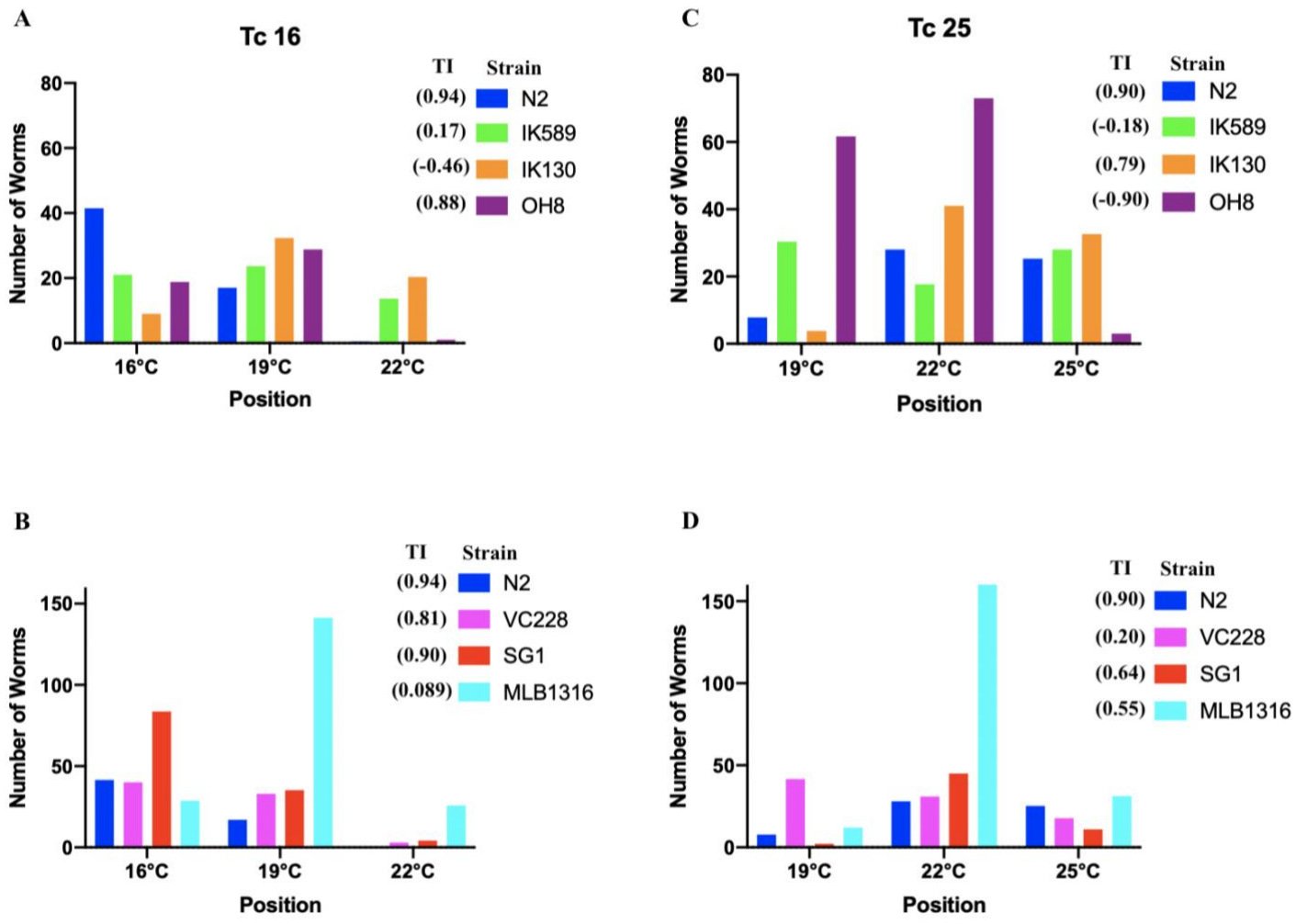


Figure 9. Thermotaxis assay positions of *C. elegans* control and mutant strains after 30 minutes. Cultivation temperatures 16°C (Tc16) in A-B and 25°C (Tc25) in C-D are shown. Average TI is shown in parentheses next to strain name. (A) Positions of control strains after 30 minutes on a 16°C-22°C temperature gradient. (B) Positions of mutant strains compared to wildtype N2 after 30 minutes on a 16°C-22°C temperature gradient. (C) Positions of control strains after 30 minutes on a 19°C-25°C temperature gradient.

(D) Positions of mutant strains compared to wildtype N2 after 30 minutes on a 19°C-25°C temperature gradient.

Chemotaxis data

All control and mutant strains were repelled by the chemorepellent 1-octanol, as depicted by a negative CI value, but *nrx-1* mutants were notably less repelled than other strains (CI= -0.1105). Wildtype N2 worms were more repelled by 1% 1-octanol (CI= -0.6085) than the athermotactic strain IK589 (CI= -0.1702) and the *nrx-1* mutant strain SG1 (CI= -0.1105) (Table 1C, D). The *nlg-1* mutant strain VC228 was only slightly less repelled by 1-octanol compared to N2 (CI= -0.5500). The double mutant strain MLB1316 was also almost as repelled as wildtype and VC228 (CI= -0.4044).

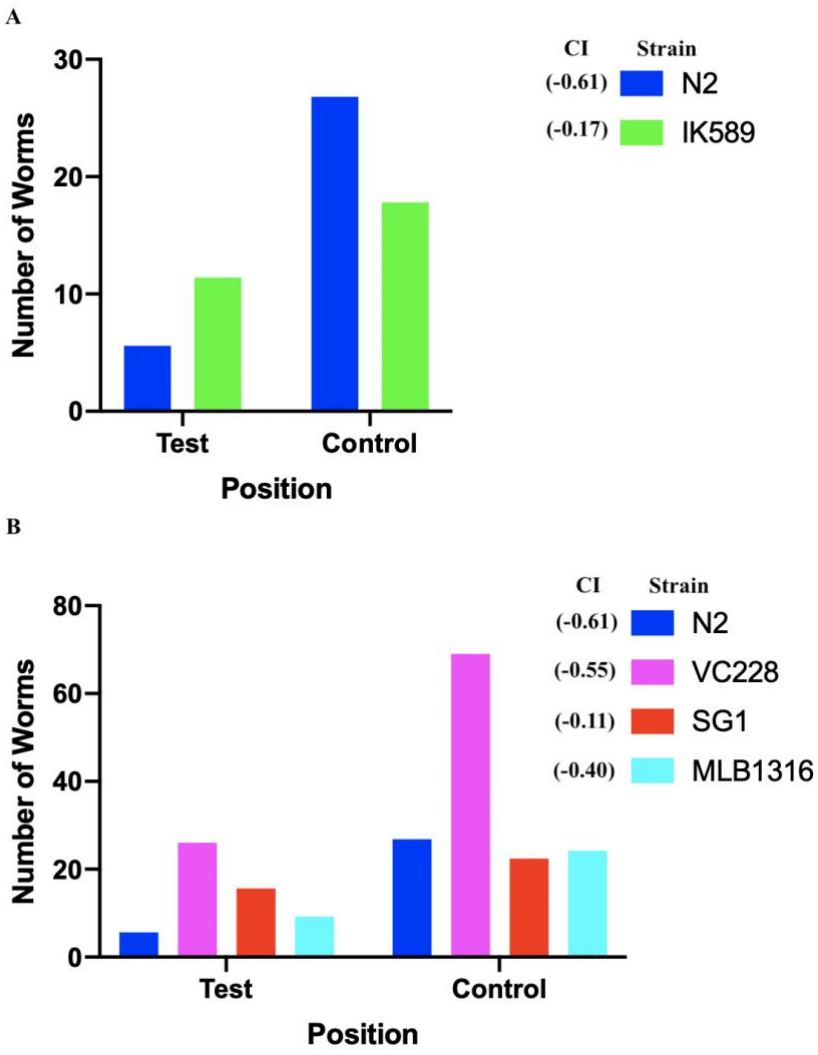


Figure 10. Chemotaxis assay positions of *C. elegans* control and mutant strains after 30 minutes exposed to 1-octanol. Test population represents worms that moved towards 1-octanol, the control population represents worms that moved towards ethanol. Average CI is shown in parentheses next to strain name. (A) Positions of control strains. (B) Positions of mutant strains compared to wildtype N2.

Table 1. Average thermotactic and chemotactic indices and SD between repetitions.

A

Strain	Tc	Average TI	SD	n
N2	25	0.9034	0.0505	6
N2	16	0.9445	0.0766	5
IK589	25	-0.1766	0.2431	3
IK589	16	0.1744	0.3580	3
IK130	25	0.7908	0.1847	5
IK130	16	-0.4592	0.4616	3
OH8	25	-0.9045	0.0891	6
OH8	16	0.8810	0.2062	4

B

Strain	Tc	Average TI	SD	n
VC228	25	0.2034	0.6400	5
VC228	16	0.8108	0.1596	4
SG1	25	0.6364	0.6298	5
SG1	16	0.8971	0.0903	5
MLB1316	25	0.5490	0.3317	4
MLB1316	16	0.0893	0.0578	4

C

Strain	Average CI	SD	n
N2	-0.6085	0.1739	5
IK589	-0.1702	0.1920	5

D

Strain	Average CI	SD	n
VC228	-0.5500	0.2674	5
SG1	-0.1105	0.4953	5
MLB1316	-0.4044	0.1142	4

Number of assay repetitions is noted by column "n." (A) Average thermotactic indices for control strains: N2=wildtype, IK159=athermotactic control, IK130=thermophilic control, OH8=cryophilic control. (B) Average thermotactic indices for mutant strains: VC228=*nlg-1* mutant, SG1=*nrx-1* mutant, MLB1316=*nlg-1/nrx-1* double mutant. (C) Average chemotactic indices for control strains: N2=wildtype, IK589=1-octanol indifferent control. (D) Average chemotactic indices for mutant strains: VC228=*nlg-1* mutant, SG1=*nrx-1* mutant, MLB1316=*nlg-1/nrx-1* double mutant.

Table 2. Summary of *C. elegans* strains used.

Strain	Type	Genetics	Phenotype
N2	Wildtype	16 exons and 845 amino acids (nlg-1) 28 exons, 1716 amino acids (nrx-1)	Migrates toward Tc, repelled by 1-octanol
IK589	Athermotactic/achemotactic	*ttx-7* (nj50), 1 exon deletion	No preference of thermal migration, not repelled or attracted to 1-octanol
IK130	Thermophilic	*pkc-1* (nj3), substitution	Migrates toward warmer temperature regardless of Tc
OH8	Cryophilic	*ttx-3* (mg158), substitution	Migrates toward cooler temperature regardless of Tc
VC228	*nlg-1* (ok259)	2,341 bp deletion (exons 8-13) and 334 bp insertion	Migrates toward cooler temperature at Tc 16, but little to no thermal preference at Tc 25. Repelled by 1-octanol
SG1	*nrx-1* (dx1)	1,498 bp deletion (exons 3-6) and 135 amino acids out of frame	Migrates toward cooler temperature at Tc 16, but little to no thermal preference at Tc 25. Not repelled or attracted to 1-octanol
MLB 1316	*nlg-1* (ok259), *nrx-1* (dx1)	VC228 + SG1	Migrates toward warmer temperature at Tc 25, but little to no thermal preference at Tc 16. Repelled by 1-octanol

Summary of the genetics and phenotypic responses of the *C. elegans* strains used in this study. Behavioral control strains are denoted by grey boxes.

Statistical analysis

Data was entered into GraphPad Prism 8 and analyzed. Using the raw data concerning the number of worms at each position after 30 minutes, a 2-way ANOVA was done for the Tc 16 population, the Tc 25 population, and the chemotaxis (CTX) assay populations. All populations are significantly different: the strain of a worm has a large effect on its position ($p<0.05$) (Figure 11). A QQ test was done for each to confirm normality for each set, indicating the correct usage of ANOVA (Figure 12).

A

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Position x Strain	39941	12	3328	F (12, 42) = 5.756	P<0.0001
Position	17248	2	8624	F (1.719, 36.10) = 14.91	P<0.0001
Strain	22925	6	3821	F (6, 21) = 6.998	P=0.0003
Subject	11466	21	546.0	F (21, 42) = 0.9442	P=0.5430
Residual	24288	42	578.3		

B

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Position x Strain	58760	12	4897	F (12, 60) = 7.318	P<0.0001
Position	28197	2	14099	F (1.962, 58.86) = 21.07	P<0.0001
Strain	26696	6	4449	F (6, 30) = 3.608	P=0.0082
Subject	36991	30	1233	F (30, 60) = 1.843	P=0.0222
Residual	40150	60	669.2		

C

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Position x Strain	2251	4	562.9	F (4, 19) = 2.954	P=0.0469
Position	4066	1	4066	F (1, 19) = 21.34	P=0.0002
Strain	7643	4	1911	F (4, 19) = 3.417	P=0.0289
Subject	10626	19	559.2	F (19, 19) = 2.935	P=0.0118
Residual	3620	19	190.5		

Figure 11. 2-way ANOVA of thermotaxis and chemotaxis assay populations. (A) Tc16 population of control and mutant *C. elegans* strains analyzed for significance. Corrected for sphericity by the Greenhouse-Geisser procedure. (B) Tc 25 population of control and mutant *C. elegans* strains analyzed for significance. Corrected for sphericity by the Greenhouse-Geisser procedure. (C) Chemotaxis population of control and mutant *C. elegans* analyzed for significance.

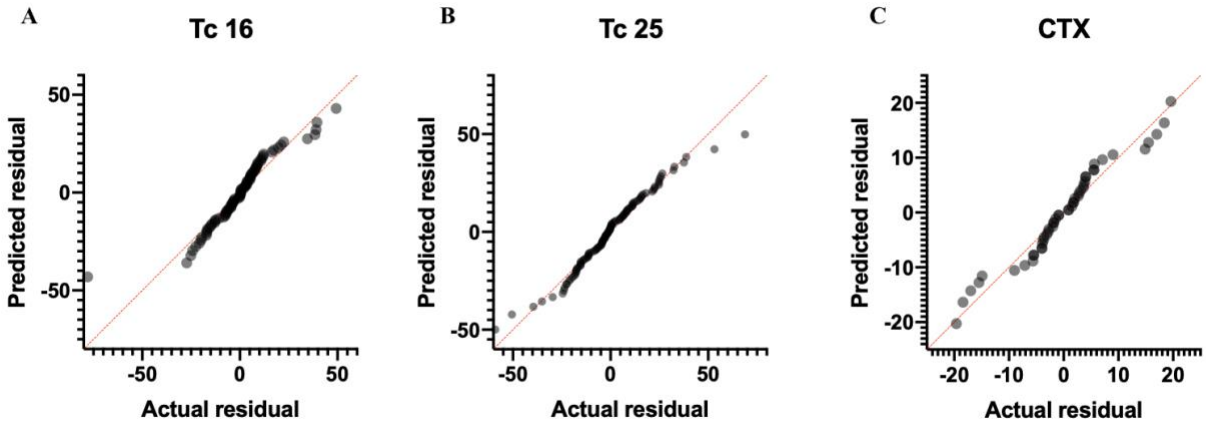


Figure 12. QQ plots for thermotaxis and chemotaxis assay populations. (A,B,C) Tc 16, Tc 25, Chemotaxis populations respectively analyzed for normality, showing that data near the mean are more frequent in occurrence than data far from the mean.

Discussion

This study employed *C. elegans* as a model system to study behavioral defects associated with mutations in the neuroligin and neurexin genes, which in humans have been found to be linked to various autism spectrum disorders (ASDs). Based on

conclusions made in previously published literature, this study hypothesized that worms with mutations in neuroligin and neurexin genes would behave differently from wildtype in both thermosensory and chemosensory abilities (Table 2).

Observing the behaviors of *nlg-1* and *nrx-1* mutant strains VC228 and SG1 respectively, along with the double mutant strain MLB1316, we were able to compare them to the wildtype N2 strain in both migration toward cultivation temperature when placed on a gradient and repulsion from 1-octanol. There was found to be a significant difference between the thermotactic behavior of both single and double mutant *nlg-1* and *nrx-1* worms compared to that of wildtype N2 worms. Additionally, there appeared to be a difference in thermosensory ability between worms raised at 16°C and those raised at 25°C. All control and mutant strains were repelled by the chemorepellent 1-octanol, but *nrx-1* mutants were notably less repelled than other strains. These findings are in line with what has been previously concluded in the literature, as neuroligin-deficient mutants of *C. elegans* were found to be defective in specific sensory behaviors and sensory processing (Hunter et al). However, the *nrx-1* strain SG1 as well as the newly constructed double mutant strain (MLB1316) have not yet been observed in the literature for behavioral deficits. In this study, the *nrx-1* mutant is most noticeably divergent in chemosensory abilities and the double mutant is one of the most defective in thermosensory processing. This proposes interesting questions regarding not only the role of single gene mutations, but the function of compounding mutations and their prevalence in and effect on both neurodevelopment and ASD. Two opposing “drives” for

upward and downward thermal migration, might be a viable explanation for this finding (Hedgecock and Russel 1975).

Using the adapted thermotaxis and chemotaxis assays described in the methods, wildtype N2 and mutants VC228 (*nlg-1*, ok259), SG1(*nrx-1*, dx1), and MLB1316 (*nlg-1*, ok259; *nrx-1*, dx1) were observed for thermotactic and chemotactic behavioral differences. The *nlg-1(ok259)* allele in the VC228 strain used in this study was also observed for behavioral deficits by Hunter et al. Through our adapted thermotaxis assay, *nlg-1* mutant worms were found to differ from wildtype in thermosensory ability, but this conclusion appeared to be affected by cultivation temperature. For example, VC228 worms raised at 25°C in particular had a notably low TI (0.2034) as compared to a TI of 0.8108 when raised at 16°C (SD 0.1596). However, the 25°C cohort had a very high standard deviation between assay repetitions (0.6400). This wasn't the only case of divergence seen between worms of the same strain raised at different temperatures, so it brings up the question of how significant the trend is and if cultivation temperature is somehow affecting thermosensory ability.

This divergence is also seen in the thermotactic indices of the double mutant strain, MLB1316. While *nlg-1, nrx-1* double mutants raised at 25°C had a TI of 0.5490 (SD 0.3317), worms of the same strain raised at 16°C showed a TI of 0.0893 (SD 0.0578). This is also interesting, as the divergence observed in TI is inconsistent by Tc. As seen in Table 1, most strains show a higher TI when raised at 16°C rather than at 25°C, indicating that a lower cultivation temperature causes *C. elegans* to migrate more toward their Tc when placed on a temperature gradient. This could be due to a limitation

in assay set-up, or it could suggest that colder temperatures have less of an effect on aberrant thermosensory behavior. Previous thermotactic experiments, such as the ones completed by Hunter et al., only performed behavioral measurements on young adult *C. elegans* raised at ~22°C. Hedgecock and Russel, however, experimented with worms raised at 16°C, 20°C, and 25°C. Their study found that migration of *C. elegans* to a preferred temperature may involve two opposing "drives," one for upward and one for downward thermal migration. Furthermore, if either "drive" is eliminated via mutation, the resulting worm should behave in a thermophilic or cryophilic manner. The authors also concluded that the thermal response of thermophilic mutants (strains that tended to move more toward warmer temperatures) was strongly modulated by Tc whereas cryophilic mutants (strains that tended to move more toward cooler temperatures) were insensitive to Tc. Interestingly, the thermotaxis assay repeats in this study done with cryophillic strain OH8 were more robust than those done with thermophillic strain IK130. Not only did the OH8 worms move more consistently toward their Tc, the deviation between assay repetitions was smaller than that of IK130 worms. Similarly to the observation of divergent outcomes based on Tc, assays done with cryophillic and thermophillic strains raised at 25°C were more robust and consistent than those done with the same strains raised at 16°C. These data suggest that cultivation temperature of *C. elegans* might have a larger effect on synapse development and memory. Additionally, perhaps expression of mutations in the neuroligin and neurexin genes specifically is influenced by temperature.

In this study, known mutant *C. elegans* strains were used as controls to validate the robustness of our assays. It has been previously determined that N2 wildtype worms will migrate toward their Tc and will be repelled by 1-octanol. Thermophillic, cryophillic, and athermotactic/achemotactic strains were used in addition to wildtype to reinforce the robustness of, and conclusions made from, the thermotaxis and chemotaxis assays. IK589, a previously determined athermotactic and achemotactic strain, showed little to no preference of migrating toward a specific temperature regardless of Tc. They also were not very strongly repelled by 1-octanol during the chemotaxis assay. Thermophillic strain IK130 showed a large preference towards warmer temperatures. Raised at 16°C or 25°C, these worms had a high chance of migrating toward the warmer end of the thermotaxis plate. Cryophillic strain OH8 conversely showed a preference towards colder temperatures. Raised at 16°C or 25°C, OH8 worms had a high chance of migrating toward the colder end of the thermotaxis plate. The utilization of these mutant control strains allowed confirmation of experimental conditions and robustness of assay set-up. Since the expected results were found with the control strains, we are accurately and dependably able to make conclusions based on the results obtained through the thermotaxis and chemotaxis assay for wildtype and mutant strains.

The chemotaxis assay used in this study compared the ability of wildtype N2 to be repelled by 1-octanol to the abilities of mutant neuroligin and neurexin *C. elegans* strains. Although all strains were repelled by the chemical (denoted by a negative CI in Figure 10), the mutant strains appeared to be less repelled than the wildtype. The IK589 strain served as the achemotactic control, and was robust with a CI of -0.1702. Interestingly, the

nlg-1 single mutant (CI= -0.5500) and the *nlg-1, nrx-1* double mutant (CI= -0.4044) were more repelled than the *nrx-1* mutant (CI= -0.1105). This conclusion leads to posing the question of structural and synaptic differences between *nlg-1* and *nrx-1* and whether the *nlg-1* mutation is somehow rescuing chemosensory ability in the double mutant. The *nlg-1* mutant strain VC228 was found to have a 2,341 base pair (bp) deletion including exons 8-13 and a 334 bp insertion while the *nrx-1* mutant strain SG1 was found to have a 1,498 bp deletion with exons 3-6 deleted and 135 amino acids out of frame. Considering that the two proteins in question interact as binding partners, this finding proposes the question of whether Nrx is inhibiting Nlg in this sensory signaling pathway. While further experimentation needs to be done to confirm this "rescue" hypothesis, the double mutant could be potentially acting in a more or less behaviorally deficient manner as compared to the single mutant strains due to the genetic structure of compounding the *nlg-1* and *nrx-1* mutations in the *C. elegans* genome.

Kimata et al. concluded that in *C. elegans,* chemosensory responses share neuronal connections with thermosensory responses. The results found in this study support this conclusion, as the TIs and CIs of mutant strains were lower than those of the wildtype strain. This suggests that a single mutation (or double, in the case of MLB1316) is causing two separate phenotypes.

In *C. elegans,* interneurons, sensory neurons, and motor neurons all come together in the nerve ring shown in Figure 2. This puts all neurons, whether it is AFD in charge of thermal stimuli, AWB, ASH, and ADL in charge of odorant stimuli (repellents to be exact), or a motor neuron such as RIM, in close proximity to interact with one another.

Previous studies have attempted to map out the mode of transmission of thermal and/or chemosensory stimuli in the *C. elegans* nervous system (Aoki and Mori 2015, Bargmann 2006, Mori and Ohshima 1995). While there is speculation about interneurons AIY, AIZ, and RIA receiving and passing on the sensory message from AFD and AWC to a motor neuron, the intricacy of this system in terms of neurexin and neuroligin in particular needs further study to be conclusive.

The conclusion that neurons act together to respond to sensory stimuli is also in line with the nature of ASDs in humans, as there can be many distinct behavioral deficits associated with the disorder, whether there exists a single mutation or compounding mutations. However, a complex neurological disorder cannot be fully understood by one or two behavioral assays using an animal model.

Although much is known about the structure and function of synapse circuitry, there is still difficulty in understanding the precise ways in which cognitive diseases arise from changes in neural circuits. Subtle changes, as opposed to large impairment of all synapses, can just as well result in disease. Thus, the same molecular alteration may produce different changes in circuitry. These changes can then result in neurological symptoms that are classified as distinct cognitive diseases, such as ASD.

Conclusion and Further Direction

This study found a significant difference between the thermotactic behavior of both single and double mutant *nlg-1* and *nrx-1* worms compared to that of wildtype N2 worms. Additionally, there appeared to be a notable difference in thermosensory ability

between worms raised at 16°C and those raised at 25°C, leading to further questions regarding the effect of cultivation temperature on neurodevelopment and thermosensory capacity. All control and mutant strains were repelled by the chemorepellent 1-octanol, but *nrx-1* mutants were notably less repelled than other strains. These findings open the door to investigating the synaptic differences that exist between *nlg-1, nrx-1* single and double mutant *C. elegans* strains.

By characterizing neuroligin and neurexin mutant *C. elegans*, this study aimed to identify and confirm behavioral deficits compared to wildtype N2 worms. These results suggest that *C. elegans* can effectively be used as a model system for the behavioral response to human ASD-associated genetic mutations. These findings will support future studies to generate knock-out mutants of *nlg-1* and *nrx-1* by CRISPR/Cas9, and eventually be able to replace *C.elegans nlg-1* and *nrx-1* genes with human genes and/or variants associated with ASD. It would additionally be interesting to create mutant strains by mating the *nrx-1, nlg-1,* and the cryophilic/thermophilic strains to see how they are related in the thermo- and chemotactic pathways. The resulting behavior can then be observed in our constructed strains, and genes or compounds to reverse the effects of the mutations can be screened for. A future outlook might also include the development of a GFP reconstitution across synaptic partners assay (GRASP) to screen for restoration of normal function (Feinberg et al. 2008). This is a system that can be used to label membrane contacts and synapses between two cells in living animals. Future projects can also be instituted to examine not only the connection, synaptic or otherwise, on thermal

and chemo-response, but the opposing “drives” of migration and the effects of mutations on such drives.

ASD and neurological disease research is important not in the thought of finding a “cure,” but in being able to piece together the disease mechanism of action and pinpoint specific genes and neurons involved. This study and its future projects allow researchers to come to a better understanding of ASD in the hope of mapping the disorder’s genotypic and phenotypic characteristics in both *C. elegans* and humans.

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