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Regulation of Glutamate Receptor (GLR-1) Under Endoplasmic Reticulum Stress in Caenorhabditis elegans

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REGULATION OF GLUTAMATE RECEPTOR (GLR-1) UNDER ENDOPLASMIC RETICULUM STRESS IN CAENORHABDITIS ELEGANS

By

Janie Aguilera

Accepted in Partial Completion
of the Requirements for the Degree
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Master's Thesis

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Janie Aguilera

August 13, 2020

REGULATION OF GLUTAMATE RECEPTOR-1 (GLR-1) UNDER ENDOPLASMIC RETICULUM-STRESS IN CAENORHABDITIS ELEGANS

A Thesis
Presented to
The Faculty of
Western Washington University

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Of the Requirements for the Degree
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Abstract

Neurons communicate with other cells to elicit outputs that include memory and movement. Cells, including neurons, create proteins every day for specific functions and in particular, neurons produce proteins that enable their communication. Proteins found in cellular membranes are synthesized at the endoplasmic reticulum (ER). However, up to 30% of new proteins are improperly folded and must be removed from the cell. A build-up of misfolded proteins can trigger the Unfolded Protein Response (UPR) which initiates other pathways of protein quality control and can determine the fate of a cell. ER-associated protein degradation (ERAD) is a ubiquitin-dependent process in eukaryotic cells that helps alleviate protein accumulation by breaking down misfolded proteins and targeting them to for degradation at the proteasome. The ERAD system is well described in yeast but is less well studied in multicellular systems. We used *Caenorhabditis elegans* (*C. elegans*) as a model organism to examine the trafficking of a single transmembrane protein through the ER under stressed conditions and as part of the ERAD pathway. The glutamate receptor, GLR-1, is a model membrane protein expressed in a subset of interneurons in *C. elegans* at the ventral nerve cord (VNC) to observe if GLR-1 is a candidate substrate for ERAD. Assessing GLR-1's accumulation in the absence of ERAD E3 ubiquitin ligases in *C. elegans* can determine the protein's regulation in ERAD. Three putative ERAD E3 ligases in *C. elegans* are: HRD-1, MARC-6, and HRDL-1. Previous results showed that deletion of most of the gene encoding HRDL-1, caused an increased accumulation of GLR-1::GFP in the VNC. HRDL-1 may have a role in regulating GLR-1 degradation at the ER and GLR-1 may be a candidate endogenous substrate for ERAD. In order to understand how ERAD effects GLR-1, we used a chemical stressor, tunicamycin, which blocks ER-dependent glycosylation and initiates the UPR. Tunicamycin treatment induces ER stress, which activates a UPR-reporter construct *hsp-4*p::GFP in *C. elegans*. The *hsp4p*::GFP reporter responds to

tunicamycin at lower doses when animals harbor the mutation gene *hrdl-1*. We used quantitative fluorescence imaging and immunoblotting to determine whether the GLR-1::GFP accumulated due to underlying ER stress caused by the lack of HRDL-1. Contrary to previous results, we found no difference in fluorescently-tagged GLR-1 in the VNC in animals lacking hrdl-1, though overall levels of tagged GLR-1 were increased. GLR-1 with a point mutation that caused ER accumulation was not affected by the loss of *hrdl-1*. We propose that another ER E3 ligase may be involved in GLR-1's regulation in the VNC that further investigation of cell bodies may uncover mechanisms of GLR-1 regulation at the ER.

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Introduction

Cells create proteins for specific functions necessary for an organism's survival. However, some proteins are improperly folded and must be removed to prevent abnormalities. The cell has a control system to remove nascent proteins that have failed to fold or assemble properly (Fujita et al., 2007; Cooper, 2000). The control system is the unfolded protein response (UPR). Part of the UPR is a mechanism that takes place in the ER, where the cell recognizes cellular stress from an accumulation of misfolded proteins and control stress through selective degradation (Araki & Nagata, 2011). ER-associated degradation (ERAD), is a process for ER quality control that targets misfolded proteins in the ER to be ubiquitylated for degradation. Ubiquitin is a small protein that is expressed in eukaryotes and can be added to target a protein as a signal to be broken down in the proteasome (Atkin & Paulson, 2014). The proteasome is a protein complex that breaks down proteins into smaller fragments. Studying the pathways that regulate protein aggregation and reducing cellular stress may help understand the causes of neurodegenerative disorders, which can be caused by protein aggregation, for example, Alzheimer's and Parkinson's Disease (Mittal et al., 2015).

Using *Caenorhabditis elegans* (*C. elegans*) as a model organism may bring new light to the cellular bases of neurodegenerative disorders (Juo & Kaplan, 2004). Because glutamate receptors are vital for neural communication, including memory and learning (Rose et al., 2003; Juo & Kaplan, 2004; Maricq et. al, 1995). *C. elegans* is a promising model for discovering how ERAD affects specific neurons and their cell biology. Learning more about the cell biology of *C. elegans* neurons and their neurotransmitter receptors under normal as well as stressed or diseased states can give more insight into the basic functions of the human nervous system at a molecular level.

The Unfolded Protein Response (UPR) and ER- associated Degradation (ERAD)

The UPR is a response by which proteins at the ER membrane initiate pathways that upregulate UPR gene expression. The protein chaperone, BiP, plays an important role in the UPR to control cellular stress in the ER. BiP is a member of the family HSP70 molecular chaperones involved in quality control and folding of proteins (Gething, 1999). The chaperone binds to newly synthesized proteins in the ER as well as misfolded proteins to be properly degraded by the proteasome (Gething, 1999). *C. elegans*, has an ortholog gene to the mammalian ER-localized chaperone BiP called *hsp-4* (Taylor & Dillin, 2013). The *hsp-4* gene can function as a reporter for UPR activation and can be tracked with activation using Green Fluorescent protein (GFP) (Taylor & Dillin, 2013; Calfon et al., 2012). However, when unfolded proteins accumulate in the ER, BiP is crucial for activation of three ER transmembrane proteins to induce the UPR (Calfon et al., 2012; Xiang et al., 2017).

The activation of UPR genes is dependent upon three ER transmembrane proteins called IRE1 (Inositol Requiring Enzyme), double-stranded RNA-activated PERK (Protein Kinase-like ER (PKR) Kinase), and ATF6 (Activating Transcription Factor 6) (Calfon et al., 2002). These transmembrane proteins are in an inactive state when BiP is bound in unstressed conditions and BiP is removed when ER stress is induced due to competition of unfolded proteins accumulating in the ER to be bound to BiP (Carrara et al., 2013). The ER proteins can sense conditions of ER stress and each has different functions for UPR gene expression (Carrara et al., 2013; Storm et al., 2016). The UPR's transmembrane sensors are involved to repress the function of protein synthesis and is the first response to regulate protein homeostasis (Chen & Brandizzi, 2013). Each transmembrane protein work independently to generate UPR gene expression from their cytosolic domains (Figure 1, Storm et al., 2016).

The cytosolic domains of the ER sensors are activated when ER stress is detected. IRE1 RNase cytosolic domain becomes activated through conformational changes to target the X-box binding protein (Xbp1) mRNA for alternative splicing to activate UPR target genes in the nucleus of the cell. The UPR genes are encoded for proteins involved in protein folding, maturation, and degradation (Carrera et al., 2013). PERK's cytosolic domain phosphorylates the eukaryotic initiation factor 2α (eIF2α) to inhibit protein translation and overload of misfolded proteins at the ER lumen (Carrara et al., 2013). The last ER sensor in the UPR is ATF6 where it becomes translocated from the ER to the Golgi to undergo proteolytic cleavage creating a transcription factor ATF6 p50 (Chen & Brandizzi, 2013; Carrera et al., 2013). The transcription factor migrates to the nucleus of the cell to initiate the expression of UPR genes. However, the UPR genes made are responsible for activating responses that determine the fate of the cell (Figure 1). Prolonged ER stress is associated with induced specific cell death pathways and is suggested to be a molecular mechanism responsible for human pathological disorders including neurodegenerative diseases (Dibdiakova et al, 2018). One class of UPR genes that is still under investigation are those involved with ERAD and the biological process it undergoes.

ERAD involves enzymes to bring ubiquitin to polyubiquitylate a misfolded protein so that the target protein can be transported to the proteasome and be broken down into smaller peptides. ERAD is a process that targets misfolded proteins that have failed the "folding cycles" in the ER (Vembar & Brodsky, 2008). Proteins that do not fold properly can lead to improper cellular function leading further to disease. The ER has multiple checkpoints for misfolded, damaged, or incomplete proteins via a process known as the ER quality control (Vembar & Brodsky, 2008; Figure 2). When misfolded proteins are identified, ERAD is initiated to target these proteins and are marked for degradation with a protein called ubiquitin. Inactivation of

ERAD leads to an accumulation of misfolded luminal and membrane proteins that can induce ER stress such as heat shock or chemical stress (Walter & Ron, 2011). The process of ERAD is important for proteins to be properly refolded or degraded via ubiquitination.

In eukaryotic cells, ERAD uses a polyubiquitination to target misfolded proteins in the ER creating a ubiquitin chain to be targeted and broken down by the proteasome. Ubiquitin is a small protein that involves E1, E2, and E3 ligases for protein degradation in the ERAD pathway (Alberts et al., 2014; Figure 3). Ubiquitin participates in an enzymatic cascade that is initiated by an E1 ubiquitin-activating enzyme (E1) (Hershko et al., 1980). After activation, ubiquitin is transferred by an E2 ubiquitin-conjugating enzyme (E2). Finally, ubiquitin becomes attached to a substrate protein from an E3 ubiquitin ligase (E3). This creates a ubiquitin chain that directs the misfolded protein to be degraded in the proteasome at the cytoplasm of the cell (Haas et al., 1982; Woelk et al., 2007; Figure 3). The proteasome breaks down the protein into smaller fragments or peptides. Thus, ERAD helps re-establish protein homeostasis for the cell after the involvement of the UPR and E3 ligases by removing accumulated, misfolded proteins from the ER (Shen et al., 2004).

In ERAD, E3 ligases are enzymes that recognize misfolded proteins or substrates when they are retained in the ER. E3 ligases are involved in ER quality control for additional proofreading for proteins to be properly degraded into small peptides to be refolded correctly (Meusser et al., 2005). Moreover, E3 ligases play an important role in substrate selection for misfolded proteins to be properly degraded because there are hundreds of genes specific to target misfolded proteins (Alberts et al., 2014).

This project focus on a single E3 ligase, HRDL-1, in *C. elegans* since E3 ligases play an important role in substrate recognition of misfolded proteins. The *C. elegans* genome has three

putative ERAD E3 ubiquitin ligases known as HRD-1, HRDL-1, and MARC-6. Depletion of these ligases caused delayed growth in the worms using different concentrations of tunicamycin (Sasagawa et al., 2007). An E3 ligase of interest in *C. elegans* is HRDL-1 where it may play a role for protein degradation in ERAD knowing it is an enzyme responsible for protein specificity and regulation (Sasagawa et al., 2007).

Experimental system

Using *C. elegans* as a model organism will provide important data for understanding the process of protein degradation in neurodegenerative diseases and regulation of membrane proteins. *C. elegans* is a simple model organism to understand more about single synapses and neurobiology. *C. elegans* can easily be manipulated to create mutants with defects in specific genes and they were the first multicellular organisms to have their genome sequenced (Brenner, 1974). *C. elegans* have been used as a simple model being identified as nematode worms to observe behavioral effects of ER stress and understanding the ERAD pathway (Sasagawa et al., 2007; Taylor & Dillin, 2013). There are 300 neurons identified in the *C. elegans* nervous system and a subset of those neurons are orthologous to neurons in the human brain (Brenner, 1974). These nematode worms can provide useful information to analyze single neurons associated with protein degradation and understanding pre-diseased states of neurodegenerative disorders.

The *C. elegans* Glutamate Receptor: GLR-1

The glutamate receptor GLR-1 in *C. elegans* is a transmembrane protein found in the AVA interneurons (Wang et al., 2012). GLR-1 is expressed in the interneurons at the ventral nerve cord (VNC) (Zheng et al., 1999; Hart et al., 1995; Maricq et al., 1995). The glutamate receptor is orthologous to human AMPA type glutamate receptor subunit GluR1 (GluA1) (Yao et al., 2011). The neurotransmitter glutamate plays a pivotal role in synaptic transmission,

synaptic plasticity, and neuronal excitation at the post-synaptic surface (Bissen, et al., 2019). The receptor, GLR-1, is a good candidate to observe expression at the VNC in *C. elegans* which can be tagged with either GFP or Yellow Fluorescent protein (YFP) at the synapse. This can also help us observe the process of how membrane proteins in neurons are regulated at the ER.

The project is focused on the receptor GLR-1 to understand how it becomes regulated in the ER and if it is an endogenous substrate for ERAD. Studies found that GLR-1 was regulated by ubiquitin signaling at the synapse to be endocytosed in neurons located at the VNC (Kowalski et al., 2011). Once the receptor is internalized from the cell surface, ubiquitinated GLR-1 can undergo either degradation or deubiquitination from deubiquitinating enzymes (Kowalski et al., 2011). In other words, removing the ubiquitin chain to prevent the GLR-1 receptor from being degraded. Another study found that GLR-1 can be recycled to the synapse from endosomes and can be assembled in a four-part complex in the ER (Brockie et al., 2013). The receptor, GLR-1, was still expressed in the plasma membrane of the synapse but is not recycled in endosomes. Structural mutations of GLR-1 were used to observe trafficking of the receptor which led to a dramatic decrease of GLR-1 expressing at the synapse and retention at the ER (Grunwald & Kaplan, 2003).

GLR-1 trafficking and export from the ER require components in the UPR signaling pathway. Studies found that the UPR pathway is required for GLR-1 exit in the ER even with the absence of ER-stress inducing agents such as heat shock or chemical agent tunicamycin (Shim et al., 2004). Suggesting there may be a basal-level requirement for UPR signaling. The basal-level requirement can be the ER sensor IRE-1. The strain *sel-1*(e1948) in *C. elegans* a mutant for ERAD is upregulated by XBP-1 in ER stress and UPR (Shim et al., 2004). To observe the trafficking of GLR-1 with the *sel-1* mutants, the IRE-1/XBP-1 pathway is required for glutamate

receptor trafficking in ER stress environments. This suggests that GLR-1 may have an essential role in ERAD in ER stress to observe what components in the quality control system are necessary for glutamate receptor assembly and activity.

To confirm if GLR-1 is an endogenous substrate of ERAD, stressing the ER with a chemical stressor can help determine if the receptor is functioning in the pathway. The ER stressor, tunicamycin, initiates the UPR in *C. elegans* and induce ER stress in cells. Tunicamycin is a bacterial toxin that inhibits glycosylation sites in the protein at the ER lumen (Bull & Thiede, 2012; Dibdiakova et al., 2018). Glycosylation is important at the lumen of the ER for proteins to fold correctly and mature to be transported to the cellular membrane of the cell. This process is known to generate a diverse amount of proteins and have different properties during folding (Alberts et al., 2014). Glycosylation inhibition can lead to protein aggregation and cell death (Dibdiakova et al., 2018). The protein, GLR-1, becomes glycosylated in the ER when it is properly folded with the help of ER lectin-like chaperones calnexin and calreticulin (Peterson et al., 1995; Grunwald & Kaplan, 2003). By treating the animals with tunicamycin the process may be that GLR-1 will not be glycosylated and will therefore be retained in the ER, activating the ERAD pathway.

A series of experiments done in the Dahlberg lab was conducted observing three E3 ligases in *C. elegans* (HRDL-1, HRD-1, and MARC-6). Removing the E3 ligase *hrdl-1* in *C. elegans,* caused increased accumulation of GLR-1::GFP suggesting *hrdl-1* has a role in regulating GLR-1 degradation based on western blotting experiments (Figure 4; Witus, 2016 unpublished data from Honors Thesis). Imaging analysis was conducted between two E3 ligase mutants: *hrd-1* and *hrdl-1*. The average puncta width (neurites at the VNC) was significantly wider in *hrdl-1* mutants compared to the wildtype expressing GLR-1::GFP supporting that GLR-

1 is being accumulated in the VNC without functional HRDL-1 (Witus, 2016 unpublished data from Honors Thesis; Figure 5). These findings suggest that GLR-1 may be an endogenous substrate for ERAD, however, knowing where the membrane protein is accumulating is still unclear.

An endoglycosidase H (EndoH) experiment was conducted to determine the location where GLR-1 is accumulating without functional HRDL-1. The purpose of the EndoH experiment was to observe GLR-1::GFP glycosylation patterns either at the ER or in the Golgi apparatus of the cell. Based on the data, the *hrdl-1* mutants displayed GLR-1 receptor was being retained in the ER compared to wildtype GLR-1::GFP (Hassell and Hulsey-Vincent, 2019 unpublished data; Figure 6). This suggests that removing functional HRDL-1 causes GLR-1 to accumulate in the ER and the pathway the protein is being targeted for degradation may be under ERAD with a specific ubiquitin E3 ligase HRDL-1. By inducing ER stress to the GLR-1 protein using a chemical stressor tunicamycin will help determine if the animals carrying the mutant strain *hrdl-1* were initially ER stressed or inducing more stress will develop a severe phenotype.

Using tunicamycin in *C. elegans* without functional HRDL-1 is a great model to study whether if GLR-1 is essential for being targeted as misfolded or inactivated. Previous work has been done to understand the process where tunicamycin induced cellular stress and what pathway the stressor is activating. In the unfolded protein response, there are three ER sensors: IRE1, PERK, and ATF6 (strom et. al, 2016). Tunicamycin activates in the IRE-1 pathway to conduct alternative splicing of XBP-1 a transcription factor for protein quality control and activate the ERAD pathway (Carerra et. al, 2013 & Taylor and Dillin, 2013).

Questions and hypotheses

In this study, an ER chemical stressor tunicamycin was used with *C. elegans* that express a fluorescent derivative of GLR-1, GLR-1::GFP. The main question we hoped to answer in this study was: Are the effects of *hrdl-1* mutation due to increased ER stress? The question was subdivided into three questions to help answer if HRDL-1 is an enzyme necessary to maintain the health of neurons expressing GLR-1 or does it require other proteins to help promote the function of HRDL-1 for proper GLR-1 degradation.

Q1: Does loss of *hrdl-1* sensitize animals to ER stress?

The first aim is directed in the unfolded protein response following the molecular chaperone BiP using tunicamycin to induce ER stress. The protein BiP or *hsp-4* in *C. elegans* was used as an indicator when it is activated in the presence of tunicamycin by GFP in a previous study observing loss of ERAD E3 ligases (Sasagawa et. al, 2007). Using the protein, *hsp-4*, to study ER stress, there can be a similar pattern with an ERAD E3 ligase HRDL-1. There are two hypotheses regarding if the loss of HRDL-1 will sensitize the worms to ER stress. If *hrdl-1* mutation causes ER stress on its own, then BiP (*hsp-4*) should be upregulated under basal conditions. Meaning without the presence of a chemical stressor. The second hypothesis is to determine if *hrdl-1* mutants become sensitized to ER stress, then BiP (*hsp-4*) should be upregulated much more than wildtype in the presence of a stressor (tunicamycin). This question was addressed discerning if worms carrying the *hrdl-1* mutation and expressing *hsp-4p*::GFP will

be used as a model to observe ER stress using tunicamycin. The strain *hsp-4p*::GFP will be used as a reporter gene for UPR activation in the ER (Taylor & Dillin, 2013).

Q2: Does loss of *hrdl-1* in combination with ER stress disrupt GLR-1 regulation?

Work was previously done to observe GLR-1 regulation with the loss of ERAD E3 ligases: HRD-1, HRDL-1, and MARC-6 (Sasagawa, et al., 2007). In prior work on the E3 ligases in *C. elegans*, one E3 ligase showed a difference in GLR-1 expression (Witus, 2016 unpublished data from Honors Thesis). Worms with loss of HRDL-1 showed a greater abundance of GLR-1::GFP and had wider puncta expression of the protein at the ventral nerve cord (Witus, 2016 unpublished data from Honors Thesis). The aim is to determine if the animals with *hrdl-1* mutation are initially ER stressed or will develop a severe phenotype by inducing ER stress. The hypothesis is if *hrdl-1* mutation in the presence of ER stress disrupts GLR-1 regulation, then more severe GLR-1 phenotypes will be observed: increase abundance of GLR-1::GFP protein and different localization than wildtype. This would indicate that GLR-1 is regulated by two different mechanisms. One that is dependent on *hrdl-1* and one that is dependent on ER stress levels. If we don't see differences in GLR-1::GFP between wildtype and mutant strains in the presence of tunicamycin, this would indicate that tunicamycin and the *hrdl-1* mutant work in the same pathway for GLR-1 regulation.

Q3: Does a mutation that affects the structure of GLR-1 with loss of *hrdl-1* disrupt protein regulation at basal conditions?

Disrupting the structure of a protein or receptor to express at the cellular membrane, the protein will be inactivated and will not be present at its destination either at synapses or in

membrane bound organelles. Previous work by Grunwald and Kaplan (2003) showed changes at the ligand binding and pore domains of GLR-1. The mutation at the ligand binding region prevents GLR-1 from exiting the ER. It is not known if a mutation preventing GLR-1 to exit in the ER with loss of HRDL-1 will have the same effect based on localization and abundance of the protein. I hypothesized that if a mutation at the ligand binding site of GLR-1 prevent regulation of the protein exiting the ER, then the point mutation in *hrdl-1* would result GLR-1 phenotypes to have high abundance of GLR-1::YFP and different localization than wildtype.

Methods

C. elegans maintenance and crossing:

C. elegans strains were grown on Nematode Growth Medium agar (NGM) with cholesterol, $MgSO_4$, and $CaCl_2$. *OP50 E. coli* a strain of bacteria was grown on NGM plates as a food source for the worms. The *C. elegans* strains (Table 1) were grown in incubators at 20˚C for 3 days from larval stage to adult worms. To create different genetic hybrids, wild type males were mated with hermaphrodites that will be homozygous for the allele of interest, for example, SJ4005 transgenic worms. The F1 males were then mated with hermaphrodites that were homozygous to the second allele of interest to create strains that were contain two alleles of interest. The alleles were then followed with another two more generations to obtain worms that will be homozygous for the desired allele.

The newly made hybrid crosses were tested and confirmed using the Polymerase Chain Reaction (PCR). The hybrid crosses were *hsp-4p*::GFP; *hrdl-1*,GLR-1::GFP; *hrdl-1*, and GLR-1::YFP; *hrdl-1* (Table 1). Primers in table 2 were used to detect the mutation of *hrdl-1*.

Tunicamycin Assay:

Adult L4 stage worms were collected and transferred to experimental plates 5 µg/mL of tunicamycin containing NGM agar. During preliminary experiments, different concentrations of tunicamycin were used. The concentrations of tunicamycin were 0 µg/mL, 2 µg/mL, 5 µg/mL, and 7.5 µg/mL. At 5 µg/mL, changes between genotypes were easily differentiated and was decided to use 5 µg/mL as an optimal concentration for tunicamycin between genotypes of interest. A single concentration of tunicamycin was added to the molten NGM agar and a control plate with ethanol (EtOH). Each plate with 5 µg/mL of tunicamycin and ethanol were allowed to

cool for at least 8 hours at room temperature and stored in a tub. The next day, 75 µl of cultured *OP-50 Escherichia Coli* was added to each treatment plate and was allowed for the bacteria to grow another 2-3 days. On experiment day, adult worms were washed with M9 buffer and were collected in Eppendorf tubes. Remaining M9 buffer was removed and 11 µL of concentrated worms were pipetted to a Whatman Filter paper "stamp". The worms collected on the "stamp" were transferred to each treatment plate for 6 hours. After the 6 hours, all the worms' images using a fluorescence microscope were captured collecting approximately 15-20 worms from each treatment. For western blotting, worms were washed with M9 buffer to collect lysates from each treatment to observe protein abundance of GLR-1 or *hsp-4p::*GFP (Sasagawa et al., 2007 ;Tcherpakov et al., 2008; Darom et al., 2010; Taylor & Dillin, 2013 and Waldherr et al.,2019).

Immunoblotting (Western Blot):

Young adult *C. elegans* on NGM agar plates or 5 µg/mL tunicamycin were washed into Eppendorf tubes with M9 solution. M9 buffer was removed from the solution to gather concentrated worms at the bottom of the Eppendorf tube.4x SDS sample buffer and 100 mM β-mercaptoethanol (BME) was added in the tube containing the concentrated worms. The animals suspended in SDS loading buffer and BME were frozen at -20˚C for storage. Once lysates were ready to use, 1 mM phenylmethylsulphonyl fluoride (PMSF) protease inhibitor in ethanol was added to the lysates. The solution was boiled for 2 minutes at 95˚C on a heat block and was centrifuged at 14k RPM in a microcentrifuge to collect the supernatant. Proteins from the lysate was separated from a denaturing 8-10% polyacrylamide gel in 1x SDS running buffer and was then transferred on a nitrocellulose membrane overnight at 4˚C with primary antibodies (Table 3).

The nitrocellulose membrane was blocked in 5% milk dissolved in Tris buffer for 5 minutes. The blots were incubated in a 1:1000 dilution of primary antibody in 5% milk in TBS overnight at 4˚C. The membrane was washed 3 times for 5 minutes each in TBS-T. The nitrocellulose membranes were incubated in a 1:10,000 dilution of secondary antibody (sheep α mouse or donkey α rabbit) in 5% milk in TBS-T for 3 hours at 4 ˚C. The membranes were washed 3 times for 5 minutes each in TBS-T. Protein bands was visualized using a chemiluminescent substrate. Images was acquired using a LI-COR Odyssey Fc imager.

Quantitative analysis of GLR-1::GFP or YFP and *hsp-4p*::GFP immunoblotting:

ImageStudio® software was used to measure GLR-1::GFP or YFP and free GFP protein bands. Tubulin was used as a loading control to determine the population of worms collected from each lysate and to determine the ratio of GLR-1 (~130 kDa) or free GFP (~30 kDa) expression between genotypes of interest. Wildtype strains were normalized to 1 and mutant strains were compared based on whether the protein was abundant compared to wildtype. The statistical analyses to compare GLR-1::GFP or GLR-1::YFP protein abundance were T. tests between conditions of ethanol and tunicamycin.

Imaging GLR-1::GFP and GLR-1::YFP localization:

All fluorescence imaging in GLR-1::GFP experiments were performed using a DeltaVision microscope with Olympus IX70 base and a 60x oil UPlan Apo NA 1.4 (oil immersion) objective equipped with an Xenon lamp to detect GFP or YFP using FITC filter. L4 stage hermaphrodites were paralyzed on glass coverslips in a drop of 30 mg/mL 2,3-butanedianoe monoxamine (BDM) in M9 buffer for 5-6 minutes prior to imaging. The

immobilized worms will be placed on a 2% (w/v) ddH_2O agarose pads on glass slides. Images were captured with a Photometrics HQ2 Cool Snap camera using a SoftWoRx Software with exposure at 0.600 ms and image field was set as 1024x1024. All ventral nerve cord (VNC) images in Z-stack (optical section 0.20) were taken in the anterior VNC posterior to the RIG neuronal cell bodies as a landmark.

Image Analysis of GLR-1::GFP and GLR-1::YFP fluorescence:

Quantitative analysis of the VNC of the worm was accomplished on Z-stacks that were comprised of 15-20 images and were deconvolved without any saturation to quantitatively analyze puncta intensity, density, and width. The individual stack images that were out-of-focus were deleted and condensed into maximum intensity projection using FIJI® an open source ImageJ program. A macro feature written in FIJI® was used to measure background fluorescence, puncta width, cord length and number of puncta identified at the VNC (See Appendix I). To measure puncta width and intensity at the VNC, an adaptive threshold was used with Phalkansar in FIJI® to help isolate puncta individually. All line scan images to measure puncta intensity, density, and width were taken mid-anterior at the VNC from the RIG neuronal cell bodes. Tukey's HSD statistical analysis for fluorescence quantification was used to test significant differences between strains for GLR-1::YFP. A Tukey HSD statistical test was used to compare varying levels of GLR-1::GFP fluorescence between wildtype strain and mutant strain without the functional HRDL-1 gene in each condition (ethanol or tunicamycin).

Results

hsp-4p::GFP are more sensitive to tunicamycin compared to wildtype:

To better understand the ER stress inducing agent tunicamycin using *hsp-4p*::GFP with *hrdl-1* mutants, a dose-response experiment was conducted to compare GFP expression in wildtype *hsp-4p*::GFP expressing animals and the E3 ligase mutant animals. Preliminary experiments were conducted to observe the sensitivity of tunicamycin using the *zcIs4* (*hsp-4p::*GFP) and a *hrdl-1* mutant strain (*zcIs4*;*hrdl-1*(*gk28*)). The *zcIs4* strain has the heat shock protein-4 (*hsp-4*) an ortholog of BiP at the promoter that is expressed with GFP (green fluorescent protein) serving as a reporter gene to observe expression of cellular stress in the UPR pathway at the ER (Sasagawa et al., 2007; Taylor & Dillin, 2013) (Figure 1 & Figure 15). Based on the data in Figure 7, the tunicamycin concentration 5µg/mL was a concentration selected to observe optimal differences between the *zcIs4* wildtype and *hrdl-1* mutant at 6 hours. The *zcIs4; hrdl-1* mutant strain had increased expression of the *hsp-4* gene which indicated that tunicamycin induced the UPR in the worm (Figure 7). In further experiments, 5µg/mL concentration of tunicamycin was used to observe GFP protein abundance with *hrdl-1* mutation.

hsp-4p::GFP abundance increase without functional *hrdl-1*:

To confirm that based on levels of high *hsp-4p*::GFP expression in *hrdl-1* mutants qualitatively, a western blot experiment was conducted. Whole cell lysate of young adult worms being exposed to either ethanol (vehicle control) or 5 µg/mL of tunicamycin were collected after 6 hours of exposure. Lysates were run on an SDS-PAGE gel and transferred to a nitrocellulose membrane. Expression of *hsp-4p*::GFP on ethanol and tunicamycin were probed with anti-GFP monoclonal antibodies and β-tubulin were probed using anti-β-tubulin as a loading control

(Figure 8 A). We observed that the zcIs4; *hrdl-1* mutants on tunicamycin expressed more GFP abundance that wildtype significantly (Figure 8 B).

This demonstrates using tunicamycin as a ER stressor to observe the effects of *hrdl-1* is an initial step to use GLR-1::GFP to observe GLR-1 regulation in the ERAD pathway. The *hrdl-1* gene is a putative loss-of-function mutation (*gk28*) with a 1981 base pair deletion and it has been previously demonstrated that the loss of the E3 ligase showed more GLR-1::GFP abundance with the absence of ER stress (Witus, 2016 unpublished data in Honors Thesis). Using tunicamycin as an ER stress inducing response in *hrdl-1* mutants, data can be predicted to observe ERAD using GLR-1 and its regulation.

GLR-1::GFP; *hrdl-1* does not display a severe phenotype in the presence of tunicamycin compared with wildtype GLR-1::GFP

GLR-1 is regulated by the IRE-1/XBP-1 pathway (Shim et al., 2004). To confirm that GLR-1 requires E3 ligase HRDL-1, we aimed to observe if the GLR-1::GFP; *hrdl-1* mutants would display a severe phenotype or will function in the IRE-1 pathway to upregulate UPR genes for ERAD. To investigate the regulation of GLR-1, tunicamycin an N-linked glycosylation inhibitor that induces ER stress was used to observe the abundance of GLR-1::GFP between wildtype and GLR-1::GFP; *hrdl-1* mutants (Taylor & Dillin, 2013). Data from Figure 4 was consistent with the fact that GLR-1::GFP; *hrdl-1* mutants displayed more GLR-1 abundance based on the fold change between wildtype GLR-1::GFP with the absence of ER stress (Figure 9 B). In contrast, there was no difference between wildtype GLR-1::GFP and the *hrdl-1* mutant in the presence of ER stress (Figure 9 B and C). The fold change between GLR-1::GFP on tunicamycin was 2.56 and GLR-1::GFP; *hrdl-1* was 2.36 respectively (Figure 9 B). This data

may suggest that HRDL-1 may be a candidate substrate for GLR-1 regulation in ERAD. However, GLR-1::GFP; *hrdl-1* mutants did not display a more severe phenotype in the presence of the ER stressor, which means that loss of *hrdl-1* did not further disrupt GLR-1 regulation. The data presented does not describe where GLR-1 is accumulating either at the cell body of the neuron or at the synapses because whole cell lysate was collected from each sample. GLR-1::GFP localization was taken into consideration as part of our observation of GLR-1 at the neurites (see below).

GLR-1::GFP localization does not change in the presence of tunicamycin:

After observing there is no significant difference between wildtype GLR-1::GFP and mutant in ER stress, images mid-anterior at the ventral nerve cord (VNC) were collected to observe changes in GLR-1 expression (Figure 10). The approach is to observe if the *hrdl-1* mutants may present different localization patterns at the VNC compared to wildtype in the presence of an ER stressor. Images of the worms were captured after 6 hours of tunicamycin or ethanol exposure. The RIG interneurons were used as a landmark to observe GLR-1::GFP puncta along the VNC. The VNC was chosen because the ubiquitin-dependent regulation of GLR-1 puncta can be observed here (Burbea et al., 2002). We expect more GLR-1::GFP puncta expression at the VNC with *hrdl-1* in the presence of an ER stressor.

We quantified GLR-1::GFP to observe changes in localization or expression at the VNC in order to determine if there are changes in GLR-1::GFP wildtype compared to *hrdl-1* mutants in the presence and absence of tunicamycin. The *hrdl-1* mutants without the presence of an ER stressor displayed lower fluorescence than compared to wildtype, but this result was not significant. In the presence of tunicamycin, the *hrdl-1* mutants displayed lower puncta intensity,

but there was no significant difference with wildtype GLR-1::GFP (Figure 11). However, observing puncta density and width there were no significant changes between *hrdl-1* and wildtype in the presence of tunicamycin (Figure 11). This may be an indication that *hrdl-1* is not a E3 ligase specific for GLR-1 regulation in ERAD and there may be other postsynaptic elements being recruited for GLR-1 (Burbea, 2002).

Previous data gathered from GLR-1::GFP and *hrdl-1* mutants showed that the puncta width in *hrdl-1* mutants had wider puncta than wildtype without an ER stressor (Figure 5). However, using our imaging and analysis protocol, we were not able to confirm these data. Following statistical analysis for puncta intensity, density, and width we did not find any significant difference between wildtype GLR-1::GFP and *hrdl-1* mutants with and without tunicamycin. Observing the quantification of mean puncta intensity, density, and width; the regulation of GLR-1 was not disrupted with the loss of *hrdl-1* in combination of ER stress at the VNC. While tunicamycin, puncta width did slightly increase in wildtype GLR-1::GFP as well as the *hrdl-1* mutants, these values were not significantly different (Figure 11). The same trend applies to puncta density between wildtype and *hrdl-1* mutants (Figure 11).

hrdl-1 **mutation does not disrupt the regulation and localization of mutant GLR-1 (ligand-binding mutant, E770A)**

A ligand binding mutant was used to observe GLR-1 regulation of a receptor that is trapped at the ER. We crossed the *hrdl-1(gk28)* allele into *nuIs114* GLR-1::YFP and *nuIs96* GLR-1(E770A)::YFP (Table 1 & Figure 15). The GLR-1(E770A)::YFP strain is a transgene that contains KP#685 which the strain has a E770A mutation changing glutamate to alanine preventing GLR-1 from exiting the ER (Kaplan & Grunwald, 2003). The ligand binding mutant

has decreased abundance at the synapses compared to wildtype GLR-1::YFP (Kaplan & Grunwald, 2003). To visualize the distribution of GLR-1::YFP with ligand binding mutant and *hrdl-1* mutant, VNC images were acquired to view differences between both genotypes (Figure 12). Qualitatively, the ligand binding mutant with the *hrdl-1* mutation (E770A; *hrdl-1*) expressed different GLR-1 abundance comparted to wildtype (Figure 11). The E770A; *hrdl-1* mutant displayed less abundant GLR-1 compared to wildtype, E770A, and *hrdl-1*. GLR-1::GFP puncta intensity, density, and width were measured using quantitative fluorescence.

After measuring GLR-1::YFP puncta at the VNC, statistical analysis was conducted to observe differences in puncta intensity, density, and width between *hrdl-1* mutants. Comparing the puncta intensity with wildtype GLR-1::YFP, the ligand binding mutant E770A and E770A; *hrdl-1* mutant displayed significantly reduced GLR-1 puncta intensity (Figure 13). While GLR-1::GFP; *hrdl-1* had no significant difference to wildtype. In puncta density, the E770A ligand binding mutant was the only transgene that displayed significantly denser GLR-1 puncta at the VNC while the *hrdl-1* mutants shared no significant difference to wildtype GLR-1::YFP (Figure 13). Comparing puncta width there was no significant difference between E770A, E770A; *hrdl-1*, and GLR-1::YFP; *hrdl-1* to wildtype (Figure 13). The data suggest that *hrdl-1* does not disrupt GLR-1 localization in mutant GLR-1 (E770A). We expect to observe more GLR-1::YFP abundance in E770A; *hrdl-1* mutants, but a different phenotype was observed with less abundant GLR-1 compared to wildtype and E770A ligand binding mutant.

Mutant GLR-1(E770A)::YFP decrease in abundance with *hrdl-1* mutation

To confirm the difference in GLR-1::YFP expression and localization with E770A; *hrdl-1*, a western blot analysis was conducted to observe total GLR-1 abundance. Normalized GLR-

1::YFP abundance was compared to wildtype and E770A ligand binding mutant with *hrdl-1* mutation (Figure 14 A). The GLR-1::YFP; *hrdl-1* mutant displayed more abundant GLR-1 compared to wildtype that was expected from previous western blot analysis with GLR-1::GFP (Figure 9 A). However, the E770A; *hrdl-1* mutant displayed less abundant GLR-1 compared to GLR-1(E770A)::YFP mutant. The low abundance of GLR-1 in E770A; *hrdl-1* correlate with the results observed previously from imaging analysis when comparing to wildtype GLR-1::YFP (Figure 12 and Figure 14 B). The data presented further supports that a structural mutation at the ligand binding domain in GLR-1 with *hrdl-1* mutation does not further disrupt regulation.

Discussion

The ERAD system is well described in yeast but is less well studied in multicellular systems. *C. elegans* is used as a model organism to examine the trafficking of a single membrane protein through the ER under stressed conditions in the ERAD pathway. The glutamate receptor GLR-1 has not been well studied to understand protein regulation in ER stressed conditions in neurons using *C. elegans*. In previous work in our lab, the mutation of ERAD E3 ubiquitin ligase *hrdl-1* influenced GLR-1 localization and expression without the presence of an ER stressor. We followed up this finding using an ER stressor (tunicamycin) and following the abundance and localization of GLR-1. My results differ from previous results, because I found no difference in GLR-1 localization in the *hrdl-1* mutant, in the presence or absence of tunicamycin. This could suggest that another E3 ligase in ERAD is upregulated to target misfolded or inactivated GLR-1 protein.

Previous experiments in the Dahlberg lab, one of the three E3 ubiquitin ligases identified, HRDL-1, may be an enzyme specific for GLR-1 in neurons using *C. elegans*. Western blot experiments done to observe GLR-1 abundance showed an accumulation of GLR-1::GFP in *hrdl-1* mutants as did fluorescence microscopy experiments. However, the western blot experiment also suggest that HRD-1 may have a role in GLR-1 regulation from having the second most abundant GLR-1::GFP (Figure 4). Further studies are needed to confirm if HRD-1 is another E3 ligase works in concert with HRDL-1 for GLR-1 regulation in ER stress conditions using double mutants.

Because *hrdl-1* displayed a disruption in GLR-1 expression and localization in *C. elegans* without the presence of an ER stressor, we wondered whether HRDL-1 is a candidate enzyme for

GLR-1 regulation in ERAD. We developed an assay to observe the role of *hrdl-1* in the presence of an ER stressor using tunicamycin. The *hrdl-1* mutant was crossed with the *hsp-4p*::GFP transgene to observe if the mutants are sensitized. Based on the stress response of the *hsp-4p*::GFP; *hrdl-1* mutants, we determined that *hsp-4* gene activity was moderately upregulated at basal conditions and highly upregulated at 5 µg/mL of tunicamycin compared to wildtype *hsp-4p*::GFP (Figure 7). This suggests that the *hrdl-1* mutants are sensitized to tunicamycin and that *hrdl-1* mutants could serve as a good model for observing role of ERAD in GLR-1 regulation.

Our ER stress experiments with GLR-1 using tunicamycin showed that *hrdl-1* mutants did not display a severe GLR-1 phenotype, which indicates that the *hrdl-1* mutants are not inhibiting normal GLR-1 regulation in ERAD. Our model indicates that tunicamycin initiates the UPR activating the ER sensor IRE-1 upregulating UPR genes for ERAD causing GLR-1 to accumulate in the ER because tunicamycin prevents GLR-1 to get glycosylated to be trafficked out of the ER (Sasagawa et al., 2007; Taylor & Dillin, 2013). But from our results, an E3 ligase other than HDRL-1 is compensating for the accumulation of GLR-1. Thus, misfolded, inactivated, or immature GLR-1 receptors are getting ubiquitylated in ER stressed conditions even with *hrdl-1* mutation. In addition, regulation by clathrin-mediated endocytosis seems unaffected since there was no significant difference in puncta density and width at the neurites at the VNC in *C. elegans* (Burbea et al., 2002 and Figure 11). An experiment to confirm the trafficking of GLR-1 in ER stress conditions is to use the *unc-11* gene that encodes a clathrin adaptin protein AP180 to recruit clathrin for protein trafficking in cells (Burbea et al., 2002).

Another option to explain why GLR-1 displayed no change in regulation in ER stressed conditions is our use of a novel imaging and analysis protocol. Puncta intensity, density, and width were measured differently from previous data collected with *hrdl-1* mutants in GLR-

1::GFP background. The imaging analysis done previously used MetaMorph (Version 6.0) to generate linescans and used a custom written software using Igor Pro (Wavemetrics) to analyze GLR-1 puncta at the VNC (Dahlberg & Juo, 2014; Kowalski et al., 2011). Our imaging analysis protocol used a macro feature written in FIJI® to isolate GLR-1 puncta was created than manually setting up a threshold to isolate the neurites expressed on the VNC. A different image acquisition and deconvolution was used with the DeltaVision microscope in this study.

Another important way in which image analysis was different was that GLR-1::GFP puncta mid-anterior at the VNC was observed rather than observing puncta posterior from the RIG neuronal cell bodies. The data presented in the current experiments show that GLR-1::GFP puncta mid-anterior at the VNC does not disrupt GLR-1 regulation with the loss of *hrdl-1* (Figure 11). However, representative images of GLR-1::GFP between ethanol and tunicamycin, the GLR-1::GFP; *hrdl-1* mutants do show wider GLR-1 puncta than wildtype at the far anterior of the VNC (Figure 10). Instead of isolating GLR-1 puncta mid-anterior at the VNC and focusing puncta close to the cell bodies, puncta width data can change between both treatments (ethanol and tunicamycin) and wildtype. If this result holds true where loss of *hrdl-1* does disrupt GLR-1 regulation based on puncta width and intensity, GLR-1 may be an endogenous substrate in the ERAD pathway. Meaning, we can expect to observe more GLR-1 localization in the *hrdl-1* mutants at the VNC and a disruption of GLR-1 regulation.

Because the *hrdl-1* mutant did not display a severe phenotype and because there was no disruption of GLR-1 regulation in ER stress conditions, HRDL-1 may function redundantly with or downstream of HRD-1 for promoting degradation with misfolded or inactivated proteins. A study observed the interaction between gp78 (HRDL-1) and Hrd1 (HRD-1) work in concert for ERAD for both luminal and membrane substrates (Zhang et al., 2015). GLR-1 is a

transmembrane protein made in the ER and is regulated in ERAD, and could therefore be a target for luminal or membrane directed ERAD (Rubenstein et al., 2012). In the study, model ERAD substrates were chosen: TCRα (T-cell receptor α), a type I membrane protein that is overexpressed in tissue culture cells and major histocompatibility complex (MHC) class I heavy-chain molecule (MHC 1-147) a luminal protein that require gp78 for degradation (Zhang et al., 2015). The study suggested that Hrd1 is essential and gp78 can maintain normal ERAD function if gp78 is permanently deactivated. Also, gp78 functions downstream with the Hrd1 complex using the BAG6 complex (Zhang et al., 2015). BAG6 is a gp78 interacting chaperone that facilitates regulation of ERAD substrates to the proteasome for degradation. Taking a closer look in HRDL-1 (gp78) and the accessory proteins like BAG6 in ERAD can help understand which protein is important for recruiting a specific E3 ligase in *C. elegans*. This may be true if observing *hrdl-1*; *hrd-1* double mutants for GLR-1 regulation in ER stressed conditions.

We wanted to further understand the potential role of HRDL-1 in ERAD using GLR-1 by preventing intracellular trafficking of the protein. We used ligand binding mutants that prevent GLR-1 from exiting the ER were used based on an EndoH analysis, which can help determine where GLR-1 is accumulating with loss of *hrdl-1*. Based on the glycosylation patterns of GLR-1::GFP from animals with the *hrdl-1(gk28)* mutation, a larger proportion of GLR-1 is retained in the ER, in the absence of *hrdl-1* (Figure 6). We made transgenic strains by crossing GLR-1::YFP and GLR-1::YFP(E770A), and visualized differences in GLR-1::YFP puncta and protein abundance (Figure 15 and Table 1). In a study to observe the regulation of GLR-1::YFP, mutations at the ligand binding and pore domains controlled the exit of glutamate receptors from the ER in *C. elegans* (Grunwald & Kaplan, 2003). The ligand-binding mutant (E770A) was shown to control exit of GLR-1 based on low puncta fluorescence, density, and width compared

to wildtype GLR-1::YFP (Grunwald & Kaplan, 2003). We hypothesized that the *hrdl-1* mutation would affect GLR-1(E770A) phenotypes, potentially by increasing the abundance of GLR-1::YFP. However, the E770A; *hrdl-1* mutants expressed less GLR-1::YFP compared to wildtype and looked the same as the E770A mutant (Figure 12, 13, and 14). This suggest that the loss of *hrdl-1* in E770A ligand-binding mutant does not further disrupt, nor does it rescue, protein regulation of the glutamate receptor in *C. elegans*. Importantly, there is also evidence that the ER does not extend far out of the cell body of the GLR-1 interneurons in *C. elegans* (Rolls et al., 2002). During imaging, cell bodies of animals expressing the E770A mutant were extremely bright—so bright that images had to be taken excluding the cell bodies, in order to enable us to view VNC puncta. Because the ER is mainly in the cell bodies, when GLR-1 is prevented from leaving the ER, it will accumulate in cell bodies. It will be important to observe the cell bodies in *C. elegans* with the ligand binding mutants to observe differences in GLR-1::YFP expression in area outside of the VNC (ie, the cell body).

Acquiring images of the ligand binding mutant and E770A; *hrdl-1* mutant expressed brighter cell bodies than wildtype GLR-1::YFP. This can be an important experiment to test whether GLR-1 is a substrate specific for HRDL-1 in protein regulation and to understand more about E770A; *hrdl-1* phenotype. Because the E770A; *hrdl-1* mutant express fewer GLR-1::YFP expression at the VNC compared to wildtype (Figure 12).

Introducing tunicamycin upregulates the unfolded protein response, which activates ERAD genes via IRE-1/XBP-1. One ERAD gene in this pathway is the E3 ligase, *hrdl-1*. In the absence of *hrdl*-1, we expected to see evidence that *hrdl-1* is important for regulating GLR-1 during ER stress, but we did not. The findings in this study suggests that although a *hrdl-1* mutation sensitizes *C. elegans* to ER stress, via tunicamycin, it does not disrupt GLR-1

regulation in the presence of that ER stressor. The GLR-1 receptor still has specificity to be targeted by a specific E3 ubiquitin ligase in ERAD. Indicating that *hrdl-1* can work in concert with another E3 ligase *hrd-1* to degrade membrane proteins that are either misfolded or inactivated in the ERAD pathway in ER stress conditions.

Future Directions

To clarify the findings of the data with GLR-1::GFP; *hrdl-1* mutants in ER stress conditions, the Dahlberg lab plans to do a re-analysis of the raw data acquired, in the presence and absence of tunicamycin, to observe most anterior VNC analysis than mid-anterior. This will help answer whether our differences in findings are due to changes in analysis protocols. In this case, we would expect to find evidence that GLR-1 regulation is disrupted in animals with the *hrdl-1* mutation, through differences in puncta width. Our new analysis tool will again be used to observe GLR-1 puncta at the VNC to compare intensity, density, and width most anterior (see Appendix I).

Another approach is to observe a different E3 ligase (*hrd-1*) in ERAD to identify if the loss of one E3 ligase, another E3 ligase can get upregulated for GLR-1 in ER stress conditions. HRD-1 is another putative ubiquitin E3 ligase for ERAD in *C. elegans*. There is evidence that HRD-1 works alongside with BiP (*hsp-4*) in gonad formation and ERAD (Sasagawa et al., 2007). Also, HRD-1 may have a role for promoting degradation with misfolded or inactivated proteins alongside with HRDL-1.

Although *hrdl-1* is expected to be upregulated in response to ER stress, there is currently no evidence for this in the GLR-1-expressing interneurons in *C. elegans*. One way we could test if *hrdl-1* is downstream of UPR activation (via tunicamycin) is to see whether *hrdl-1* transcription is upregulated in response to tunicamycin. Comparing *hrdl-1* gene expression to *hsp-4* and *hrd-1* expression would allow us to understand if and how these genes are regulated in response to ER stress in *C. elegans*. One way to do this would be to construct a new promoter-

reporter using the promoter of *hrdl-1* driving GFP. That would give us information about if the gene is activated, and in which cells.

In order to determine how ER-retention might be affected by ER stress, we also plan to repeat our ER stress response experiments with the ligand binding mutant and assay them via immunoblot and fluorescent image analysis. This approach would allow us to determine whether inducing ER stress will effect differences in phenotypes for GLR-1(E770A)::YFP expression and abundance. The Dahlberg lab also plans to cross the ligand binding mutant, in the presence and absence of the *hrdl-1* mutation, with *hrd-1* to create novel single and double mutants with which to explore the roles of those two E3 ligases in ERAD for GLR-1 regulation.

Furthermore, it is also important to investigate the appearance of the interneuron cell bodies that have increased intensity in ligand binding mutants. Both GLR-1(E770A)::YFP and E770A; *hrdl-1* have cell bodies with increased fluorescence (Figure 12). More importantly, E770A mutants show increased puncta density (of GLR-1::YFP) at the VNC compared with wild-type GLR-1::YFP animals, while E770A; *hrdl-1* animals do not. *hrdl-1* could be a factor in neuronal development. For example, one study focused on a kinase CDK5, a cyclin-dependent kinase for neuronal development and the kinase regulates the abundance of GLR-1 at the VNC (Juo et al., 2007; Monteiro et al., 2012). Loss of CDK5 activity resulted a decreased abundance of GLR-1 at the VNC and an accumulation of the glutamate receptor in neuronal cell bodies (Juo et al., 2007). Crossing the ligand binding mutants and/or *hrdl-1* mutants with *cdk-5* mutants could help further our understanding about the regulation of GLR-1.

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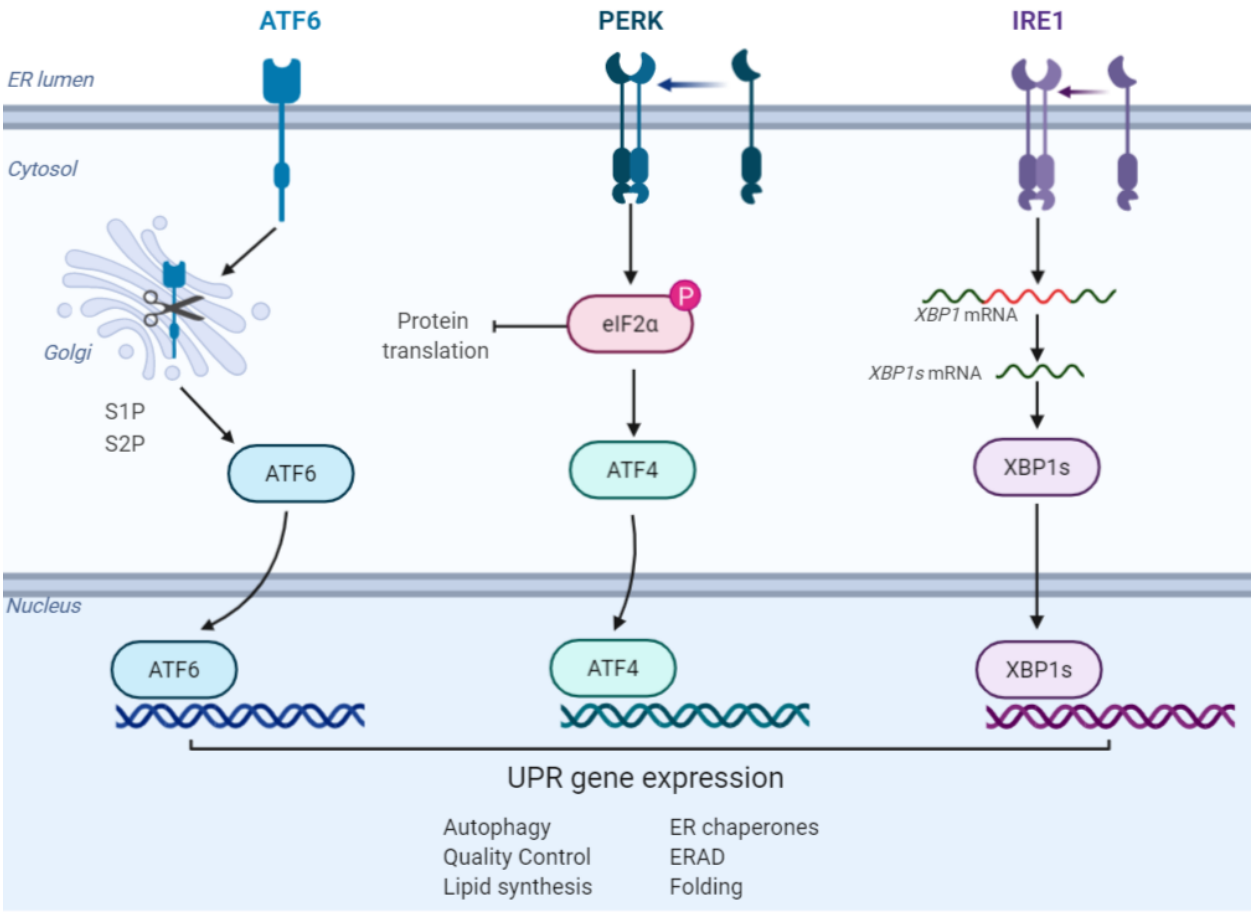


Figure 1. UPR gene expression pathways from IRE1, PERK, and ATF6 ER stress sensors. Each sensor is the first response to initiate the UPR to relieve proteins accumulating in the ER and allow BiP to bind to the misfolded protein (Storm et al., 2016).

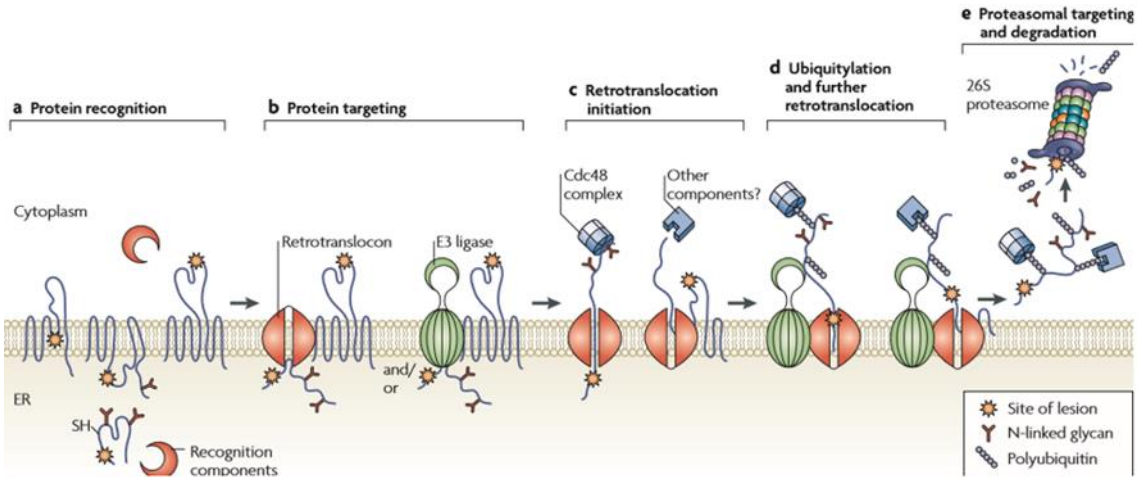


Figure 2. Figure 2 The step-by-step process of ER-associated degradation (ERAD). a. Protein recognition were cytoplasmic or luminal chaperones in the ER come in to recognize misfolded proteins. b. Protein targeting for ERAD to target misfolded proteins to the retrotranslocon or ER ligases. c. Retrotranslocon initiation for proteins to be ubiquitinated. Once ubiquitinated, misfolded proteins/substrates will be degraded from the proteasome. e. Proteasome targets and degrades tagged misfolded proteins. The misfolded protein is broken down into peptides (Figure used with permission from Vembar and Brodsky, 2008).

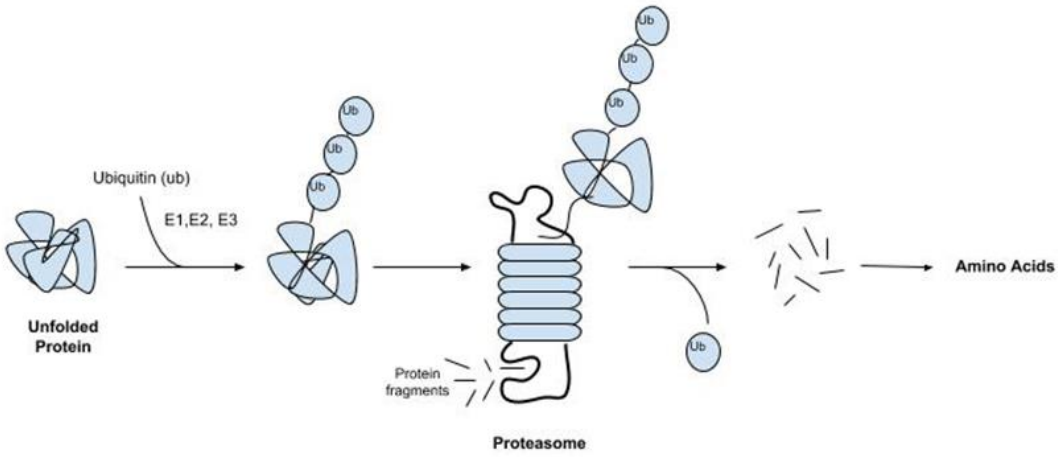


Figure 3. Ubiquitin-mediated degradation pathway where a misfolded protein becomes tagged by ubiquitin with the help of ligases to be degraded by the proteasome. The misfolded protein is broken down into protein fragments and then to amino acids to be refolded once again.

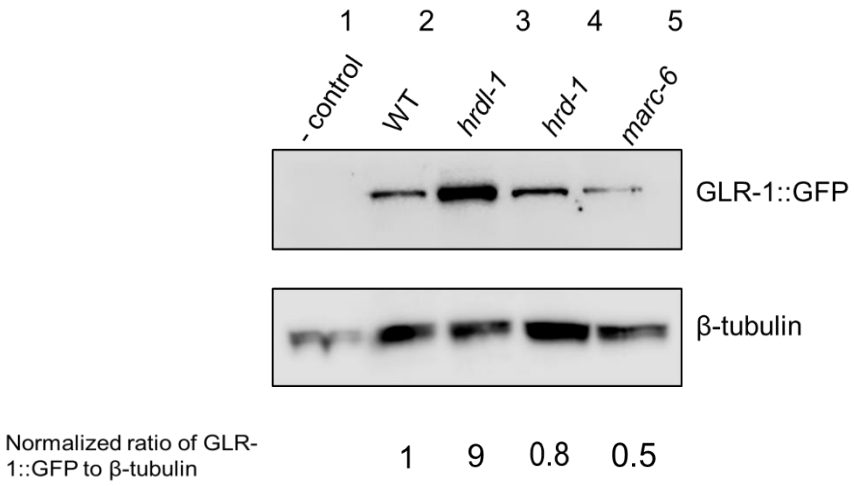


Figure 4. Western Blot representative image displaying GLR-1::GFP is affected by mutation of ERAD E3 ubiquitin ligases. The *hrdl-1* mutant expresses more protein abundance of GLR-1 compared to the last two E3 ubiquitin ligases (Witus, 2016 unpublished data in Honors Thesis).

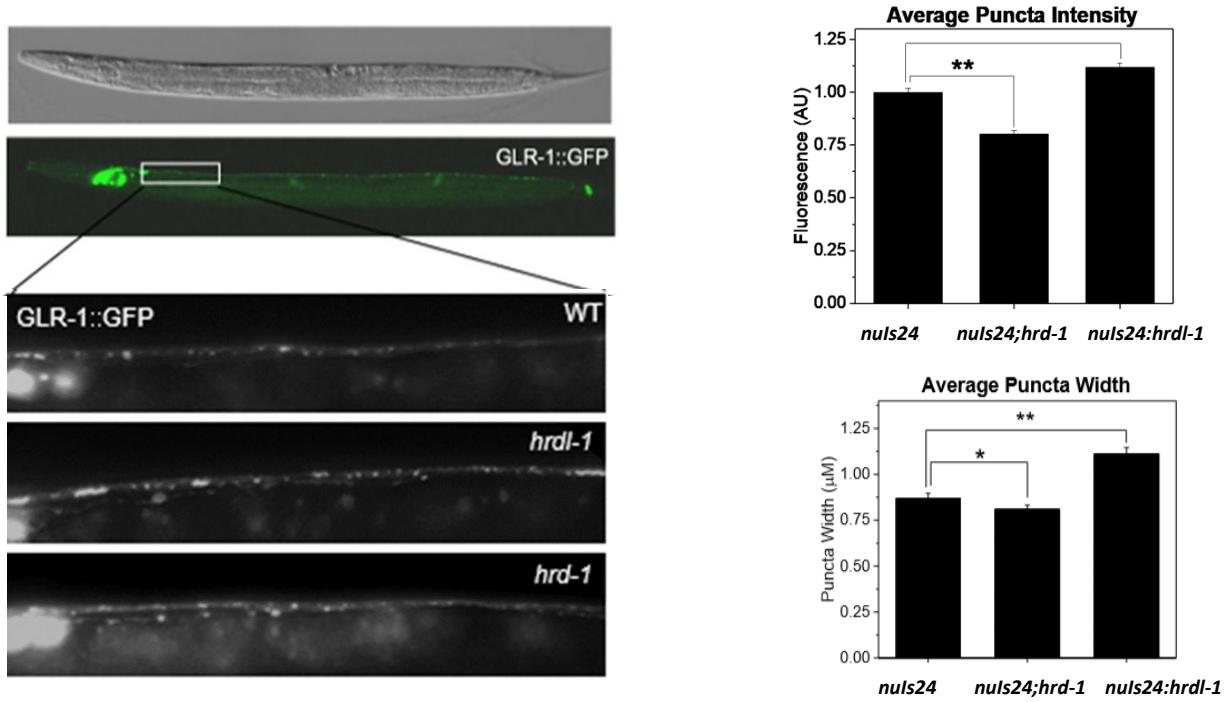


Figure 5 Ventral Nerve Cord (VNC) imaging analysis displaying GLR-1::GFP in *C. elegans* is regulated by *hrdl-1* an ERAD E3 ubiquitin ligase. (Witus, 2016 unpublished data in Honors Thesis). GLR-1::GFP images were taken anterior of the worm at the VNC comparing puncta abundance from wildtype (*nuIs24*).

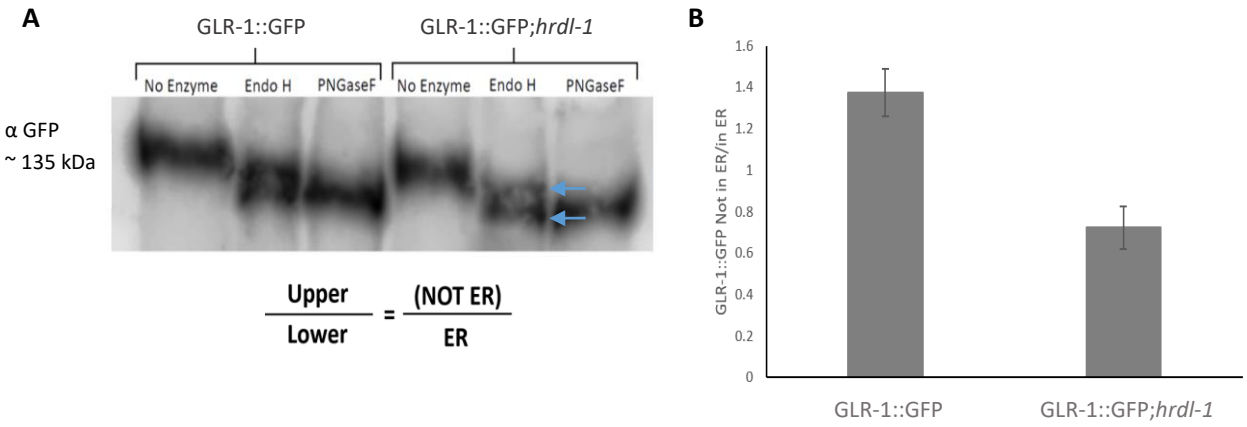


Figure 6. Endoglycosidase H (Endo H) analysis to observe GLR-1::GFP glycosylation patterns with and without functional *hrdl*-1. A) Wildtype animals displayed GLR-1::GFP to be glycosylated and *hrdl-1* mutants did not. (B) Density ratio was measured to observe upper and lower bands (blue arrows) between wildtype and *hrdl-1* mutants. The *hrdl-1* mutants displayed misfolded GLR-1 receptor to be retained in the ER.

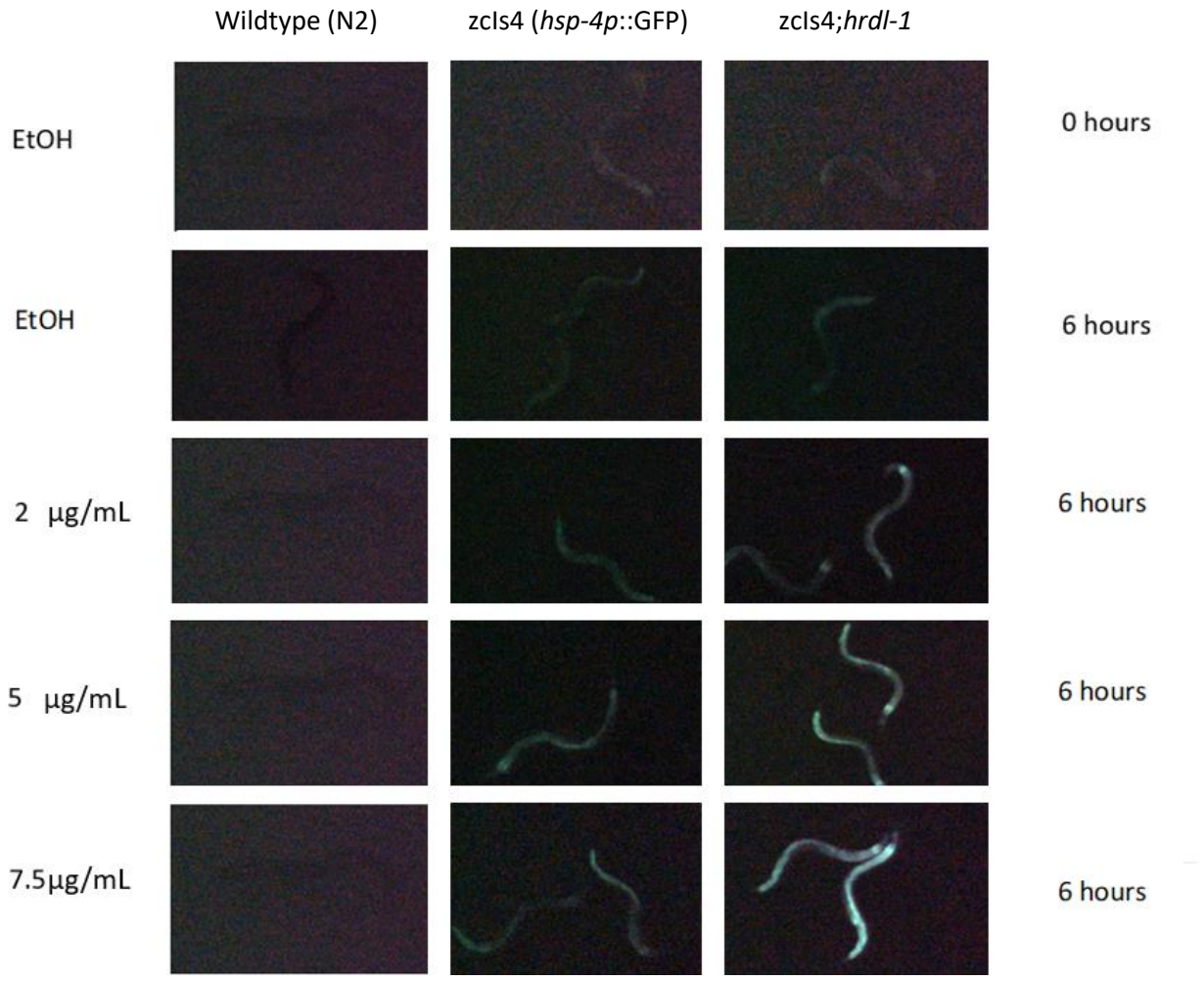


Figure 7. The ER stress reporter *hsp-4p*::GFP is active in the presence of high concentrations of tunicamycin. Animals that have the *hsp-4p*::GFP reporter with the mutant gene *hrdl-1* respond to tunicamycin at lower doses of tunicamycin.

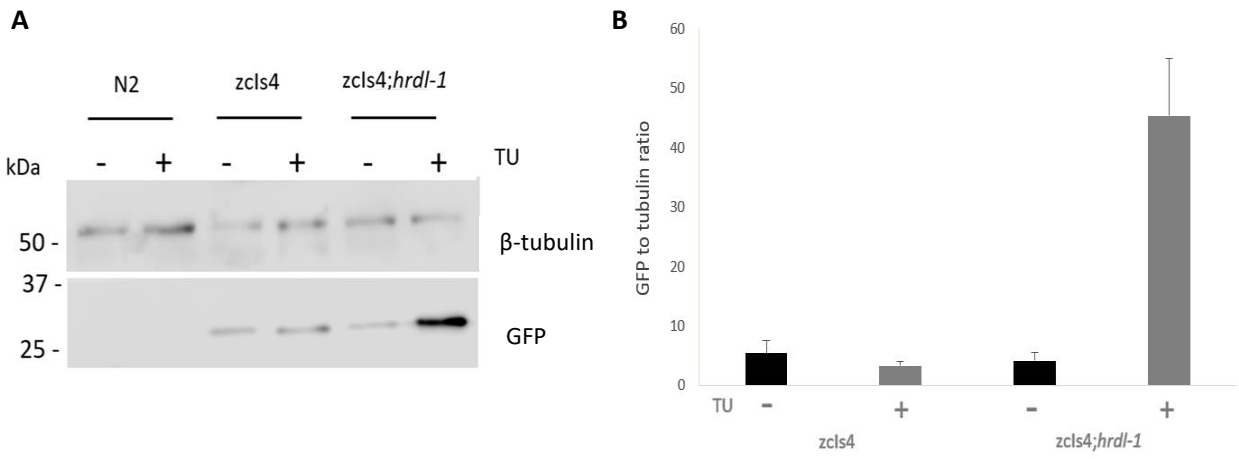
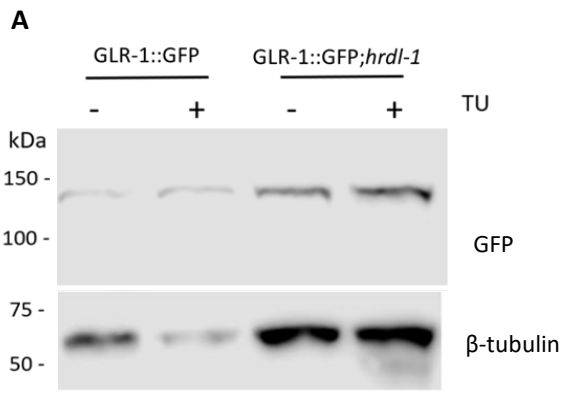


Figure 8. Western blot of *hsp-4p*::GFP and tubulin as a loading control. A) *zcIs4*; *hrdl-1* mutants showed more GFP in the presence of tunicamycin compared to *zcIs4* and N2. B) Normalized protein levels of *zcIs4* and *zcIs4*; *hrdl-1*. zcIs4; *hrdl-1* mutants had 30x more GFP without functional HRDL-1. Ethanol was used as a vehicle control to compare with tunicamycin……



B

	GLR-1::GFP		GLR-1::GFP;*hrdl-1*	
Average normalized ratio of GLR-1::GFP to tubulin	EtOH	TU	EtOH	TU
	1	2.56	2.63	2.36

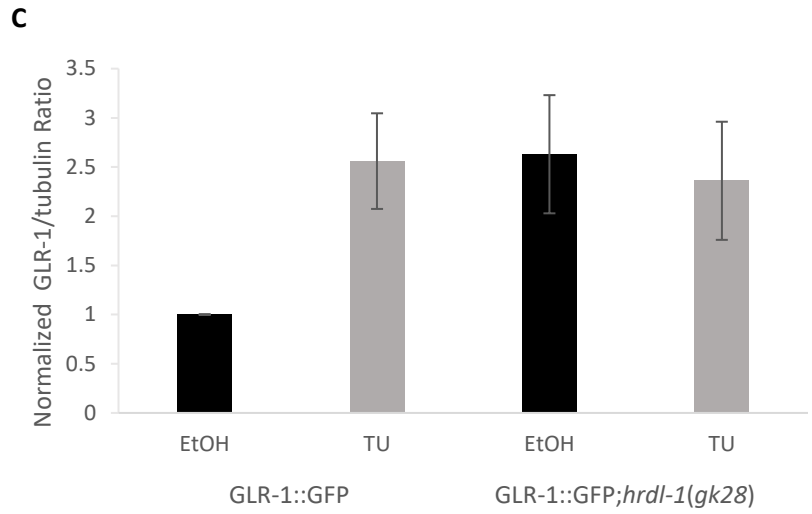


Figure 9. Western blot measuring GLR-1::GFP abundance in wildtype and *hrdl-1* mutants +/- tunicamycin. A) Whole cell lysate was obtained from both ethanol (vehicle control) and 5 µg/mL of tunicamycin. Blots were incubated with GFP monoclonal antibodies to probe GLR-1::GFP abundance (top) and anti-β-tubulin polyclonal antibodies as loading control (bottom). B) Table of average normalized ratio of GLR-1::GFP to observe fold change between *hrdl-1* mutants and wildtype with tunicamycin. C) Fold change of GLR-1::GFP between wildtype and *hrdl-1* mutants with and without tunicamycin. Results were shown relative to fold change levels of GLR-1::GFP abundance with error bars indicating standard deviation.

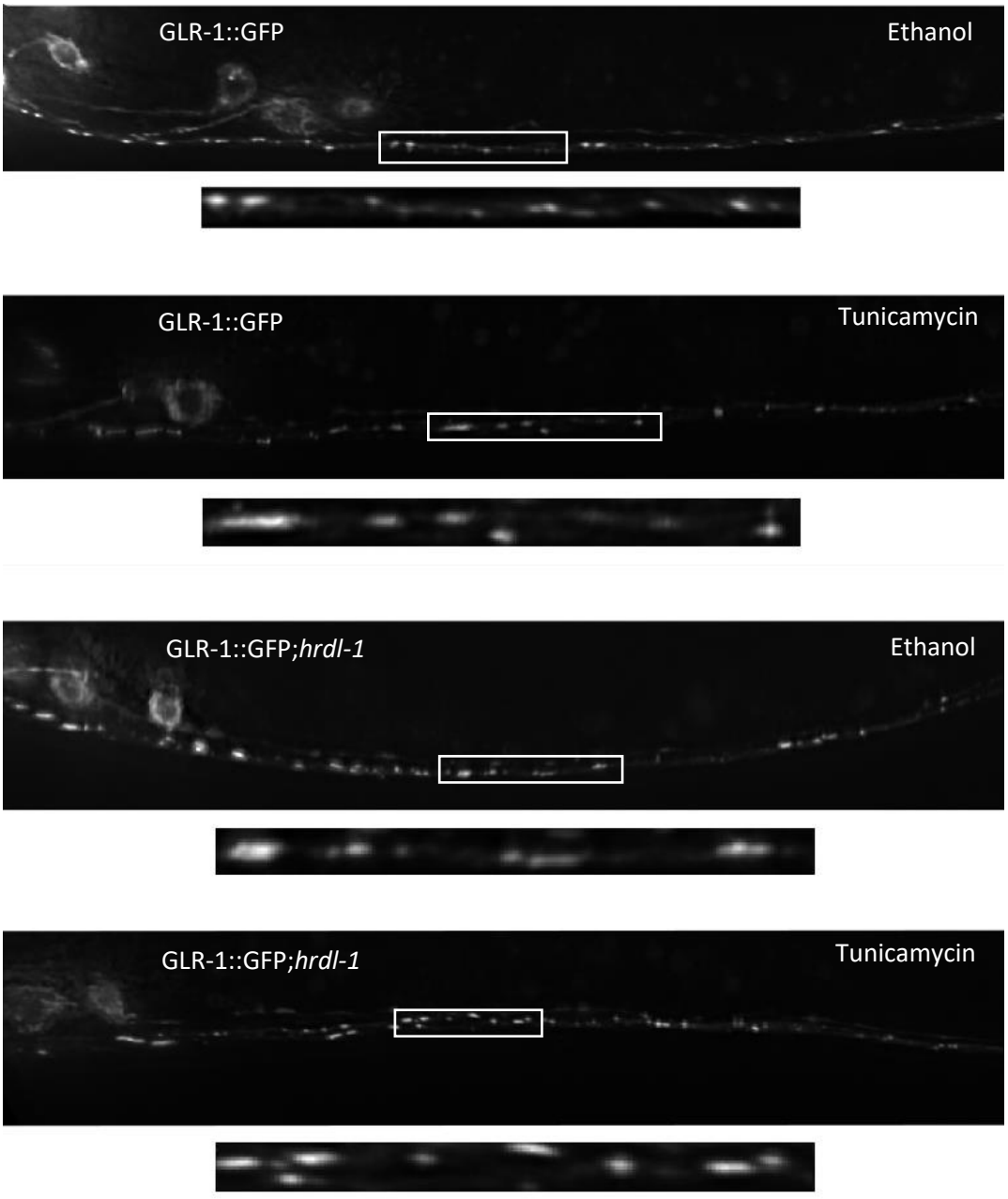


Figure 10 Representative ventral nerve cord (VNC) images of *hrdl-1* mutants in GLR-1::GFP background with tunicamycin and an image close up of the puncta below each image (zoom factor: 200%) (GLR-1::GFP ethanol n= 15, GLR-1::GFP tunicamycin n= 16, GLR-1::GFP; *hrdl-1* ethanol n= 17, and GLR-1::GFP; *hrdl-1* tunicamycin n= 24). 95% ethanol was used as a vehicle control to compare between genotypes. Images were taken on the DeltaVision at 60X oil objective magnification. Images set at maximum intensity on FIJI® to observe puncta fluorescence.

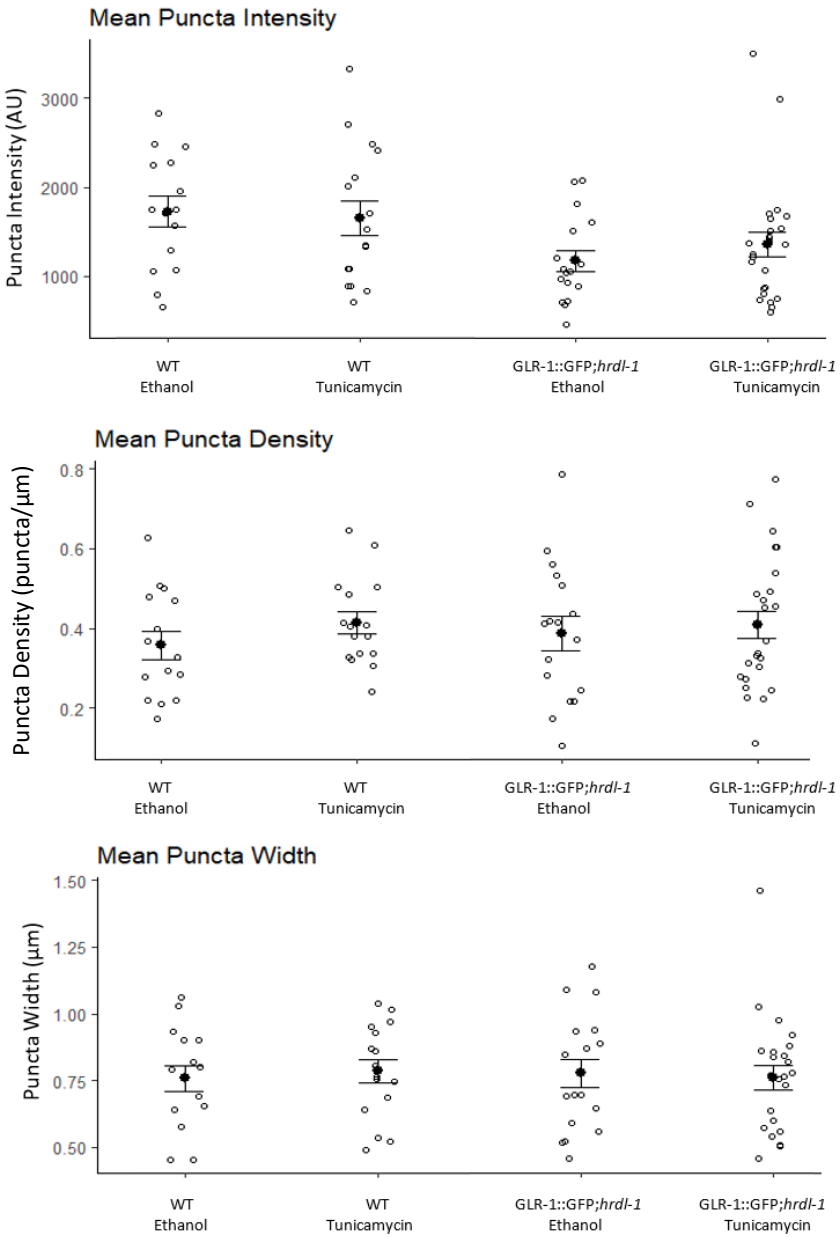


Figure 11. Quantification of mean puncta intensity, density, and width between wildtype GLR-1::GFP and *hrdl-1* mutant. A Tukey HSD test was calculated between genotype and treatment. Error bars represent standard deviation. L4 stage worms were randomly selected and imaged per strain on the DeltaVision and quantified via FIJI® analysis. Error bars represent standard error.

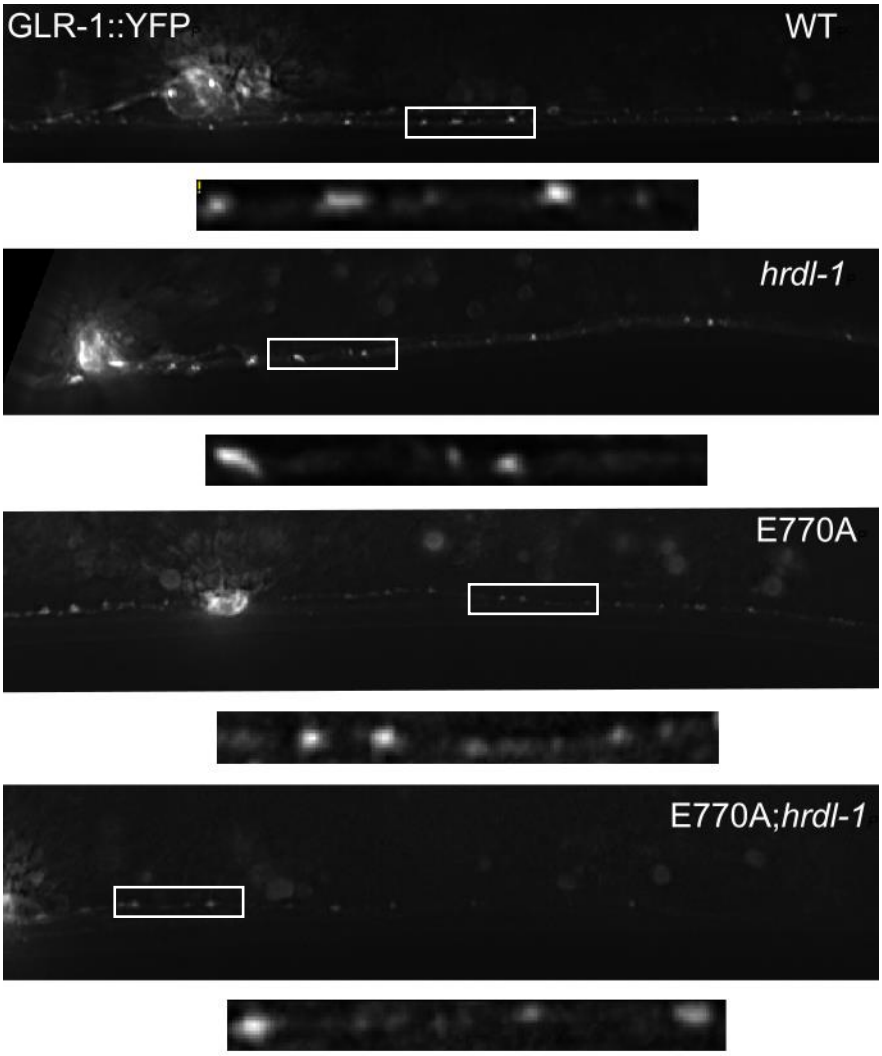


Figure 12. Representative ventral nerve cord (VNC) images of *hrdl-1*mutants and ligand-binding mutant in GLR-1::YFP background (Wildtype n= 20, *hrdl-1* n= 18, E770A n= 17, and E770A; *hrdl-1* n= 18). Images taken on DeltaVision at 60X oil objective magnification and image close up of the puncta below each image (zoom factor: 200%). Images set at maximum intensity on FIJI® and brightness are adjusted to enable fluorescence viewing between each genotype. Each image displayed localization patterns observed between wildtype and *hrdl-1* mutant.

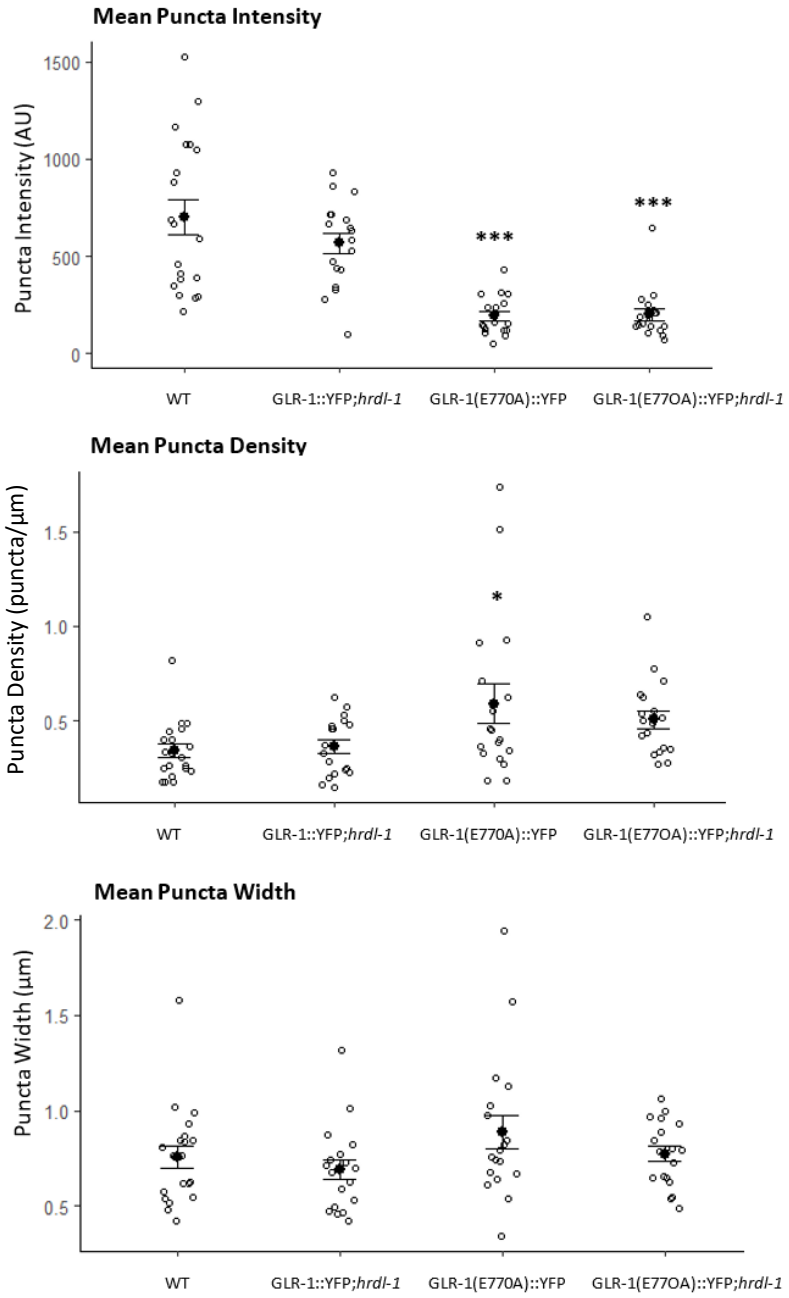


Figure 13. Quantification of mean puncta intensity, density, and width from *hrdl-1* mutants in GLR-1::YFP background. Strains analyzed were wildtype (GLR-1::YFP), *hrdl-1*, E770A, and E770A; *hrdl-1*. L4 stage worms were randomly selected and imaged per strain on the DeltaVision and quantified via FIJI® analysis. Error bars represent standard error. *** = $p < 0.001$ and * = $p < 0.05$ in comparison to wildtype followed by Tukey's HSD test.

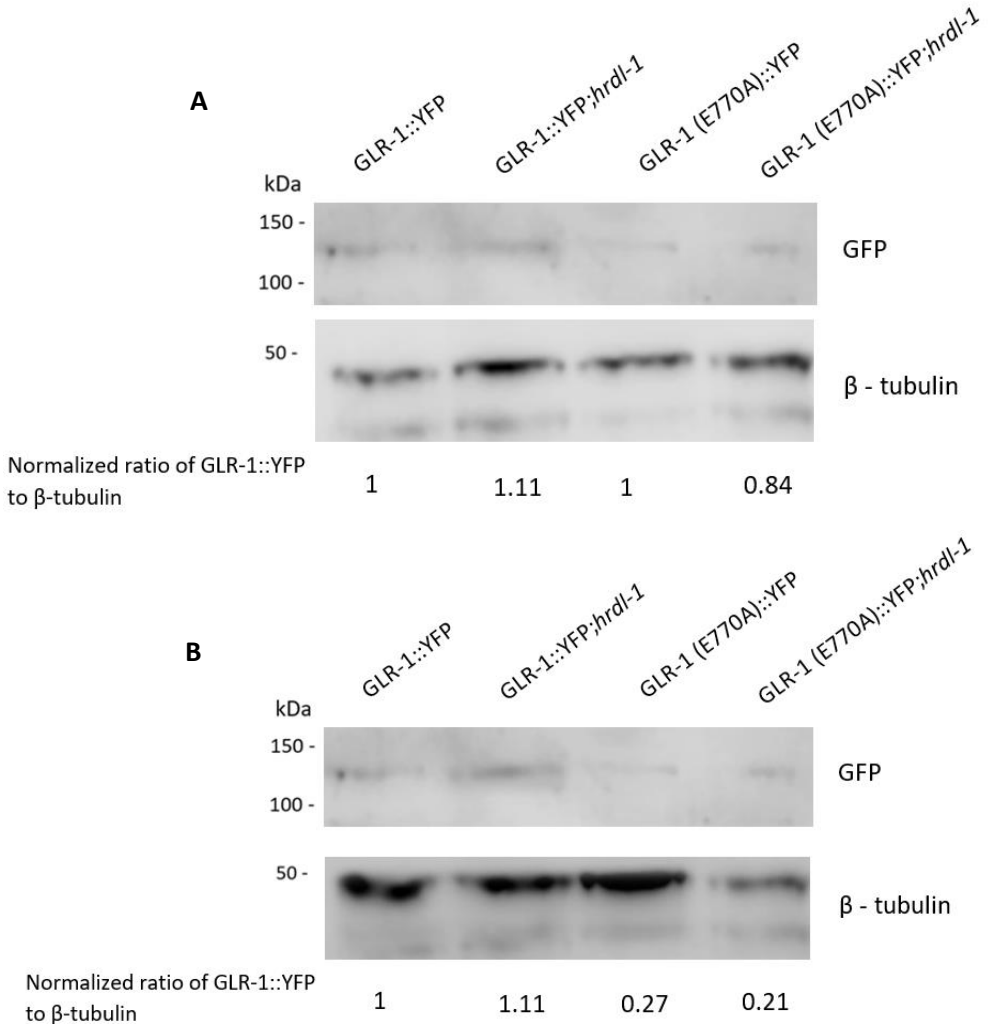


Figure 14. Western blot analysis measuring GLR-1::YFP and *hrdl-1* mutants. GLR-1(E770A)::YFP is the ligand- binding mutant preventing glutamate from binding. Whole cell lysate was collected from a mixed population of developmental staged animals. Blots were incubated with anti-GFP antibodies to probe GLR-1::YFP abundance (top) and anti-β-tubulin antibodies (bottom). Quantification indicates fold change between GLR-1::YFP abundance and β-tubulin as a loading control. A) Comparing normalized GLR-1::YFP between wildtype and ligand binding mutant with *hrdl-1*. B) Comparing normalized values for GLR-1::YFP for all three genotypes against wildtype GLR-1::YFP.

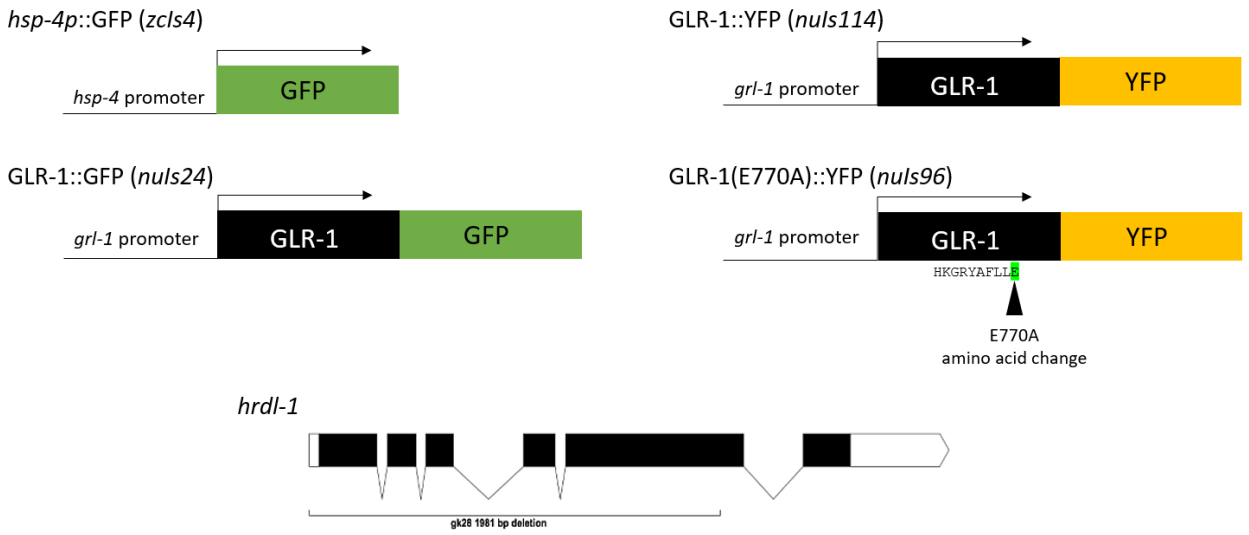


Figure 15. Genetic maps of strains used in this study. *hsp-4p*::GFP is a transgenic strain used as a reporter gene of *hsp-4* in ER stress conditions. GLR-1::GFP is a transgene with a *glr-1* promoter with a fusion reporter to observe expression of GLR-1. GLR-1::YFP contains a fusion reporter of yellow fluorescent protein (YFP) for expression of GLR-1. GLR-1(E770A)::YFP a transgene with a E770A amino acid change at *glr-1* promoter creating a structural mutation of GLR-1 at the ligand binding domain. *Hrdl-1* (*gk28*) is a E3 ligase harboring a 1981 base pair deletion in the first 5 exons and is crossed with all four of the transgenes to observe protein regulation in ERAD.

Strain name	Genotype	Description
Bristol N2	Wildtype	Wild type strain used to create all other strains
SJ4005	*zcIs4* *hsp-4p*::GFP	*hsp-4*::GFP is an ER stress reporter. Worms express low levels of GFP at basal conditions at the gut and hypodermis. GFP increase in the presence of tunicamycin
FJ354	*nuIs24* Pglr-1::GLR-1::GFP	Glutamate receptor-1 (glr-1) fused with GFP located at the ventral nerve cord
FJ863 F26E4.11 (gk28)	*hrdl-1**^{6}(*gk28*)	*hrdl-1* putative null allele and E3 mutant. Strain crossed with SJ4005, CLD83, and CLD84
FJ864	*nuIs24*; *hrdl-1**^{6}(*gk28*)	Transgenic worm strain created by Dr. Lina Dahlberg. The strain does not have the functional E3 ligase HRDL-1 and has expressed GLR-1::GFP
KP2592	*nuIs114* GLR-1::YFP	Wildtype GLR-1 receptor protein to compare with the ligand-binding mutants known to be retained in the ER. The receptor is tagged with yellow fluorescent protein
KP2267	*nuIs96* GLR-1[E770A]::YFP	Ligand binding mutant containing an amino acid change from glutamate to alanine. GLR-1 receptor known to retain in the ER.
CLD 77	*hsp-4p*::GFP; *hrdl-1**^{6}(*gk28*)	*hrdl-1* in *hsp-4p*::GFP background used for tunicamycin assays
CLD83	*nuIs114*; *hrdl-1**^{6} (gk28)	*hrdl-1* in GLR-1::YFP background
CLD84	*nuIs96*; *hrdl-1**^{6} (gk28)	*hrdl-1* in GLR-1[E770A]::YFP background

Table 1. List of *C. elegans* strains used in the study. Strain name, genotype, and description to provide background information about each strain and their purpose in the study.

Primer name	Primer sequence	Gene detection
ama-1 (Forward)	5’ GGATGGAATGTGGGTTGAGAATCAG 3’	*ama-1* used as control primers
ama-1 (Reverse)	5’CGCACAACGTCTTCCGAG 3’	
GFP-start (Forward)	5’ CGGCTAGCAAAGCAAAGGAGAAGAAC 3’	Green Fluorescent Protein (GFP) to determine animals tagged with GFP
GFP-NOT (Reverse)	5’ ACCCTAGCGGCCGGTATAGTTCATCC 3’	
LD51(gk28) (Reverse)	5’GTGTGCCCGGACAATGCTTC 3’	*hrdl*-1 to screen mutant hybrids from genetic crossing
hrdl-13-16 (Forward)	5’ CGCTCCGATGTTACGCGTCG 3’	

Table 2. Primers used for detecting a mutation with E3 ligase gene *hrdl-1* when crossing mutation in *hsp-4p*::GFP, GLR-1::GFP, and GLR-1::YFP background.

Antibody name	Antibody type	Antibody species	Antibody isotype	Detection
JL-8	Monoclonal primary antibody	Mouse	IgG	GFP
β-tubulin	Polyclonal primary antibody	Rabbit	IgG	β-tubulin
Sheep α mouse	Secondary antibody HRP conjugate	Sheep	IgG	Fluorescent tag to JL-8 antibody
Donkey α rabbit	Secondary antibody HRP conjugate	Donkey	IgG	Fluorescent tag to β-tubulin antibody

Table 3. Antibodies used for Western Blotting with GLR-1::GFP, GLR-1::YFP, and free GFP. β-tubulin was used as a loading control and abundance ratio to GLR-1 or free GFP.

Appendix I:

Quantitative analysis of *C. elegans* GLR-1::GFP synaptic puncta using ImageJ/Fiji

Neriah Alvinez, Nick Galati, Caroline Dahlberg

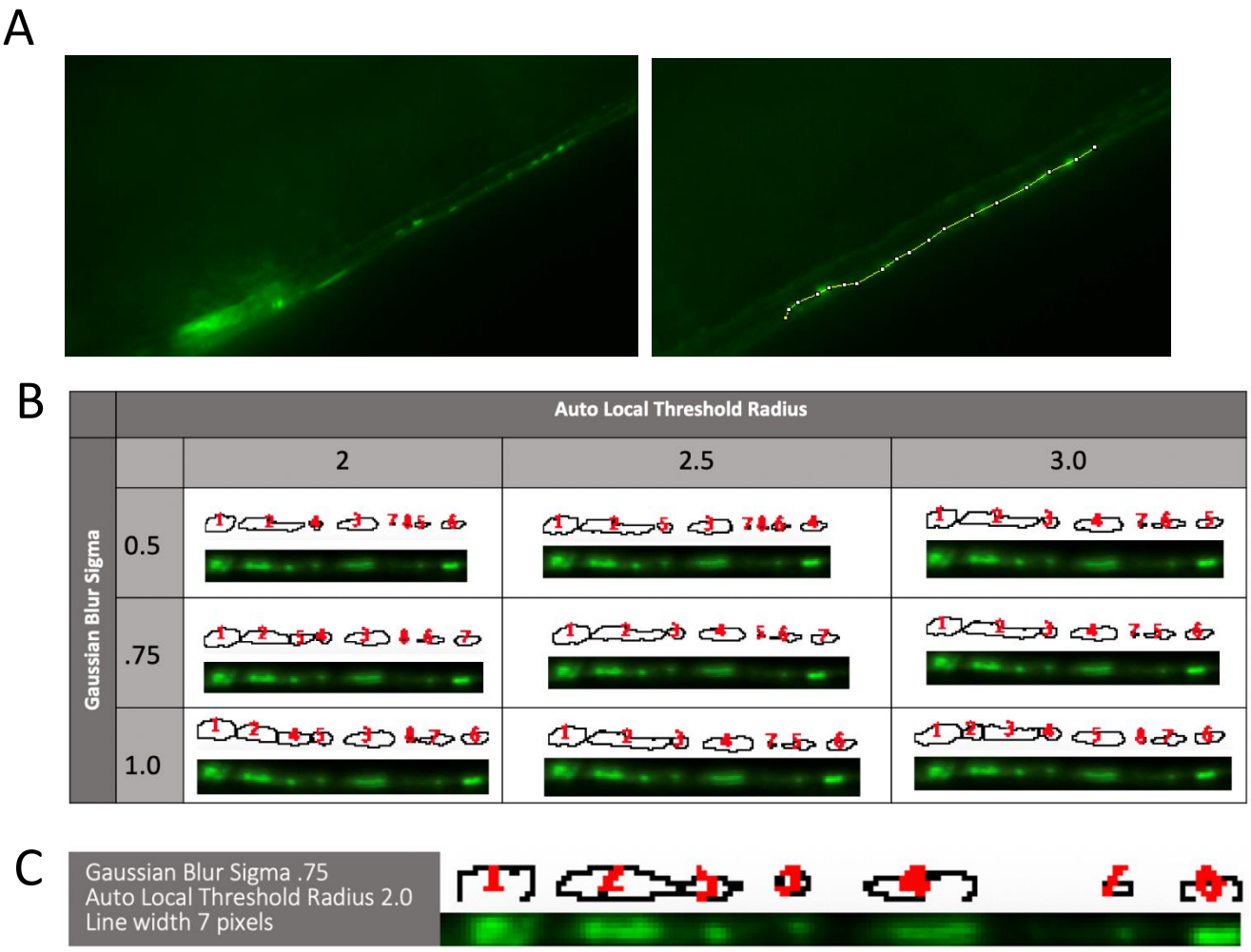


Figure 1: (A) A line selection is manually selected over a segment of interest in the ventral nerve cord to measure GLR-1::GFP puncta parameters. This allows exclusion of much of the background fluorescence. **(B)** Comparisons of different Gaussian blur and auto local threshold radii were done to analyze accuracy of size and detection of puncta among varying Gaussian blur and auto local threshold radius. This was repeated across 3 other images. **(C)** The optimal readout of data from selection made after the FIJI macro applied Gaussian blur sigma of 0.75, Phansalkar Auto Local threshold algorithm with a radius of 2.0, and Watershed.

Description

The quantitative analysis of synaptic puncta can allow us to identify genotypes that affect neural synapse pattern and morphology. We observe the glutamate receptor GLR-1 fused to green fluorescent protein (GLR-1::GFP) to understand the effects of ubiquitin-mediated degradation on an interneuron receptor (Dahlberg & Juo, 2014). Prior GLR-1::GFP analyses used the custom puncta program in Igor Pro (Dittman & Kaplan, 2006), but because it relies on proprietary software, it may not be accessible to all researchers. We currently approach puncta analysis using auto local thresholding in the free, open source ImageJ program run as the distribution FIJI, (Schindelin et al. 2012) allowing for a reproducible and less-biased data readout for puncta analysis.

To facilitate semi-automatic analyses of fluorescent puncta from live-imaged stacks, we created a macro in ImageJ. This allows us to create a segmented line down the ventral nerve cord of the animal and enables analyses of animals that are in curved or straight orientations (Figure 1A). We can then define a spline pixel width that is closest to the puncta to exclude more background fluorescence. The macro then filters the data with a Gaussian Blur to allow the auto local threshold Phansalkar to accurately detect puncta while the Watershed filter separates puncta in close proximity. The macro also generates drawings of the filtered data, allowing users to double check the puncta detection accuracy (Figure 1B).

Through comparing various Gaussian blur sigma values and auto local threshold radii among three different images, we determined a blur sigma of 0.75 and threshold radius of 2.0 to be optimal with a line selection closest to the ventral nerve cord (Figure 1BC). The resulting measurements that are taken allow users to calculate puncta density, fluorescence intensity, area, and width of the puncta. The source code can be modified to suit other puncta data similar to GLR-1::GFP, such as synaptobrevin-1 (SNB-1). Users can change resulting measurements taken, adjust blur sigma and auto local threshold radius, and the line selection is adjustable upon starting the macro program.

The macro code and written protocol are available here (at some dropbox link).

A video tutorial is available on YouTube (at some link that will be here).

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Author Contributions

Neriah Alvinez: Methodology, Conceptualization, Software, Validation, Visualization, Writing – original draft

Nick Galati: Methodology, Software

Lina Dahlberg: Supervision, Conceptualization, Writing – review & editing

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