Characterization of neuronal specific responses to induced misfolded protein stress in Caenorhabditis elegans

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Characterization of Neuronal Specific Responses to Induced Misfolded Protein Stress in

*Caenorhabditis elegans*

An Honors College Project Presented to
the Faculty of the Undergraduate
College of Science and Mathematics
James Madison University

by Claire Gormley

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Abstract

Misfolded protein stress has been associated with many types of disease, including neurodegenerative disorders like Alzheimer’s, Parkinson’s and Huntington’s disease. When a cell accumulates misfolded proteins in the endoplasmic reticulum, misfolded protein stress occurs and the unfolded protein response (UPR) is triggered to induce mechanisms that will allow the cell to either survive or undergo cell death. The nascent polypeptide associated complex (NAC) is a co-translational chaperone and α/β heterodimer that manages protein folding and localization, and protects against misfolded protein stress; changes in NAC function have been linked to both neurodegeneration and cancer. In these studies, I depleted the βNAC homologue ICD-1 from Caenorhabditis elegans and characterized changes in cell viability and nature, specifically in neurons, which are known to be susceptible to misfolded protein stress in the worm. I hypothesized that the levels of apoptotic cell death would increase in neurons in response to the misfolded protein stress. RNA interference (RNAi) was used to generate a range of icd-1 mRNA depletion levels, and the effects were evaluated at the microscopic level in worm strains expressing neurons marked by fluorescent proteins. Variations in the level of ICD-1 depletion led to a range of phenotypes, including the hypothesized increased apoptosis in embryonic neurons, as well as migration defects and changes gene expression patterns and dendrite and axon growth in adult neurons. These results may provide significant insights into how the NAC functions in healthy cells and how it malfunctions during the development of disease.
Introduction

Environmental stressors, such as heat, toxins, nutrient deficiencies and aging, can greatly reduce the ability of a cell to perform daily functions, such as protein folding. Improper protein folding can lead to the formation of protein aggregates and the onset of misfolded protein stress (Figure 1), which can cause major damage within the cell. For example, protein aggregates can inappropriately interact within organelles and other cellular compartments, causing disturbances in their functions. Misfolded protein aggregates can also interact with other types of proteins, causing them to fold incorrectly. In the event of misfolded protein stress in the Endoplasmic Reticulum (ER), the cell activates the Unfolded Protein Response (UPR), which consists of three branches in most organisms: PERK, IRE1, and ATF6 (Figure 2). These three branches act independently and cooperatively to initiate cell-saving measures, such as slowing protein translation, increasing the size of the ER, increasing levels of misfolded protein degradation and/or increasing the levels of chaperone proteins. If the damage caused by misfolded protein stress is too acute or chronic, the UPR will initiate cell death, typically by apoptosis (Hetz 2012).
Figure 1. A schematic free energy diagram depicting the progression of protein folding from productive folding events to aggregation events that occur when cell stress is introduced into the system. The formation of intramolecular interactions allows proteins to move toward a more kinetically favorable native state. Intermolecular interactions occur when several molecules fold simultaneously in the same cellular compartment, resulting in improper interactions between residues of individual proteins. Intermolecular interactions will lead to the formation of amorphous aggregates, toxic oligomers or ordered amyloid fibrils. Chaperones function to prevent the formation of aggregations through assisting and accelerating the formation of intramolecular interactions (Hartl et al 2011).
Figure 2. The Unfolded Protein Response (UPR) mechanism consisting of three branches: PERK, ATF6, and IRE1α. The UPR is triggered during misfolded protein stress in the ER lumen and engages several different mechanisms in the cytoplasm to save the cell, through increased chaperone expression (ATF6), increased protein degradation or decrease in transcribed mRNA (PERK), and increased ER surface area (IRE1α), or induces apoptosis when cell-saving mechanisms fail to resolve the misfolded protein stress (Hetz et al 2009).

Chaperones are a class of proteins that assist in the proper folding and localization of other proteins, comprising the proteostasis network that prevents the accumulation of misfolded and damaged proteins. As such, chaperones are a central component of the UPR’s cell-saving activities (Brehme et al 2014). One important contributor to proteostasis is the Nascent-polypeptide Associated Complex (NAC), a highly conserved, co-translational chaperone complex that aids in the proper localization and folding of nascent peptides as they emerge from the ribosome. The complex consists of two subunits: αNAC (ICD-2 in C. elegans) and βNAC
(ICD-1 in *C. elegans*) that work together to assist in folding (Beatrix *et al* 2000). However, under misfolded protein stress, the complex is thought to dissociate, with each subunit performing as yet unknown functions. It is speculated that the independent subunits work as transcription factors to stimulate and/or assist the UPR and combat protein aggregation (Kogan, Gvozdev 2014). Depletion of either subunit of the NAC results in increased ER-specific misfolded protein stress in *C. elegans* (Arsenovic *et al* 2012).

Management of protein folding is essential to cell health; misfolded protein stress and protein aggregation are associated with neurodegenerative diseases, while overactive UPR functions are associated with cancer. Protein aggregation in neurons is diagnostic of diseases like Alzheimer’s, Parkinson’s and Huntington’s and a majority of cancers upregulate cell-saving aspects of the UPR to prevent rapidly translated proteins, that are often mutant, from aggregating and causing cell death in quickly proliferating cells (Fels & Koumenis, 2006). Interestingly, and seemingly specific to neurons, previous research has found expression levels of both subunits to be altered in neurodegenerative disease states; βNAC levels are depleted in Huntington’s disease, and αNAC is under-expressed in Alzheimer’s disease (Kim 2002; Chae 2012). Based on this information, I hypothesized that neurons of *C. elegans* exposed to misfolded protein stress through the depletion of α or βNAC, undergo apoptosis, resulting in their disappearance from the organism. My alternative hypothesis was that the depletion of α or βNAC results in a variety of neuronal defects affecting not only viability of the cell, but also the nature and location of neurons.

To determine the presence, location and nature of neurons depleted of either NAC subunit, a mutant strain of *C. elegans* was obtained that is genetically engineered to express fluorescent proteins that highlight both the cell body and extensions of all neurons in the worm.
*C. elegans* are hermaphroditic nematodes that undergo a three day reproductive life cycle. In combination with a short reproductive life cycle, and genetically identical progeny, the fate of every cell in the *C. elegan* is known, including neurons, allowing us to identify effects on cell presence, location and nature straightforwardly (Figure 3). In addition, the high conservation of both apoptotic and UPR mechanisms in the worm allows us to extrapolate our findings and interpretations to other systems, including humans.
Figure 3. Neuronal map of a wild-type adult *C. elegans* depicting the longitudinal nerve tracts and commissures in the body. The location of each neuron is represented, providing a reference to compare effects on neuronal presence and location in NAC-depleted animals (Based on White et al 1986).
Misfolded protein stress in the ER was induced through depletion of either α or βNAC via RNA interference (RNAi) (Figure 4). RNAi uses short, double stranded pieces of RNA (dsRNA) that target complementary mRNA of a specific gene, in this case icd-1 or icd-2 (Bloss 2003). The process begins by introducing dsRNA into the cell, which is recognized as foreign and cleaved by an RNase III like enzyme, DICER. The short fragments of the dsRNA, known as small interfering RNAs (siRNAs), associate with the RNA induced silencing complex (RISC) and function to recognize complementary mRNAs for degradation. Degradation of specific mRNA molecules leads to a decrease in translation of that protein, thereby reducing the activity of that specific protein. In these studies, double stranded RNA specific for either icd-2/αNAC or icd-1/βNAC was introduced to the C. elegan by feeding the worms E. coli expressing the appropriate dsRNA. As the adult C. elegans consume the α or βNAC specific RNAi, they package the dsRNAi into their embryos, thereby affecting both themselves and their progeny.
Figure 4. A schematic diagram of the mechanism of siRNA interference and miRNA interference. A) siRNA structure. B) siRNA pathway. dsRNA is introduced into the cell and is recognized as foreign by DICER, which cleaves the molecule. The short fragments of degraded dsRNA, siRNA, associate with RISC to recognize complementary mRNA fragments, which are then degraded and therefore knocked-down within the cell. C) miRNA pathway. miRNA silence protein expression similarly to siRNAs, however, they are not specific for a single mRNA and are therefore able to regulate the expression of multiple mRNA molecules (Copyright the Royal Society of Chemistry, 2003) (Tatiparti et al 2017)

In the research presented here, the depletion of ICD-1/βNAC induced a strong misfolded protein stress and subsequent UPR in the progeny of treated C. elegans. The misfolded protein stress led to a range of phenotypes in the progeny, many of them specific to neurons. In the
strain expressing fluorescent proteins in the cell body and extensions of all neurons, the ventral nerve cords of ICD-1-depleted progeny were observed and quantified by fluorescence 24 and 48 hours after their parents were exposed to RNAi. The ventral nerve cord consists of 55 neurons spanning from the anterior nerve ring/brain to the posterior anal ganglia. We observed a decrease in the number of observable neurons fluorescing in the ventral nerve cord, particularly towards the posterior of the C. elegan, which was determined through comparing images with the C. elegan neuronal map created by Sulston et al (1976) (Figure 3). The number of neurons in the ventral nerve cord varied greatly from worm to worm, implying that RNAi exposure was not uniform, but these worms consistently showed fewer neurons relative to wild type.

The reproducible loss of fluorescing neurons in the ventral nerve cord of ICD-1 depleted animals led me to question the fate of these missing cells, more specifically, whether these cells were missing due to cell death. Under strong RNAi exposure, we observed significant cell death in embryos that were unable to develop into larva. Animals experiencing weaker RNAi effects were able to develop into adults, but often showed fewer ventral nerve cord neurons, perhaps also due to cell death. Alternatively, there are a least two other explanations for a loss of fluorescing neurons in ICD-1-depleted animals: loss of ICD-1 has affected the expression of the fluorescing protein in the neuron and/or loss of ICD-1 has affected the location of the neuron to the point where it is scored as missing. The goal of my project was to determine whether one or more of these outcomes explains the neuronal phenotypes being observed in ICD-1-depleted C. elegans. My results indicate that all three outcomes, cell death, changes in gene expression and changes in cell localization, are likely affecting these worms, indicating that loss of the NAC in C. elegans, and the resulting misfolded protein stress in the ER, is affecting cell functions beyond those solely responsible for viability.
**Materials and Methods**

**Care and Maintenance of *C. elegans***

*C. elegans* strains were maintained on Nematode Growth Medium (NGM) (Carolina Biological) that were treated with 200 µl of the *Escherichia coli* strain OP50-1 as a food source. The worms were maintained at 22 °C and transferred to a new NGM plate with OP50-1 every 3-4 days.

**Preparation of Bacterial Culture**

Cultures of *E. coli* OP50-1 and RNAi strains expressing dsRNA targeting either ICD-1 mRNA were prepared using Luria Broth culture media. 15 mL of Luria Broth was incubated with a colony of the desired *E. coli* strain and 15 µl of antibiotics (streptavidin for OP50-1 cultures and ampicillin for RNAi cultures) at 50 µM to prevent growth of other microorganisms at 37 °C for 12 hours in the shaker. OP50-1 cultures required streptomycin and RNAi plasmid strains required ampicillin. After 12 hours the cultures were transferred to sterile 15 mL tubes and refrigerated at 4 °C until needed.

**RNAi plate production**

RNAi plates were prepared as follows: 4.5 g NaCl, 25.5 g agar, 3.75 g peptone, 1.5 mL of 2mg/mL uracil, 0.22 g CaCl, 0.75 g of 10mg/mL cholesterol in 1462 mL of diH₂O. The solution was autoclaved, allowed to cool, and 37.5 mL of phosphate buffer (pH 6), 1.5 mL of 1M MgSO₄, 15 µl of 0.1M IPTG (required for induction of dsRNA expression) and 1.5 mL of 25 mg/mL ampicillin (for selection of dsRNA-expressing plasmid) were added.

**RNAi assays and Observations**

Approximately 50 late larval *C. elegans* were transferred to an RNAi plate seeded with *icd-1* or *icd-2* RNAi bacterial culture. After 24 hours, the adult worms were moved to another
RNAi plate, and the progeny were observed. This process was repeated with the progeny of adults exposed to 48 hours of RNAi. Surviving progeny were allowed to develop and scored for the number of fluorescent neurons in their ventral nerve cord, as well as any other deviations in neurons throughout the body, such as expression of Rab-3, migration defects, and deviations to neuronal extension patterns. These observations were made on a Zeiss Axiophot microscope with fluorescent capabilities, and images were captured and analyzed Ocular Advanced scientific camera control, version 1.0.
Results

The purpose of this study was to understand neuronal-specific effects generated by the misfolded protein stress and subsequent UPR induced by the depletion of the NAC in *C. elegans*. I hypothesized that neurons enduring misfolded protein stress induced through the depletion of the alpha or beta subunit of the NAC are absent as a result of cell death. Alternatively, the depletion of the NAC may trigger a more complex set of responses that impacts not only viability, but also location and nature of the affected neurons. To test these hypotheses, *C. elegans* were exposed for 96 hours *icd-1* RNAi and scored for the number of neurons in their ventral nerve cord every 24 hours. Subsequently, adult *C. elegans* were exposed to a total of 48 hours *icd-1* RNAi, and their progeny were characterized 24 and 48 hours after the start of RNAi exposure under fluorescent microscopy. The number of neurons in the ventral nerve cord, the number of apoptotic cells throughout the body, and the nature and location of the neurons were recorded at these time points.

Adult *C. elegans* contain 55 neurons in their ventral nerve cord (Figure 5). Every 24 hours, for a total of 96 hours, RNAi exposed adults were scored for the number of neurons in their ventral nerve cord. On average, the decrease in the number of neurons present in the ventral nerve cord was 52 at 24 hours, 50 after 48 hours, 45 after 72 hours and 48 after 96 hours (Figure 6). Disappearance of neurons occurred most prominently at the posterior end on the worm (Figures 7).
**Figure 5.** Adult *C. elegans* contains 55 ventral nerve cord neurons. DIC and fluorescent images of an untreated adult *C. elegans* genetically engineered with neuron-specific Rab-3::rfp containing a nuclear localization signal demarcating the cell bodies of the ventral nerve cord and nerve ring neurons. A) DIC image of adult *C. elegans*. Arrows indicated the location of the ventral nerve cord and the nerve ring. B) A fluorescent RFP image of neurons within an untreated adult *C. elegans*. C) An overlay image of A and B.
Figure 6. Quantification of neurons in the ventral nerve cord of adult *C. elegans* exposed to *icd-1* RNAi over time. Each bar represents the number of neurons present in the ventral nerve cord at 24-hour time points after being treated with *icd-1* RNAi. Ventral nerve cord neurons were identified and quantified via a neuronal specific red fluorescent protein (“NRFP”), and these numbers were compared to the number of neurons normally observed in untreated adults. Standard error bars show the deviation from the mean number of neurons for each time point. Standard deviation values for the control group through 96 hours *icd-1* exposure are 4.98, 3.96, 5.12, 5.34, and 6.18, respectively. Although no significant difference is shown, the above trend justifies further investigation into the altered phenotypes of RNAi treated *C. elegans*. 
Figure 7. Neurons are most often not observed in the posterior region of the ventral nerve cord in IC-1-depleted adults. The left diagram depicts a mapped, six cell cluster at the posterior end of an untreated adult *C. elegans*. The right image was taken under fluorescent microscopy and reveals the absence of detectible signal in three neurons (VA10, VB 11, DA7) and barely detectible signal in one neuron (DB7) in an icd-1 RNAi treated adult.

The progeny of adults exposed to 48 hours of RNAi were observed from embryos to adults. Normal levels of apoptosis in untreated embryos were compared to the level of apoptosis in the embryonic progeny of icd-1 RNAi treated adults. Embryos exposed to strong levels of RNAi showed high levels of neuronal apoptosis, resulting in numerous apoptotic bodies fluorescing positively with the neuron-specific green fluorescent protein UNC-119 (Figure 8 C,D). These cell deaths were distinguishable from the corpses that normally occur during embryogenesis (Figure 8 A,B) by their high number and small, fragmented morphology. Such
embryos did not hatch into adulthood, and were considered the most strongly affected population during the icd-1 (RNAi) time course.

![Figure 8](image)

**Figure 8.** Strong depletion of ICD-1 results in high levels of neuronal cell death during embryogenesis. A) A DIC image of an untreated embryo. Arrows indicate the raised, button-like corpses of cells that have undergone apoptosis. B) Fluorescent image of the untreated embryo shown in image A showing neuron-specific green fluorescent protein UNC-119 contained in cell corpses. C) A DIC image of embryo from of an icd-1 RNAi treated adult. The embryo is misshapen compared to image A, however no cell corpses are visible. D) Fluorescent image of embryo in image C, showing numerous UNC-119::GFP-positive puncta, presumably resulting from numerous neuronal cell deaths.

Embryos exposed to lower levels of ICD-1 depletion completed embryogenesis and often developed into adults, allowing us to determine both the location and nature of the neurons in
these ICD-1-depleted animals. Using the *C. elegans* neuron map, I was able to assess the location of neurons throughout the body, and found migration of neurons to improper locations (Figure 9). Specifically, the fluorescent image of a four-cell cluster in the dorsal nerve cord of

**Figure 9.** Depletion of ICD-1 results in neuronal migration defects. Comparison of four-cell cluster in dorsal nerve cord in untreated and *icd-1* RNAi treated adult *C. elegans*. A) A mapped diagram of the PDEL, SDQL, PVDL, and PVM neurons in the dorsal nerve cord. B) A fluorescent image of the PDEL, SDQL, PVDL, and PVM neurons in an untreated adult *C. elegan* strain with Rab-3::RFP and Unc-119::GFP. C) A Rab-3::RFP fluorescent image of the four-cell cluster in the dorsal nerve cord in which SDQL has migrated to the right. D) An Unc-119::GFP fluorescent image of the adult *C. elegan* from image C. This image shows the extensions of the PDEL, SDQL, PVDL and PVM neurons.
an ICD-1-depleted adult often displayed inappropriate migration patterns, including the perpendicular migration of the SDQL neuron away from its typical position relative to the three other neurons shown in figure 9. In such a case, the inability to observe a neuron in its expected location is not due to cell death, but rather a cell migration defect, in support of my alternative hypothesis.

In addition to mislocalized neurons, adult progeny of ICD-1-depleted animals also showed a change in nature of their neurons, including changes in expression of the neuronal specific protein Rab-3. Rab-3 is a Ras GTPase that is in this case linked to a nuclear locator signal (NLS) to fluoresce specifically the nuclei of all neurons in the worm. Using DIC microscopy, I was able to visualize by morphology the presence of neurons in the heavily enervated region of the worm called the nerve ring (also referred to as the “brain” of the worm) and determine their state of Rab-3 expression using fluorescent microscopy (Figure 10). In ICD-1-depleted animals, neurons that displayed normal location and morphology in the nerve ring were no longer expressing Rab-3::GFP at detectible levels, indicating that specific, neuron-specific gene expression patterns had changed in these cells. As with the case of migration defects, the loss of a neuron-specific marker would make a neuron “invisible” to detection by fluorescence, and be scored as missing, even though it had survived and even migrated properly.
Figure 10. Depletion of ICD-1 results in changes in neuron-specific gene expression. DIC and fluorescent images comparing a nerve ring of an untreated C. elegan with the nerve ring of an icd-1 RNAi treated C. elegans. A) A DIC image of an untreated nerve ring. The black line extends the length of the nerve ring. B) Neurons fluorescing Rab-3::RFP in a nerve ring of an untreated C. elegan. Every neuron identified via morphology is also expressing Rab-3::RFP. C) The nerve ring of an icd-1 RNAi treated C. elegans. Arrows and arrowheads indicate nerve cells in DIC. D) Image C detecting expression of Rab-3::RFP. Arrowheads indicate neurons expressing significantly reduced levels of Rab-3::RFP.
Finally, in addition to effects on cell migration and neuron-specific gene expression, depletion of ICD-1 also affected the formation of axons and dendrites in surviving adults. The establishment of a properly configured and connected neuronal network relies on both the correct migration of neurons during development as well as gene expression programs that allow neurons to grow functional extensions. If both migration and gene expression are being affected in ICD-1-depleted neurons, axon and dendrite patterning could also be affected. I observed such abnormalities throughout the nervous systems of affected worms. The Anterior Lateral Microtubule neuron (ALM) was of particular focus due to its relatively simple and invariant extension structure and its sensitivity to depletion of ICD-1 (Figure 11). The ALM neuron typically has one relatively long extension directed anteriorly and one shorter posterior extension (Figure 11 A,B). In ICD-1-depleted animals, the posterior extension of the ALM was often strongly affects, often showing two extensions (Figure 11 C), or one extension containing a bifurcation (Figure 11 D). Both of these phenomena could result from inappropriate migration, a departure from normal neuronal gene expression, or both.
Figure 11. Depletion of ICD-1 affects the formation of neuronal extensions. Comparison of the ALM neuron’s extension patterns between treated and icd-1 RNAi treated adult *C. elegans*. A) A photograph of a wild-type adult *C. elegan* ALM neuron from Wormatlas. B) A fluorescent image of the ALM neuron in an untreated adult *C. elegan* genetically engineered to express Unc-119::GFP. C) Fluorescent image of the ALM neuron in the progeny of an icd-1 RNAi treated adult *C. elegan*. The image reveals a posterior branching pattern with increased length and additional extensions. D) Another fluorescent image of the ALM neuron in the progeny of an icd-1 RNAi treated adult. The image shows a bifurcated posterior branching pattern with additional extensions and increased length.
Discussion

The goal of my study was to characterize the role of the NAC in the management of misfolded protein stress in the ER of *C. elegans*. More specifically, I wanted to characterize the role of the NAC in neuron viability and function by depleting the complex and assessing the various phenotypic outcomes in embryos and adults. My original hypothesis was that depletion of the NAC results primarily in neuronal cell death, resulting in missing neurons throughout the body of the affected animals. My revised hypothesis included the possibilities that in addition to cell death, depletion of the NAC affects both neuronal migration and gene expression, generating the same phenotype as cell death: the lack of detectible neurons throughout the body of the affected animal.

The NAC is a co-translational chaperone that functions to help nascent peptides fold and locate properly within the cell. Changes in the levels of NAC subunits have been associated with numerous diseases; down regulation of αNAC is observed in neurodegenerative diseases such as Alzheimer’s, while βNAC is known to be down regulated in Huntington’s and overexpressed in breast, prostate and gastric cancers (Symes 2013; Wang 2013). When either subunit of the NAC is depleted in the *C. elegan*, misfolded protein stress is induced in the ER that triggers a strong UPR that often results in cell death, indicating that both NAC subunits play key roles in maintaining ER-specific homeostasis and cell viability (Arsenovic *et al* 2012). During misfolded protein stress, the NAC is believed to dissociate into the alpha and beta subunits and work to alleviate the stress and prevent proteins from aggregating further. For unknown reasons, neurons are more sensitive to misfolded protein stress in *C. elegans*, and the damage caused by misfolded protein stress in neurons more often leads to cell death. These phenomena mirror what is observed in human disease; protein aggregates in neurons are thought to result in cell
death, contributing to the pathology of neurodegenerative diseases, such as Alzheimer’s, Huntington’s and Parkinson’s disease. As mentioned earlier, NAC levels are depleted in a number of these diseases, therefore, understanding the role of the NAC in the management of misfolded protein stress may lead to a better understanding of the development of these devastating diseases.

Contributing to the complexity of understanding NAC function are the observations that link the individual subunits to effects on gene expression. Significant NAC depletion of either NAC subunit leads to high levels of misfolded protein stress and eventually cell death via apoptosis. Sub-lethal levels of depletion of either subunit do not induce cell death, but may allow the unaffected subunit to impact cell function not directly related to viability. Because both the α and β subunits have been linked to transcriptional effects (Kogan, Gvozdev 2014), RNAi-driven depletion of one subunit may allow the other to induce changes in gene expression, causing varying phenotypes, including mislocalization, improper extension formation, and changes in neuronal-specific protein expression.

Loss of neurons was first observed in the ventral nerve cord of adult worms exposed to icd-1 RNAi (Figures 6 and 7). Adult C. elegans normally have 55 neurons in their ventral nerve cord, clusters of neurons in the nerve ring and anal ganglia, and various motor and inter-connecting neurons throughout their bodies. Ventral nerve cord neurons were counted because individual cells were easily visible compared to the nerve ring and anal ganglia. Adult worms were observed every 24 hours and scored for number of neurons in the ventral nerve cord, as well as body shape, apoptosis and necrosis, brood size and general malformations. Longer exposure to RNAi typically resulted in a decrease in neurons and increased overall degradation. Especially strong exposure caused neurons to decrease by approximately 10 cells along the
ventral nerve cord, usually near the posterior end of the worm (Figure 6 and 7). Decrease in neurons in the nerve ring and anal ganglia were also observed, but not quantitated. The standard error bar margins are most likely due to the variability in RNAi strength and exposure to individual *C. elegans*. The variability in the control population could be due to natural loss of fluorescent signal of aged neurons.

Numerous explanations could account for the decrease in neurons. Initially, it was believed that the neurons were undergoing apoptosis, however, no fluorescent corpses or apoptotic cell bodies were observed in the region of the missing neurons (Figure 10). Apoptosis was observed in strongly affected progeny embryos, but surprisingly, no fluorescent corpses were observed in adult worms. The disappearance of neurons may therefore have been due to migration defects and/or change in nature of the neuron that prevents the expression of the fluorescent marker, in other words, the neurons were present but not in the right place and/or expressing the fluorescent markers necessary for visualization.

The progeny of RNAi-exposed adult *C. elegans* exhibited more dramatic phenotypes because the RNAi is packaged directly into embryos. Embryos that experienced strong levels of RNAi were misshapen, in some instances to the point of experiencing an enclosure defect, a bursting of an embryo’s internal fluid and materials at the seam cells, and full of fluorescent corpses and/or apoptotic bodies, chunks of dead cell. Embryos normally undergo controlled levels of apoptosis throughout development to get rid of superfluous cells, for example the sister cells of a number of neurons, however, high levels of apoptosis leads to improper development and most likely death. Embryos with high levels of NAC depletion rarely developed past the gastrula stage of development, likely due to the loss of cells necessary for embryonic viability. Unengulfed apoptotic bodies observed in some embryos may indicate that neighboring cells were
unable to dispose of dead cell materials, possibly due to overwhelming rates of apoptosis (Figure 8). That the overwhelming cell death takes place primarily in neurons is indicated by these apoptotic bodies containing the neuron-specific protein UNC-119::GFP, confirming earlier studies that neurons are particularly susceptible to death when the NAC is depleted.

Progeny embryos that experienced weaker RNAi exposure and lower levels of NAC depletion developed into larvae and adults with varying phenotypes. Once again, missing neurons were observed in the ventral nerve cord, possibly due to apoptosis during embryonic development. Improper migration of neurons was observed near the dorsal nerve cord, specifically a four-cell cluster corresponding with PDEL, SDQL, PVDL and PVM. Typically these neurons are close in proximity, but when treated with RNAi they were spread out linearly and in certain instances had neurons migrating perpendicularly or missing entirely (Figure 9). Neurons also experienced a change in nature, such that they were no longer expressing Rab-3, the neuronal specific protein fused to RFP that allowed for the visualization of the neuronal cell body; specifically, neurons in a nerve ring that were visible under DIC, did not express Rab-3::RFP (Figure 10). These same neurons continued to express the neuronal-specific cell marker Unc-119::GFP, indicating that not all neuron-specific gene expression was being affected by ICD-1 depletion. A change in axon-dendrite patterning was also observed in ICD-1-depleted progeny adults, including the number of extensions present, the length of the extensions, and the branching patterns of these extensions (Figure 11).

The various phenotypes observed in these ICD-1-depleted animals may be explained by the putative transcriptional activity of liberated ICD-2 as ICD-1 levels decreased over time. Previous work has shown that ICD-2 homologue αNAC affects gene expression during normal bone cell and blood cell differentiation, and overexpression of αNAC, but not βNAC, has been
observed in brain cancer and childhood leukemia (Hämmerle et al 2003; Jafarov et al 2012; Hekmatnejad et al 2014; Zeng et al 2014;). The ability of ICD-2 to affect gene expression in neurons would be potentiated by the loss of ICD-1, allowing this subunit to change the nature of the cell in the absence of its binding partner. Whether ICD-2 would have such an effect on neuronal gene expression could be determined through analysis of the transcriptional control regions of these genes in conjunction with reporter assays that determine the ability of ICD-2 to contribute to the control of these genes’ expression.

The migration defects and changes in extension morphology observed in ICD-1-depleted neurons could be the direct result of changes in neuronal gene expression in conjunction with ICD-1-depletion effects on other cell types, specifically hypodermal cells. Neurons rely on hypodermal cells to both localize properly and form functional extensions. Direct interactions between proteins expressed on the surfaces of both cell types help the nervous system of the worm form properly throughout development (Shaham 2015). It is clear by the misshapen bodies and enclosure defects of strongly affected embryos and larvae that hypodermal cells are susceptible to ICD-1 depletion, even though they are less likely to die under this stress relative to neurons. If changes in gene expression of neurons and/or hypodermal cells alters the nature of their cell surface proteins, cell-cell interactions could be negatively affected, resulting in neuronal mislocalization and abnormal axon and dendrite formation. We are currently assessing the effects of ICD-1 depletion on the nature and number of hypodermal cells in affected animals.

In conclusion, neurons exposed to misfolded protein stress induced through the depletion of α or β NAC respond relative to the strength of depletion. High levels of ICD-1 depletion result in increased levels of apoptosis in neurons and eventually death of the organism. Lower levels of ICD-1 depletion results in migration defects abnormal extension growth and changes in
the nature of the neurons, as observed in changes in the expression of a neuronal specific proteins. These observations support my alternative hypothesis, that a range of neuronal defects result from inducing misfolded protein stress through depletion of the NAC.

Understanding cell-specific responses to misfolded protein stress may help determine potential treatments to neurodegenerative diseases, such as Alzheimer’s, Huntington’s and Parkinson’s Disease. Neurons respond to the misfolded protein stress induced by depletion of one subunit of the NAC in two ways: in high levels of NAC depletion, by increasing levels of apoptosis, or in sub-lethal situations, by changing the nature and location of neurons. The locations of specific neurons, as well as the proteins they express will potentially change their interactions within the nervous system, and may contribute to the old-aged, and malformed phenotypes observed in icd-1 RNAi exposed adults. Determining the mechanism by which these responses happen, and the role the NAC plays in inducing certain responses, will provide necessary information to understanding the UPR and producing a treatment and/or potential cure to neurodegenerative diseases.
Future Research Investigations

Specific neuronal responses to depletion of α or β NAC have been identified, however, it is not understood why the frequency of these responses and their effects on the ability of the worm to function normally. Further observation and quantification of neuronal migration, protein expression, neuronal extension pattern deviations and neuronal death must be carried out to fully understand the significance of these findings. Studies focused on the sensitivity of treated worms to touch, their ability to recognize food, and any alterations in their movement patterns should also be conducted to understand how changes in neuronal phenotype may affect the organism’s ability to function.
References


