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Investigating the Role of DNC-2 and DLI-1 on AMPA Receptor Mediated Behaviors in Caenorhabditis elegans

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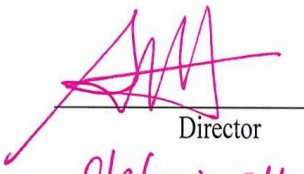

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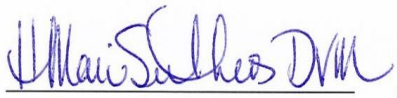

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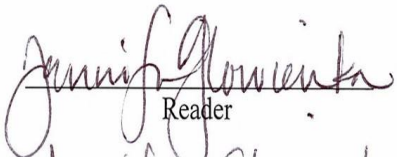

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Life and Environmental Science

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Abstract

Γ-amino-3-hydroxy-5methylisoxazole-4-proprionic acid (AMPA) receptors (AMPARs) are protein complexes involved in excitatory neurotransmission. AMPARs are tetrameric structures consisting of the pairings of GluA1, GluA2, GluA3, and GluA4 subunits. Dctn2 and Dync1li1 were found to interact with GluA2 in an immunoprecipitation screen that was performed on postnatal day 14 rat brains. DNC-2 and DLI-1 were identified as the *C. elegans* homologs of Dctn2 and Dync1li1, respectively, and are components of motor proteins that transport cargo throughout the cell. The goal of this project was to determine whether knocking down DNC-2 and DLI-1 by RNAi affects AMPA Receptor-mediated behaviors, including mechanosensation and chemosensation, in *C. elegans.* Worms with the gene knockdowns were not successfully produced but mechanosensation and chemosensation assays were performed on GLR-2 knockout (KO) *C. elegans*. The GluA2 KO worms demonstrated a lowered ability to sense and respond to both mechanical and chemical stimuli.

Introduction

Synaptic plasticity facilitates learning, memory, and cognition (Brown et. al., 2007). To maintain proper neuronal plasticity ion channels, scaffolding molecules, and signal transduction regulators need to be trafficked to the appropriate synapse (Esteban, 2008). Research focused on the mechanisms of synaptic plasticity is important as a lack of plasticity in the brain can lead to Alzheimer's Disease (Esteban, 2004), Schizophrenia (Stephan et. al., 2006), or Mental Retardation (Govek et. al., 2005). Γ-amino-3-hydroxy-5methylisoxazole-4-proprionic acid (AMPA) receptors (AMPARs) are ion channels that mediate most excitatory synaptic transmissions in the CNS and they are vital in establishing and maintaining synaptic plasticity (Brown et. al., 2007). Elucidating the mechanisms of AMPAR trafficking from the nucleus to the synapse is important for understanding the role of AMPAR function in synaptic plasticity.

AMPARs are heterotetramers composed of a combination of the subunits GluA1, GluA2, GluA3, and GluA4 (Tomita et. al., 2003). Each subunit must be transported to the dendrites, a feat that involves interactions with members of the Kinesin and Dynein motor protein superfamilies (Goldstein and Yang, 2000). After arriving at the synapse, AMPAR insertion into the synaptic membrane is regulated by Transmembrane AMPAR Regulatory Proteins (TARPs) (Chen et. al., 2000). Aside from the role of motor proteins and TARPs, the exact molecular mechanisms involved in the targeted transport of AMPARs to the synapse are largely unknown.

The focus of my research project was to determine the functional role of DNC-2 and DLI-1 in AMPAR trafficking in the model organism *Caenorhabditis elegans* (*C. elegans*). *C. elegans*, transparent nematodes, were chosen as the model organism because individuals are inexpensive, easy to maintain, easy to observe, their genome sequence is readily available, and protocols for generating gene knockdowns have been established. *DNC-2* and *DLI-1* are

homologs of the genes that encode for DCTN2 and DYNC1LI1, two proteins that were identified as GluA2 interactors in a rat brain immunoprecipitation experiment (Shanks, 2012). According to NCBI: AceView, *DNC-2* codes for a member of the Dynamitin family, which is involved in spindle morphology and alignment in embryos, nuclear movement and pronuclear migration. DLI-1, also known as dynein light intermediate chain, is associated with mitosis, pronuclear and nuclear migration, and normal function.

For my research, I hypothesized that the loss of DNC-2 and DLI-1 function by RNA interference (RNAi) in *C. elegans* would affect the trafficking of GLR-2, the *C. elegan* homolog of the mammalian GluA2 protein, to the synapse and negatively impact mechanosensation and chemosensation behaviors that rely on normal AMPAR function.

Materials and Methods

An overview of the entire project can be seen in Figure 1.

RNA Extraction→Reverse Transcription
→PCR of target gene→Insertion of target into L4440 plasmid→Transformation of RNAi feeding bacteria with plasmid→Allow *C. elegans* to consume transformed feeding bacteria→Perform mechanosensation and chemosensation assays on *C. elegans*

Figure 1. Projected procedure for the entire experiment.

Culturing and Maintenance of *C. elegans*

All *C. elegans* strains were purchased from Carolina Biological. The different strains maintained were wild type (N2) and GLR-2 knockout (KO). Stocks of original worms and future generations of each strain were kept at -80°C. When needed, vials were thawed on ice and plated onto OP50 *E. coli* seeded Nematode Growth Media (NGM) plates. Stock OP50 was also purchased from Carolina Biological and stored at -80°C.

To make NGM plates, 3.0g NaCl, 2.5g peptone, and 17.0g agar were mixed and then dissolved in 972mL of reverse osmosis (RO) water. The solution was autoclaved and then the following reagents were added aseptically: 1.0mL cholesterol in ethanol (5mg/mL), 1.0mL M $CaCl_2$, 1.0 mL M $MgSO_4$, and 25.0mL M phosphate buffer (pH 6.0). The resulting 1000mL of NGM was poured aseptically onto 35-40 petri dishes, left overnight, and then stored in a sealed container at room temperature (~23°C).

Before moving *C. elegans* onto the NGM plates, the plates were seeded with OP50 media. OP50 is an *E. coli* mutant whose growth is limited by the amount of uracil in the NGM plates and therefore allows for the visibility of the worms (Brenner, 1973). To create the media 1.0g tryptone, 0.5g yeast extract, and 0.5g NaCl were combined and dissolved in 100mL of RO water. The resulting solution was then autoclaved. After cooling to room temperature the solution was aseptically inoculated with OP50 and then incubated at 37°C overnight. OP50 media was stored at 4°C.

NGM plates were seeded by aseptically transferring 100µL of OP50 media to the center of the NGM plates and then spread around the plate while avoiding the edges to prevent the worms from drying out of the sides of the plate. The seeded plates were left at room temperature overnight and then checked for contamination.

Non-contaminated plates were then populated with *C. elegans* either from frozen stock populations or from previously populated plates. Transferring worms from a pre-existing plate to a new one involved a technique called chunking. The chunking process included removing a portion of the agar populated with *C. elegans* and placing it onto an unpopulated NGM plate.

RNA Extraction

The Qiagen RNeasy kit was used for RNA extraction following the manufacturer's protocol. To prepare for RNA extraction, *C. elegans* were washed from the NGM plates using 5mL of M9 buffer and then transferred to a 15mL conical vial. Each vial was spun at 4000rpm for 5 minutes and the supernatent was removed. After adding 600µL of Buffer RLT from the RNeasy kit, the worms were homogenized by pipetting with a 27 gauge needle and syringe for 1 minute. The homogenate was then transferred to a 1.5mL microcentrifuge tube, centrifuged for 3 minutes at 13000rpm, and the remainder of the RNeasy protocol was carried out. To elute the purified RNA from the RNeasy spin column, 50µL of RNase free water was added prior to centrifugation. The RNA concentration was then determined by using either a BioRad SmartSpec or NanoDrop spectrometer. All RNA samples were stored at -20°C.

Reverse Transcription

The reverse transcription of *C. elegans* into cDNA was carried out using the RETROscript RT kit from Thermo Fisher Scientific. The following reagents were added to sterile PCR tubes: 2µg of RNA, 1µL of Random Hexamer Primers, and enough RNase free water to make a final

volume of 12µL. The tubes were then incubated at 65°C for 5 minutes. The reagents and their amounts added next are shown in Table 1.

Table 1. Reagents added in order used for the second step of Reverse Transcription.

Reagent	Volume
5x Reaction Buffer	4µL
RiboLock RNase inhibitor	1µL
10mM dNTP mix	2µL
Revert Aid RT enzyme	1µL

The tubes were then incubated at 25°C for 5 minutes, 42°C for 60 minutes, and then at 70°C for 5 minutes. Samples were then stored at 4°C until needed.

Gene Specific PCR

The cDNA generated from the reverse transcription was used as template for the PCR amplification of *DNC-2* and *DLI-1*. The PCR amplification of *ACT-1* was used as a positive control as it is very highly expressed in *C. elegans*. For the PCRs, reagents from the GoTaqGreen PCR Master Mix from Promega were used as indicated in Table 2.

Table 2. Reagents used for PCR

Reagent	Volume
2x GoTaq Green Master Mix (Promega)	12.5µL
10µM Forward Primer (IDT)	1.5µL
10µM Reverse Primer (IDT)	1.5µL
Nuclease Free Water (BioRad)	4.5µL
cDNA	5µL

The PCR reactions were incubated in the thermocycler at the times and temperatures indicated in Tables 3 and 4 depending on the gene sequence being targeted.

Table 3. PCR temperatures and times for *DNC*-2 and *ACT-1*

Temperature (°C)	Time (min)	
95	2.0	
95	0.5	Repeat 31x
58	0.5	
72	2.0	
72	5.0	
4	Infinity	

Table 4. PCR temperatures and times for *DLI-1* and *ACT-1*

Temperature (°C)	Time (min)	
95	2.0	
95	0.5	Repeat
55	0.5	39x
72	2.5	
72	5.0	
4	Infinity	

Gel Electrophoresis

PCR samples were run on 1% Molecular Biology Agarose gel (BioRad) with 1x TBE Buffer (VWR). Invitrogen's TrackIt 1KbPlus DNA Ladder was used as the marker. Five microliters of PCR sample was loaded into the gel and it was run at 110 volts.

PCR Purification

PCR purification was performed using Qiagen's QIAquick PCR Purification Kit following the manufacturer's protocol. Six samples from the PCR were pooled together and 600µL of Buffer PB was added. Purified DNA samples were eluted from the purification columns with 30µL of RNase-free water.

Restriction Digest and Ligation

A summary of the Restriction Digests performed are presented in Table 5. Reactions were incubated at 37°C overnight in a BioRad thermocycler.

Table 5. Reagents for Restriction Digests.

Reagent	*DNC-2*	L4440 vector (Addgen) for *DNC-2*	*DLI-1*	L4440 vector (Addgene) for *DLI-1*
DNA	10 µL	*5* µL	10µL	5 µL
10x buffer 2 (New England Bio Systems)	3 µL	3 µL	0 µL	0 µL
10x buffer 3.1 (New England Bio Systems)	0 µL	0 µL	3 µL	3 µL
Nuclease Free Water	12 µL	17 µL	12 µL	17 µL
Xho-1 restriction enzyme (New England Bio Systems	0 µL	0 µL	1 µL	1 µL
Kpn-1 restriction enzyme (New England Bio Systems)	1 µL	1 µL	0 µL	0 µL
Not-1 restriction enzyme (New England Bio Systems)	1 µL	1 µL	1 µL	1 µL
BSA 10x (New England Bio Systems)	3 µL	3 µL	3 µL	3 µL

The reagents for ligations can be seen in Table 6 and Table 7 for *DNC-2* and *DLI-1* respectively.

Reagents were added together and then incubated at 16°C overnight.

Table 6. Reagents for *DNC-2* Ligations.

Reagent	Amount (µL)
10x Buffer 2	1.5
L4440 vector for *DNC-2* Restrictive Digest	2
DNC-2 Restrictive Digest	5
T4 Ligase (New England Bio Systems)	1
Nuclease free water	5.5

Table 7. Reagents for *DLI-2* Ligations.

Reagent	Amount (µL)
10x Buffer 3.1	1.5
L4440 vector for *DLI-1* Restrictive Digest	2
DLI-1 Restrictive Digest	5
T4 Ligase	1
Nuclease free water	5.5

Transformation into Competent Cells

The transformation of ligation reactions (recombinant DNA) was performed using Invitrogen's Subcloning Efficiency DH5α Competent Cells, following the manufacturer's protocol. To prepare the competent cells for planting, S.O.C Medium was added and 200µL of the resulting solution was spread onto LB AMP plates. Plates were made by combining 12.5g of LB power, 7.5g of Agar, and 500mL of RO water. After autoclaving, 500µL of 1000x Ampicillin stock was added aseptically and 10cm Petri dishes were filled three quarters full and then left overnight to set. The LB AMP plates were stored at 4°C.

Miniprep of Recombinant DNA from Transformed DH5α cells

To extract recombinant DNA from the transformed DH5α cells, Qiagen's QIAprep Spin Miniprep Kit was used, following the manufacturer's protocol. The DNA was eluted from the miniprep columns using 30µL of Buffer EB supplied in the kit.

Diagnostic Digest

To determine whether *DNC-2* and *DLI-1* ligated into the L4440 plasmid, a diagnostic digest was performed. The reagents in Table 8 were added together and then incubated at 37°C overnight in a BioRad thermocycler.

Table 8. Reagents for Diagnostic Digests.

	DNC-2	*DLI-1*
Reagent		
Not-1 Restrictive Enzyme	0.2 µL	0.2 µL
Kpn-1 Restrictive Enzyme	0.2 µL	0 µL
Xho-1 Restrictive Enzyme	0 µL	0.2 µL
10x Buffer 2	1 µL	0 µL
10x Buffer 3.1	0 µL	1 µL
10x BSA	1 µL	1 µL
Miniprep DNA	5 µL	5 µL
Nuclease free water	2.6 µL	2.6 µL

Mechanosensation Assay

Elmer's Glue was used to attach a plucked eyelash to a toothpick. After sterilizing the eyelash with 70% ethanol, each worm was stroked 10 times between the mouth and anus. The number of positive reactions to the touch were then recorded out of 10. A positive reaction was recorded when the *C. elegans* moved backwards, turned around, or curled up.

Chemosensation Assay

Chemosensation assay plates were made by combining 1.5% agar, 75mM NH_4Cl, and 10mM MOPS and then adding NH_4OH with 0.25% tween 20 until it is pH 7.2. A half circle of 150mM $CuSO_4$ was then made with 50, 1 µL drops. Wash S Basal Buffer was prepared using

100mL of 1M NaCl, 50mL of 1M potassium phosphate with a pH 6.0, 1mL of 0.5M cholesterol in ethanol, and then 849mL of RO water. The NGM plates hosting *C. elegans* were washed with 5mL of S Basal Buffer. The plates were washed a second time with 3 mL of S Basal Buffer. The second application of 3 mL S Basal Buffer was used to resuspend the worms 3-4 times. The buffer with the worms was transferred into two 1.5mL microcentrifuge tubes and centrifuged at 3500 rpm for 3 min. After centrifugation, the supernatant was removed and 100 µL of buffer remained that contained the worms. The worms were then applied to the chemosensation assay plates inside of the half circle of $CuSO_4$. A 2 µL drop of isoamyl alcohol in a 1:10 dilution in ethanol was then placed 1cm from the midline of the plate. After one hour the percent of worms that crossed the midline against the total number of worms was calculated.

Results

Gel electrophoresis results indicate successful RNA extraction, reverse transcription, and PCR amplification of *Act-1*, *DNC-2*, and *DLI-1* (Figure 2).

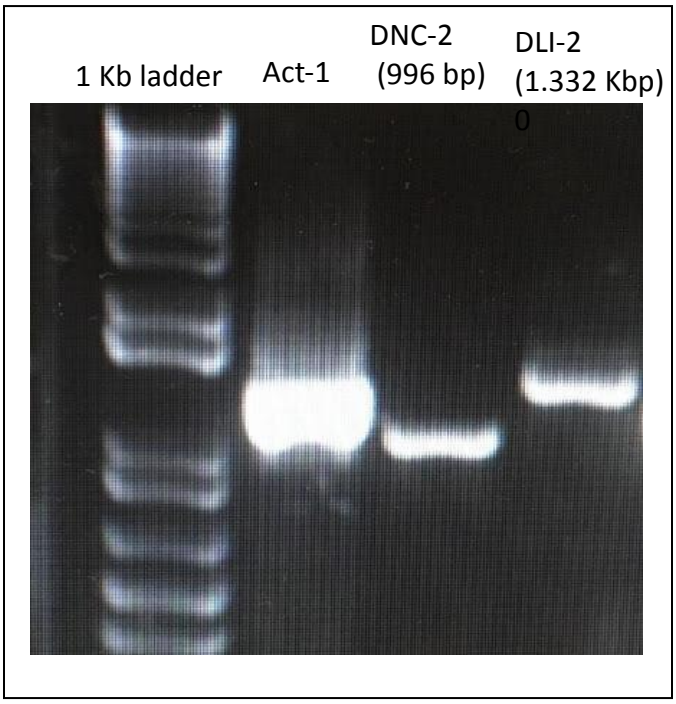

Figure 2. Gel electrophoresis of RT-PCR samples run on a 1% agarose gel. Samples from left to right are 1Kb ladder, *Act-1*, *DNC-2*, *DLI-1*.

The ligation of *DNC-2* and *DLI-1* into the L4440 vector was unsuccessful as can be seen from the presence of re-ligated vector DNA in the Diagnostic Digests performed on DH5α transforms (Figure 3).

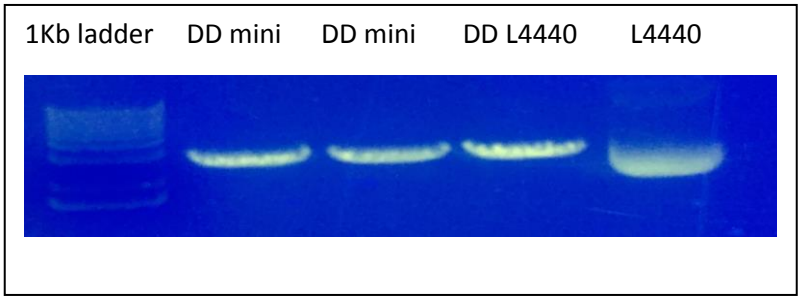

Figure 3. Gel electrophoresis of miniprepped DNA from DH5α transformations. Bands from left to right are 1Kb ladder, diagnostic digest of miniprep DNA (DD mini), separate diagnostic digest of miniprep DNA (DD mini), diagnostic digest of L4440 (DDL4440), and uncut L4440.

While mechanosensation assays of DNC-2 and DLI-1 knockdown worms could not be completed due cloning difficulties, assays were performed on GLR-2 KO worms. The response rate of GLR-2 KO worms was then compared to the rate of response of the wildtype strain (N2) of *C. elegans* (Figure 4). The GLR-2 KO *C. elegans* averaged fewer responses than the N2 *C. elegans.* Similarly, chemosensation assays were performed on GLR-2 KO worms and those results were compared to N2 worms (Figure 5). A higher percentage of GLR-2 KO worms crossed the aversive barrier than the N2 worms.

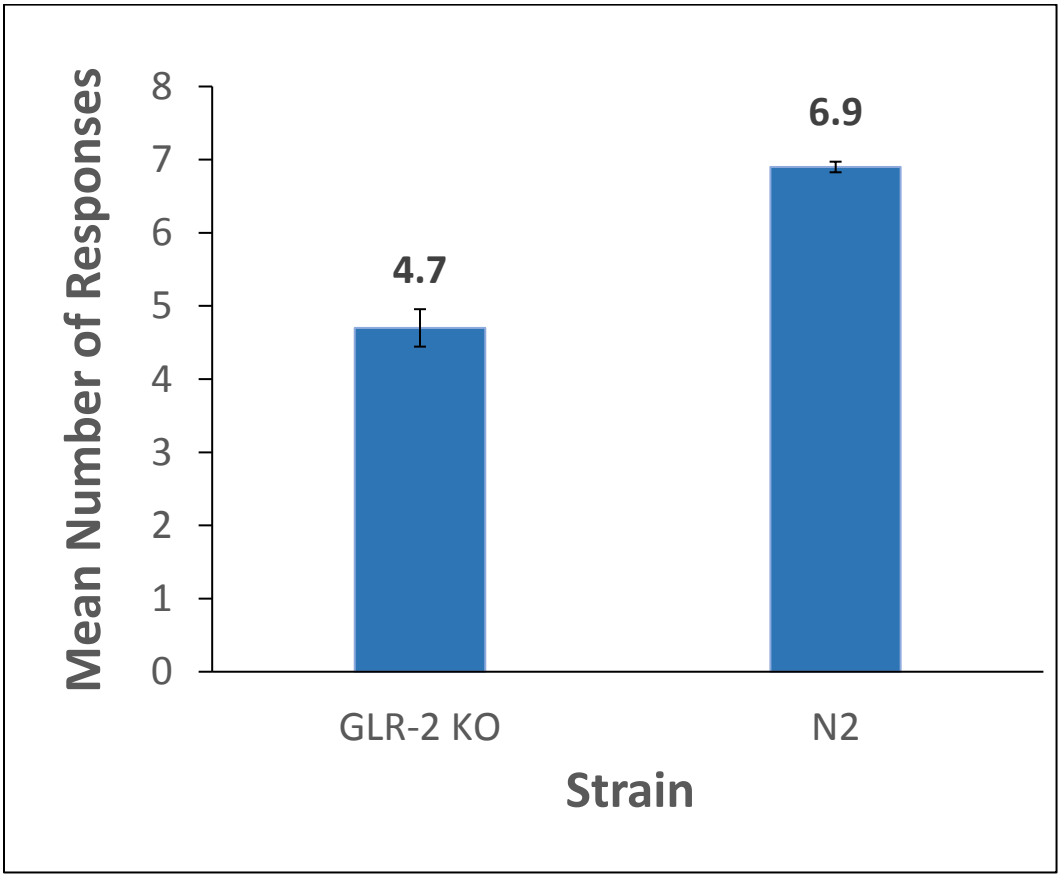

Figure 4. Mean positive responses of wildtype N2 and GLR-2 knockout *C. elegans* to mechanical stimuli. The data indicates a significant difference between the rate of response of GLR-2 knockout worms and wildtype worms (p=3.57E-11). Error bars represent standard deviations.

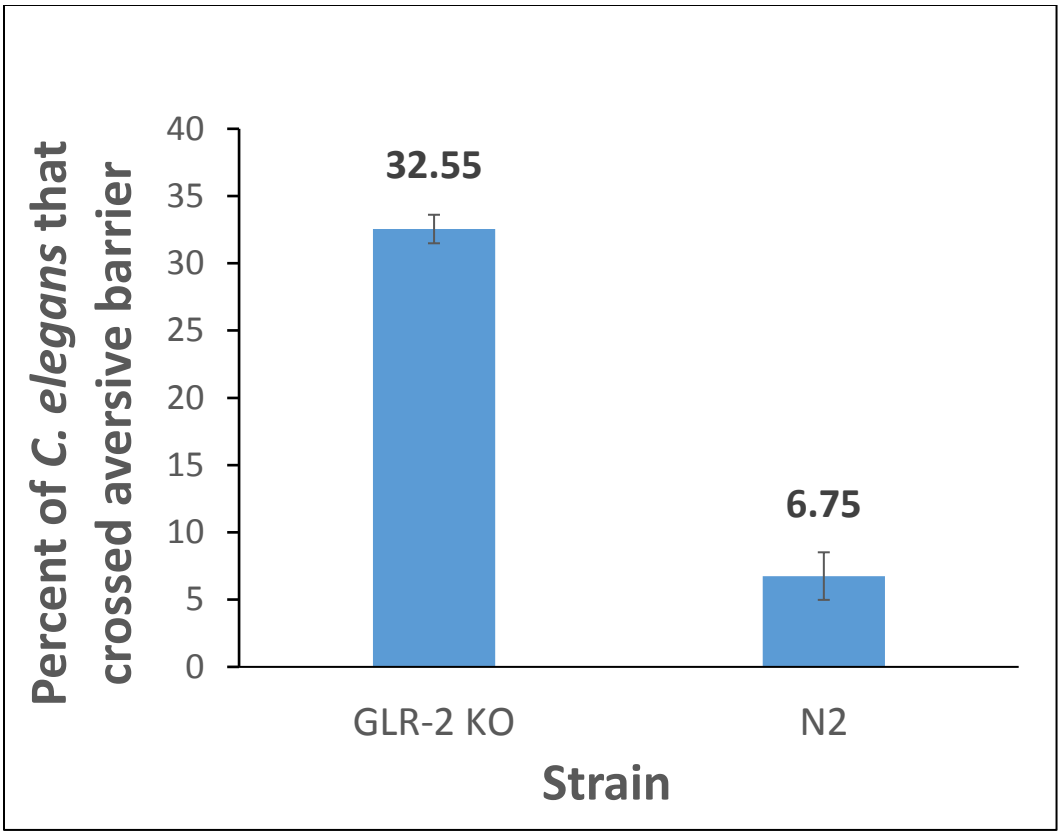

Figure 5. Percentages of GLR-2 knockout worms and wild type N2 worms that crossed the aversive barrier during the chemosensation assay. These results indicate a significant difference in responses (p = .0003). Error bars represent standard error.

Discussion

The goal of my research project was to investigate the role of DNC-2 and DLI-1 in GLR-2 trafficking to the synapse. More specifically, I hypothesized that if DNC-2 and DLI-1 facilitate the transport of the GLR-2 subunit, then loss of these proteins should result in deficits to AMPA receptor mediated behaviors, including mechanosensation and chemosensation. While I was unable to generate worms lacking DNC-2 and DLI-1 function due to cloning difficulties, I was able to carry out the mechanosensation and chemosensation assays on GLR-2 KO worms. My

results from these pilot experiments showed that the lack of GLR-2 led to a decrease in the responsiveness of worms to mechanical and chemical stimuli as shown by an average of fewer responses in the mechanosensation assay and an increased number of worms that crossed the aversive barrier in the chemosensation assay.

In past studies it has been shown that functioning AMPARs are necessary for both mechanosensation and chemosensation (Mellem et. al., 2002). Worms containing a GLR-1 mutation have a decreased responsiveness to mechanosensation, but there is no change in chemical avoidance (Hart et al., 1995; Maricq et al., 1995). Similarly, worms with the GLR-2 KO have a decreased responsiveness to mechanical stimuli (Mellem et. al., 2002). However, worms with GLR-2 GLR-1 double mutations delayed in their responsiveness to the aversive osmotic stimuli (Mellem et. al., 2002), indicating that a functioning GLR-2 subunit is necessary for chemosensation.

For future directions, the cloning of *DNC-2* and *DLI-1* into the L4440 RNAi expression vector needs to be optimized to obtain recombinant DNA. Once the cloning is successful, the recombinant DNAs can be used to transform a specialized strain of feeding bacteria, HT115, that the *C. elegans* will consume to induce knockdown of DNC-2 and DLI-1. Following exposure to the transformed feeding bacteria, mechanosensation and chemosensation assays will be performed to determine whether knockdown of *DNC-2* and *DLI-1* affects these AMPAR-mediated behaviors. In addition to the behavioral assays, RT-PCRs will be performed on the HT115-fed worms to quantify the extent of *DNC-2* and *DLI-1* RNA knockdown in comparison to wild type N2 *C. elegans.*

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